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Defez

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(54) **METHOD TO IMPROVE PHOSPHATE SOLUBILIZATION IN PLANTS**

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C12N 15/00	(2006.01)
C07H 21/04	(2006.01)
C12P 17/10	(2006.01)
C05F 11/08	(2006.01)

(52) **U.S. Cl.**

CPC ***C12P 17/10*** (2013.01); ***C05F 11/08*** (2013.01); ***C12P 3/00*** (2013.01)

(58) **Field of Classification Search**

CPC C12P 3/00; C12N 9/80; C12N 9/0069; C12N 15/73

See application file for complete search history.

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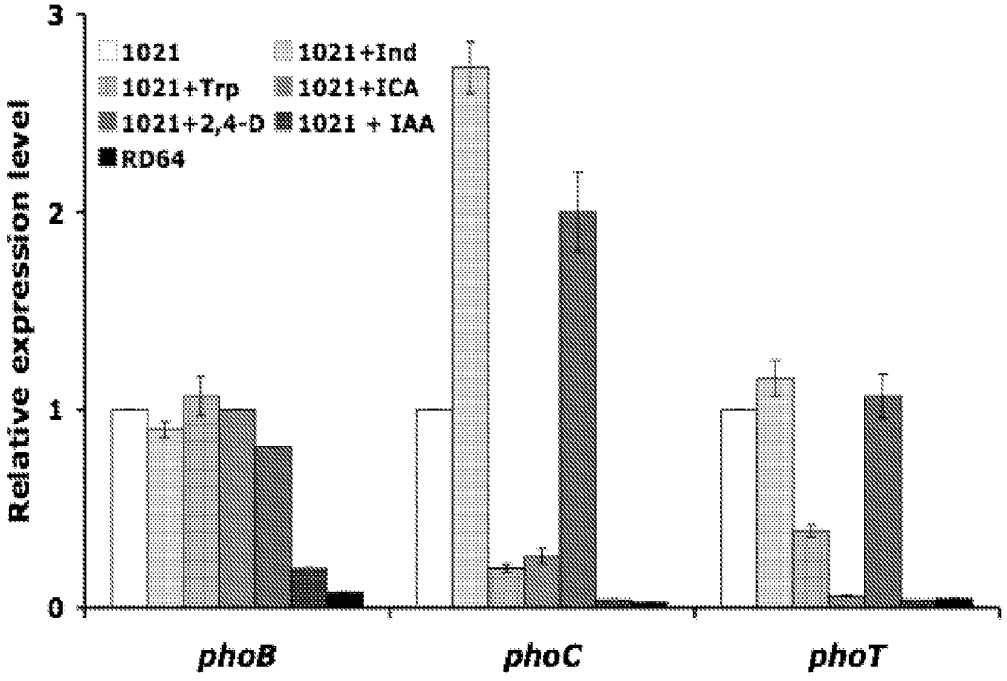
(74) *Attorney, Agent, or Firm* — Lucas & Mercanti, LLP

(57) **ABSTRACT**

The present invention relates to the use of a bacterium having an high indole-3-acetic acid (IAA) content for solubilizing phosphate rock (PR) in the ground, wherein said bacterium is obtained by transformation with a gene encoding an agent able to increase the IAA content.

3 Claims, 7 Drawing Sheets


Relative expression level
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2
1
0
1021
1021+Ind
1021+Trp
1021+ICA
1021+2,4-D
1021 + IAA
RD64
phoB
phoC
phoT

Fig. S1

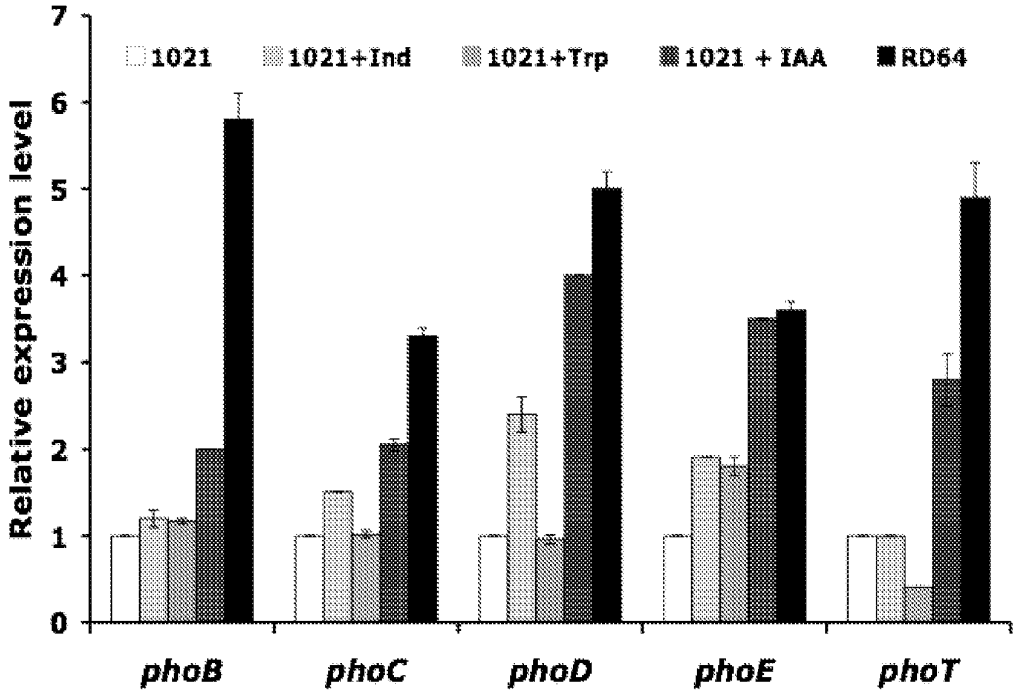
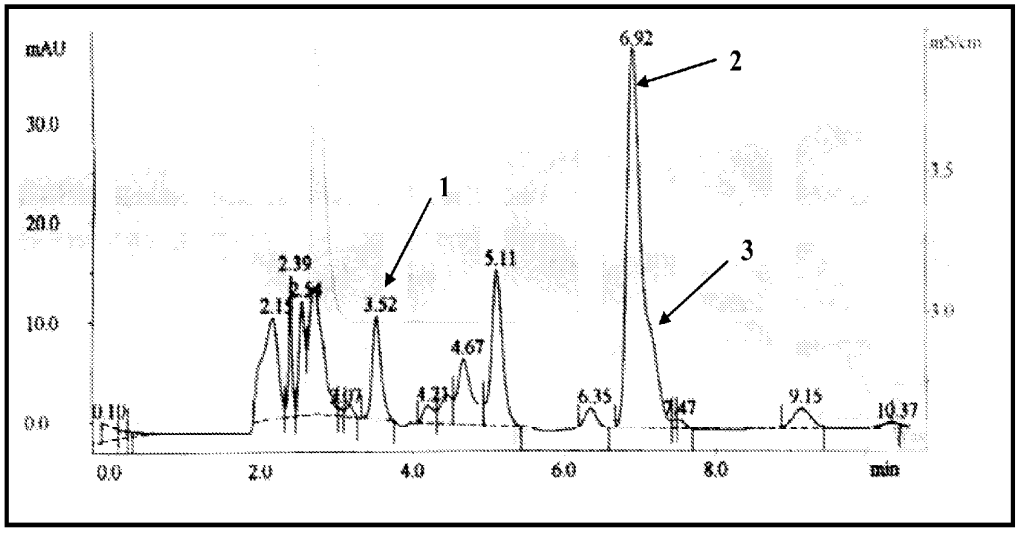
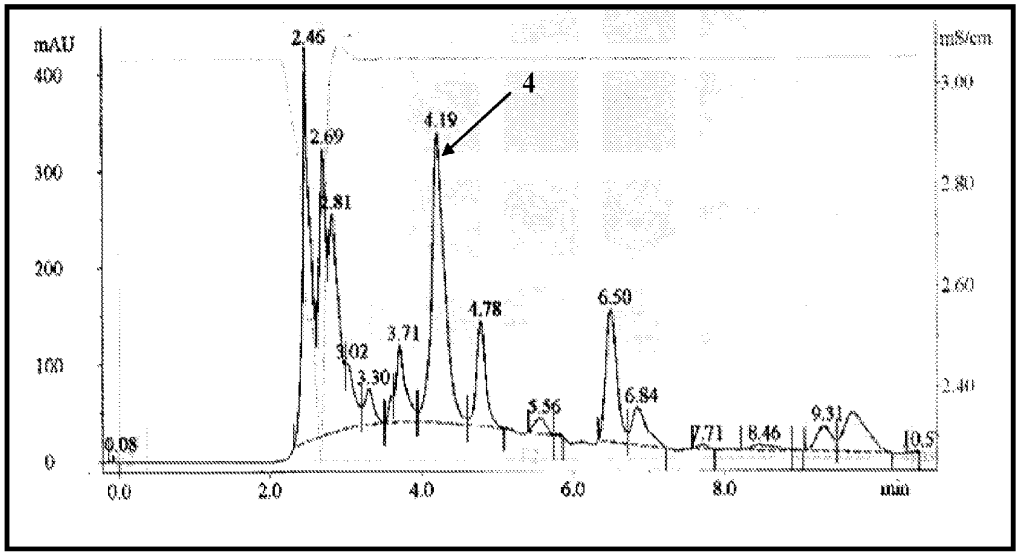

1021
1021+Ind
1021+Trp
1021 + IAA
RD64
Relative expression level
0
1
2
3
4
5
6
7
phoB
phoC
phoD
phoE
phoT

Fig. 1

A


mAU
30.0
20.0
10.0
0.0
mS/cm
3.5
3.0
1
2
3
6.92
5.11
3.52
2.39
4.67
6.35
9.15
10.37
0.0
2.0
4.0
6.0
8.0
min

B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400
300
200
100
0
mS/cm
3.00
2.80
2.60
2.40
4
2.46
4.19
2.69
2.81
4.78
6.50
3.71
3.02
3.30
5.56
6.84
9.31
0.08
7.71
8.46
10.5
0.0
2.0
4.0
6.0
8.0
min

Fig. S2

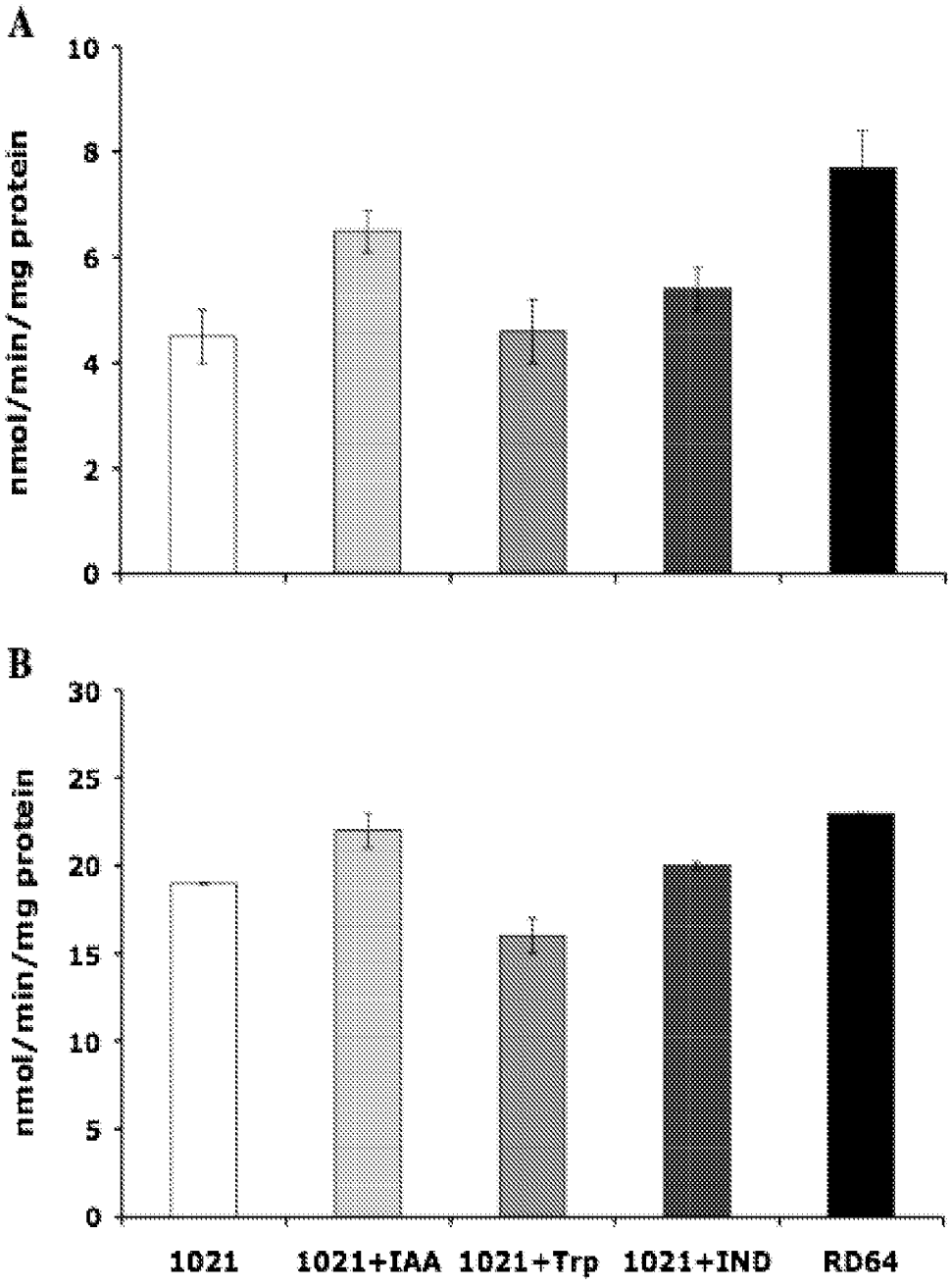
A
nmol/min/mg protein
0
2
4
6
8
10
B
nmol/min/mg protein
0
5
10
15
20
25
30
1021
1021+IAA
1021+Trp
1021+IND
RD64

Fig. 2

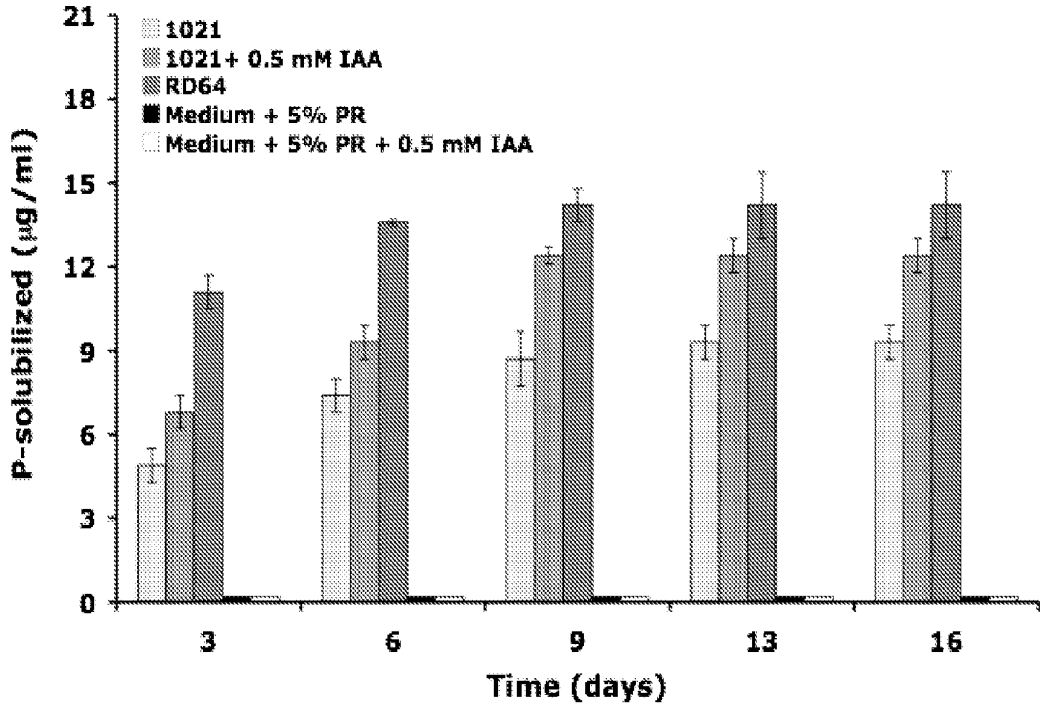
21
18
15
12
9
6
3
0
P-solubilized (µg/ml)
1021
1021+ 0.5 mM IAA
RD64
Medium + 5% PR
Medium + 5% PR + 0.5 mM IAA
3
6
9
13
16
Time (days)

Fig. 3

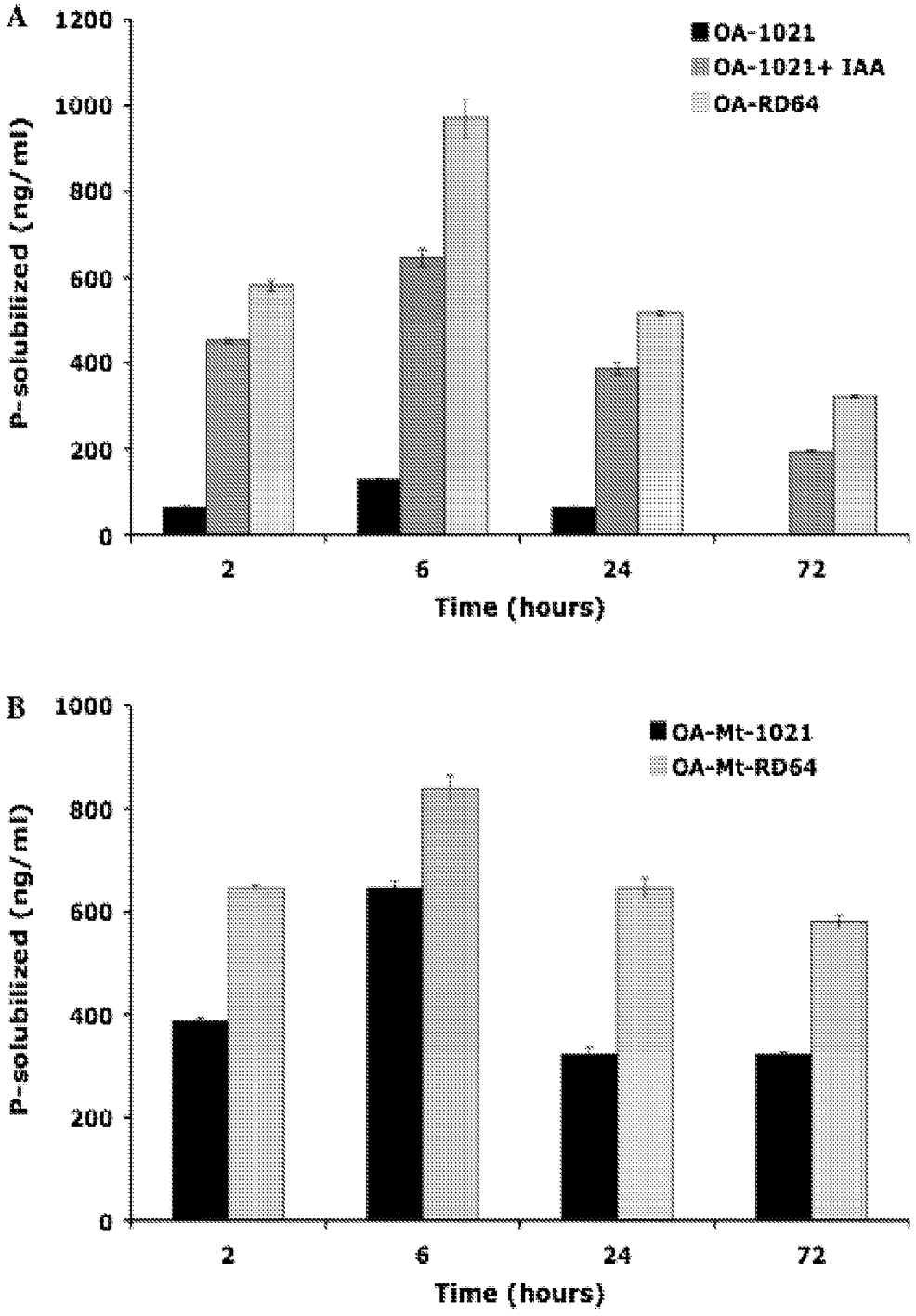
A
1200
1000
800
600
400
200
0
P-solubilized (ng/ml)
OA-1021
OA-1021+ IAA
OA-RD64
2
6
24
72
Time (hours)
B
1000
800
600
400
200
0
P-solubilized (ng/ml)
OA-Mt-1021
OA-Mt-RD64
2
6
24
72
Time (hours)

Fig. 4

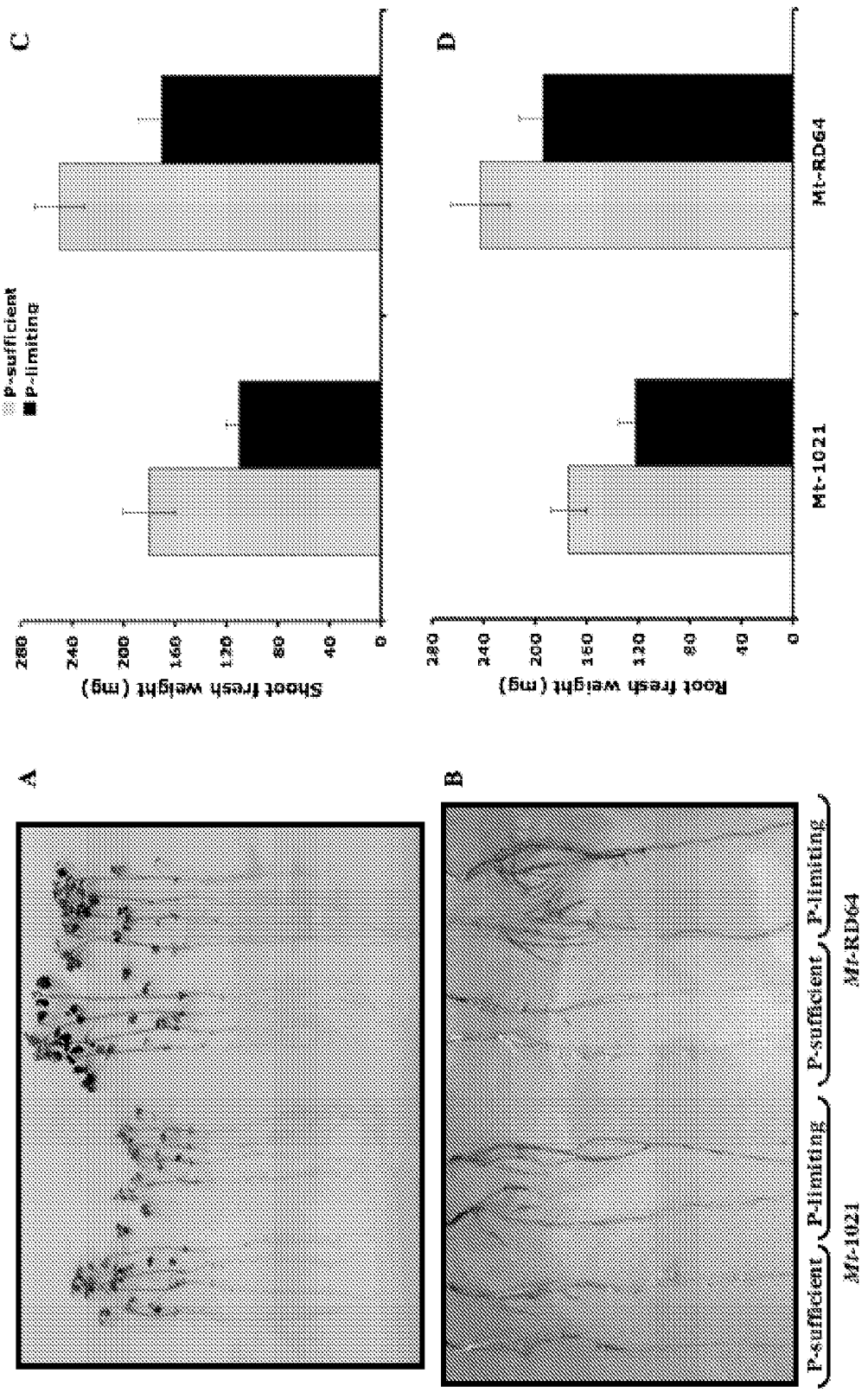
A
B
C
D
P-sufficient
P-limiting
Shoot fresh weight (mg)
Root fresh weight (mg)
0
40
80
120
160
200
240
280
Mt-1021
Mt-RD64
P-sufficient
P-limiting
P-sufficient
P-limiting
Mt-1021
Mt-RD64

Fig. 5

METHOD TO IMPROVE PHOSPHATE SOLUBILIZATION IN PLANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a 371 of PCT/IB2010/051249 filed Mar. 23, 2010, which claims the benefit of Italian Patent Application No. RM2009A000128 filed Mar. 23, 2009, the contents of each of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to the use of a bacterium with elevated levels of indole-3-acetic acid (IAA) for agricultural applications, for instance to improve agriculture yield and to increase the availability of fertilizers for vegetal growth.

BACKGROUND OF THE INVENTION

Nitrogen (N) and phosphorus (P) are the most limiting factors for plant growth. Some microorganisms improve the uptake and availability of N and P minimizing chemical fertilizers dependence.

Compared with the other major nutrients, such as nitrogen, phosphorus (P) is by far the least mobile and available to plants in most soil conditions. Although P is abundant in soils in both organic and inorganic forms, it is frequently a major or even the prime limiting factor for plant growth. Many soils throughout the world are P-deficient, because the free concentration (the form available to the plant), even in fertile soils, is generally low due to high reactivity of soluble P with calcium, iron, or aluminium that leads to P precipitation (36, 41). In addition, in developing countries chemical fertilizers, which provide the three major plant nutrients (N, P and potassium) are not widely used due to cost limitations. In these areas the direct application of ground Phosphate Rock (PR) is increasingly used, even if the P released from PR is often too low for crop growth (9, 38). It is known that many microorganisms, in particular of the genera *Pseudomonas, Bacillus* and *Rhizobium*, have the ability to change their metabolism in response to the phosphorus available for cellular growth. The switch in metabolism is mediated through the repression and induction of various genes whose products are involved in processes ranging from the uptake and acquisition of P sources to de novo synthesis of new cellular components (36, 18). Furthermore, in vitro studies showed that for some of these bacteria both the P-solubilizing activity and the production of the auxin indole-3-acetic acid (IAA) (39, 17) were observed, despite a direct correlation linking IAA production to P-solubilization was not demonstrated.

P uptake has been investigated in various microorganisms. Many bacteria, including *S. meliloti*, have at least two P transport systems, consistent with the high- and low-affinity transport systems. The high-affinity system is encoded by the phoCDET operon, and the low-affinity system is encoded by pit (in the orfA-pit operon). In *S. meliloti* the expression of genes encoding for both P transport systems is controlled by the PhoB activator. Under P-excess conditions, PhoB is inactive, and the phoCDET are not expressed. Under P-limiting conditions, the low-affinity Pit permease system is repressed by activated PhoB, while the high-affinity PhoCDET system is induced and becomes the primary mechanism of P transport (10). Many bacterial strains contain products pstSCAB homologs that function as high-affinity phosphate transporters. For *S. meliloti* 1021 a 1-bp deletion in the pstC ORF is probably responsible (via PhoB) for the moderate constitutive

activation of 12 phosphate-starvation inducible genes, observed in the absence of phosphate stress (24, 43). In both plants and microorganisms, the primary mechanisms of PR solubilization are H^+ excretion, organic acids production and acid phosphatase biosynthesis (2, 3). Organic acids, including acetate, lactate, malate, oxalate, succinate, citrate, gluconate, ketogluconate, etc. can form complexes with the iron or aluminum in ferric and aluminum phosphates, thus releasing plant-available phosphate into the soil (18, 22). Organic acids may also increase P availability by blocking P absorption sites on soil particles or by forming complexes with cations on soil mineral surface (36).

Mineralization of most organic phosphorus compounds is carried out by means of phosphatase enzymes. The major source of these enzymes in soil is considered to be of microbial origin. In particular, phosphatase activity is substantially increased in the rhizosphere. The pH of most soils ranges from acids to neutral values. Thus, acid phosphatases should play the major role in this process (36).

In the present invention, the P-solubilizing ability of a *S. meliloti* 1021 strain, RD64, and its effect on the growth of *Medicago* host plant were analysed.

The author used the *S.meliloti-M. truncatula* system since the microarrays were available for the bacterium and *Medicago* is a well recognized model system for indeterminate nodule development.

The RD64 strain has been previously engineered to overproduce IAA (11, 35), showing that it is able to release into liquid growth media up to 78-fold more IAA compared to wild type 1021 (12, 21). It was also previously reported that, as found for IAA-treated *E. coli* cells (7), RD64 is more resistant to salinity and other abiotic stresses (5). *Medicago* plants nodulated by this strain have a higher degree of protection against oxidative damage induced by salt stress (5). Furthermore, it was previously shown that IAA triggers induction of tricarboxylic acid cycle or citric acid cycle, TCA cycle enzymes in quite distant systems such as transformed human cells (15), *E. coli* (8) and *S. meliloti* (21) with a mechanism not yet understood.

To evaluate the global effects triggered by IAA overproduction in *S. meliloti* RD64, the gene expression pattern of wild type 1021 was compared with that of RD64 and 1021 treated with IAA and other four chemically or functionally related molecules by microarray analysis.

Among the genes differentially expressed in RD64 and IAA-treated 1021 cells, the author found two genes of pho operon. This unexpected finding led them to examine the mechanisms for mineral P solubilization in RD64 and the potential ability of this strain to improve *Medicago* growth under P-starved conditions. P-starved conditions are defined when bacteria, either 1021 or RD64, grow in media containing 1.0 mM K-phosphate. An increase in acid phosphatase activity and organic acids excretion was observed for RD64 strain in free-living conditions. Furthermore, the amount of organic acids exuded from the roots of *Medicago* plants nodulated by this strain was higher than that measured for plants nodulated by the 1021 wild type strain. This effect was connected to the enhanced P solubilization and plant dry weight production observed for these plants.

DESCRIPTION OF THE INVENTION

In the present invention, a strain of *S. meliloti* 1021 was engineered to over-produce the phyto-hormone IAA using a plasmid pG-Promintron-iaaM-tms2, described in WO00/28051 (strain RD64). The skilled person in the art will understand that other strains may be engineered.

It was surprisingly found, in the present invention, that RD64 is highly effective in mobilizing P from insoluble sources such as phosphate rock (PR). Under P-limiting conditions, the higher P-mobilizing activity of RD64, as compared to 1021 wild type strain, is connected with the up-regulation of genes coding for the high-affinity P transport system, the induction of acid phosphatase activity and the increased secretion into the growth media of malic, succinic and fumaric acids. P-limiting conditions relate to a concentration of 5% PR when only bacteria were grow in a defined minimal media, or 0.02% PR when *Medicago* plants nodulated by either 1021 or RD64 were grown in defined minimal media. *Medicago truncatula* plants nodulated by RD64 (Mt-RD64), when grown under P limiting conditions, released higher amounts of another P-solubilizing organic acid, the 2-hydroxyglutaric acid, as compared to the plants nodulated by the wild-type strain (Mt-1021).

It has already been shown that Mt-RD64 plants exhibited a higher dry weight production as compared to Mt-1021 plants. Here the author reports that also P-limiting Mt-RD64 plants show a significant increase both in shoot and root fresh weight when compared to P-limiting Mt-1021 plants.

The author discusses how, in a *rhizobium*-legume model system, a balanced interplay of different factors linked to the bacterial IAA over-production rather than IAA production per se stimulates plant growth under stressful environmental conditions, and in particular, under P-limitation.

Thus, a soil bacterium such as RD64, able to provide solubilised P for plant growth is particularly advantageous to improve agricultural yield, in particular in tropical areas such as sub-Saharan areas where the use of chemical fertilizers is limited and where large sources of PR are available.

It is therefore an object of the present invention the use of a bacterium having an high indole-3-acetic acid (IAA) content for solubilizing phosphate rock (PR) in the ground, wherein said bacterium is obtained by transformation with a gene encoding an agent able to increase the IAA content.

Preferably said agent able to increase the IAA content is either an indolacetamide hydrolase (iaaM) or tryptophane monoxygenase (tms2) enzyme.

In a preferred embodiment the bacterium belongs to the genus *Rhizobium*.

Preferably said bacterium of the genus *Rhizobium* is of species *S. meliloti*.

Still preferably said bacterium is able to produce the indole-3-acetic acid (IAA) phytohormone.

Yet preferably, said bacterium is contained within leguminous plant nodules.

It is a further object of the invention a method to provide solubilized phosphorus to a plant able to nodulate and/or to the soil surrounding the growth of said plant comprising inducing the nodulation of said plant with a bacterium having an high indole-3-acetic acid (IAA) content, wherein said bacterium is obtained by transformation with a gene encoding an agent able to increase the IAA content.

Preferably said agent able to increase the IAA content is either an indolacetamide hydrolase (iaaM) or tryptophane monoxygenase (tms2) enzyme.

Preferably said bacterium belongs to the genus *Rhizobium*.

Yet preferably, said bacterium of the genus *Rhizobium* is of species *S. meliloti*.

Still preferably said bacterium is able to produce the indole-3-acetic acid (IAA) phytohormone.

Preferably said bacterium is contained within leguminous plant nodules.

The invention will be now illustrated by means of non limiting examples referring to the following figures.

FIG. S1. Quantitative RT-PCR analysis of pho operon genes in *S. meliloti* cells under P-sufficient conditions (13 mM K-phosphate). The relative expression level was >1 for genes more highly expressed in RD64 and in 1021 cells treated for 3 hours with 0.5 mM IAA, Ind, Trp, ICA and 2,4-D. The relative expression level was <1 for genes more highly expressed in 1021 cells (control). Error bars represent the standard deviation from three independent biological experiments.

FIG. **1**. Quantitative RT-PCR analysis of pho operon genes expression in *S. meliloti* cells under P-starving conditions. The relative expression level was >1 for genes more highly expressed in RD64 and in 1021 cells treated for 3 hours with 0.5 mM IAA, Ind, Trp, ICA and 2,4-D. The relative expression level was <1 for genes more highly expressed in 1021 cells (control). Error bars represent the standard deviation from five independent biological experiments ($p<0.05$).

FIG. **2**. Acid (A) and alkaline (B) phosphatase activity in *S. meliloti* cells under P-starved conditions. Log-phase cells grown in a MOPS-buffered minimal medium (starting P concentration=13 mM) were washed and then resuspended in the same medium containing no added P (1021 and RD64 strains) and 0.5 mM IAA, Trp or Ind (1021 strain). Treatments were performed for 3 hours at 30° C. Values are the means±SD of four independent biological experiments ($p<0.05$).

FIG. **3**. Soluble phosphate release into *S. meliloti* cultures containing 5% PR as P source. Data are the mean±SD of four independent biological experiments ($p<0.006$).

FIG. S**2**. HPLC chromatograms at 210 nm of organic acids in (A) bacterial supernatants and (B) root exudates samples. The arrows point to peaks identified by GC-MS. The numbers correspond to the following acids: (1) malic, (2) succinic, (3) fumaric and (4) 2-hydroxyglutaric.

FIG. **4**. Changes in soluble phosphate release into the bacterial free medium containing 5% phosphate rock (PR) as P source (P-limiting conditions for bacteria growing in a minimal media with PR). The organic acids (A) fumaric, malic and succinic were added to levels that simulate the amount released by 1021 (OA-1021), 1021 treated with 0.5 mM IAA (OA-1021+IAA) and RD64 cells (OA-RD64) into the growth media. The 2-hydroxyglutaric acid (B) was added to level that simulate the amount released by Mt-1021 (OA-Mt-1021) and Mt-RD64 (OA-Mt-RD64) plants into the growth media. The amounts of each added organic acids derived from the data obtained in HPLC analysis and were reported in the Material and Methods section. Data are the mean±SD of five independent biological experiments ($p<0.006$).

FIG. **5**. Effect of bacterial IAA over-expression on *Medicago truncatula* growth. (A) Phenotype of plants grown for 4 weeks under P-limiting (0.02% PR) and P-sufficient conditions (more than 8 mM K-phosphate). (B) Roots phenotype of plants grown for 1 week as described in (A). (C) Shoot fresh weight, (D) root fresh weight of plants grown as described in (A). Data are the mean±SD ($n=30$, $p<0.001$).

MATERIALS AND METHODS

Bacterial Growth Conditions

The *S. meliloti* wild type 1021 strain and the IAA-overproducing RD64 strain containing the p-iaaMtms2 construct were previously described (12, 21). Standard mannitol minimal medium for *rhizobium* (RMM) (19) was modified to contain 1% (w/v) mannitol as carbon source, 1% (w/v) ammonium chloride, 10 mM morpholine propanesulfonic acid (MOPS; pH 7.0) to buffer and P (KH_2PO_4) added to a final concentration of 1 mM (P-starvation) and 13 mM (P-sufficient). Antibiotics were included as required (5).

P-Depletion

For P starvation experiments, cells of 1021 wild-type and RD64 strains were grown at 30° C. to mid exponential phase (OD_{600}=0.6) in RMM broth containing 1% (w/v) mannitol as carbon source and 13 mM P, washed with RMM containing 0 P, resuspended in the same medium and then divided into three cultures. No P (−P), 1.0 mM P (P-starved cells) or 13 mM P (+P cells also named P-sufficient cells) was added into the three cultures, respectively. The P-starved and P-sufficient 1021 wild type cells were treated for 3.0 hours with 0.5 mM IAA. To test the specificity of IAA-effects, other four selected compounds [indole (Ind), tryptophan (Trp), indole-3-carboxylic acid (ICA) and 2,4-dichlorophenoxyacetic acid (2,4-D)], whose acidity covers a range that goes from the acid (pH 2.9) to the weak acid (pH 6.1), were dissolved in 50% (w/v) ethanol and added to P-starved and P-sufficient 1021 wild type cells to a final concentration of 0.5 mM. The newly introduced IAA biosynthetic pathway use Trp to produce IAA, thus opening the question of whether the RD64 cells are Trp starved. Indeed, as the authors introduced in the bacteria two new genes that convert Trp into IAA, the two genes might be very efficient so that as soon as a molecule of Trp is around they convert it into IAA and there is no Trp to be included into the protein synthesis. To rule out that this bacteria could be partially starved for Trp, RD64 cells were also treated with 0.5 mM Trp and used for microarrays and RT-PCR analyses. Finally, to avoid solvent interference, control cells were treated with similar amount of ethanol solution. After 3 hours of each treatment, cell batches were collected, freezed and stored at −80° C. for use in experiments.

For phosphate solubilization experiments 5% Moroccan phosphate rock (PR) (Sigma-Aldrich, cat. No. 32) was used as P source. When 5% PR is used as P source to allow bacterial growth this is described as a P-limiting condition. At least five independent experiments were always performed.

Microarray Analysis

Previously described methods were used to compare the gene expression patterns of 1021 untreated cells (control) with those of RD64, 1021+IAA, 1021+Ind, 1021+Trp, 1021+ICA and 1021+2,4-D cells grown under P-sufficient (13 mM) conditions as reported in Imperlini et al. (21).

RT-PCR Analysis

Total RNA from P-sufficient and P-starved cells was isolated as previously described (5). cDNA were synthesized with the StrataScript™ reverse transcription reagents (Stratagene) and random hexamers as primers. Quantitative PCR was performed with the Power SYBR PCR Master Mix (Applied Biosystems). Reactions were run on the iCycler iQ (Bio-Rad). The thermo cycling condition were: 15 min at 95° C., 40 cycles of denaturation at 95° C. for 20 s, annealing (20 s) and extension (35 s) at 72° C. Specific primer pairs, designed using the Primer3 software, are shown.

phoB:
(SEQ ID N 1)
5'-TTACGTCGTCAAGCCCTTCT-3'
and
(SEQ ID N 2)
5'-CCGGTGAGGACATGAGAAAT-3';

phoC:
(SEQ ID N 3)
5'-ACTCCTGCGCATGATAAACC-3'
and
(SEQ ID N 4)
5'-TGTTGAGGACGCTCAGTACG-3';

-continued

phoD:
(SEQ ID N 5)
5'-TATCTCGTTCCCCTCGTCAC-3'
and
(SEQ ID N 6)
5'-ACCTTTGTCGACCATCTTGC-3';

phoE:
(SEQ ID N 7)
5'-GCTTCATCCTGTGCTTCCTC-3'
and
(SEQ ID N 8)
5'-AGACCTTCCTCCGGTTTCAT-3';

phoT:
(SEQ ID N 9)
5'-TGGCGTCGTTCTTTACATGA-3'
and
(SEQ ID N 10)
5'-GTCTCCTTTTCGAGCGTGAC-3';

smc02641:
(SEQ ID N 11)
5'-CGAGAGGTGATGACGGAAGT-3'
and
(SEQ ID N 12)
5'-ACCGACTTTCTCGCACAGAT-3';

smc00128:
(SEQ ID N 13)
5'-CTTCAGCATGAACGACCAGA-3'
and
(SEQ ID N 14)
5'-AAGAACCGCGTAACCTTCCT-3'.

smc02641 and smc00128 were used as housekeeping genes for data normalization in the comparative Ct method as previously described (8).

Phosphatase Activity

Alkaline and acid phophatase enzymes under P-limiting conditions were assayed as previously reported (16). Units are reported as nanomoles per minute per milligram of protein. Protein concentrations were determined by the Bradford's assay.

Phosphate Solubilization

The concentration of soluble phosphate was estimated using a modification of Fiske and Subarrow method as described by Saheki et al. (37).

Plant Growth Conditions

Seeds of *Medicago truncatula* cv Jemalong 2HA were surface sterilized, germinated and transferred into hydroponic units as previously reported (5). P-limiting conditions were achieved by providing a modified Jensen medium containing $CaCO_3$ 1 mM and KCl 1.1 mM instead of 7.3 mM $CaHPO_4$ and 1.1 mM K_2HPO_4, respectively. These plants received, only on the first week, 0.02% PR. For collection of exudates, the roots of four weeks old plants were washed, submerged in sterile water and kept in a growth chamber for 48 h. Exudates were evaporated to dryness and analysed by HPLC. The identity of peaks was confirmed by GC-MS.

Organic Acids and Phosphate Release

Based on the results obtained in the analysis of organic acids production in cultures supernatant, malic (MA), succinic (SU), fumaric (FU) and 2-hydroxyglutaric (2HG) acids were added in bacterial free medium, and soluble phosphate concentration was measured. For 1021 growth simulating conditions, 1.4 mg/l FU, 500 mg/l MA and 1 g/l SU were added. For 1021+IAA growth simulating conditions, 16 mg/l

FU, 860 mg/l MA and 860 mg/l SU were added. For RD64 growth simulating conditions, 5.6 mg/l FU, 840 mg/l MA and 3.1 g/l SU were added. For Mt-1021 and Mt-RD64 growth simulating conditions, 2HG was added at final concentrations of 49.6 mg/l and 115.2 mg/l, respectively. Bacterial free medium was also treated with 0.5 mM IAA solution. A media that simulates growth is a media without bacteria or their supernatant. The media comprises only RP and the organic acids used as purified powder commercially available (SIGMA), the organic acids concentration used are that produced by the bacteria when grown in a defined media (with IAA, or without, or from RFD64).

Measurement of Organic Acids Using HPLC

The organic acids were determined by HPLC with a reverse-phase Hypersil GOLD C18 (100×4.6 mm) column (Thermo Electron Corporation). The operating conditions and quantification were previously described (20).

GC-MS Analysis

Organic acid fractions collected from HPLC were dried, derivatized to their tert-butyldimethylsilyl (tBDMS) derivatives and analysed on a Micromass GCT mass spectrometer (Waters corp, Manchester, UK) coupled to an Agilent 6890 Series gas chromatograph fitted with 7683 auto-sampler (Agilent Technologies, Palo Alto, Calif.) and ZB-5 ms (Phenomenex, Macclesfield, UK) caplillary column (30 m×0.25 mm I.D.×0.25 μm d.f. with 5 m Guardian). Samples were injected using splitless injection technique at 250° C. and a helium gas flow of 2.0 ml min^{-1}. The oven was set at 70° C. for 2 min, then ramped at 7° C. min^{-1} to 350° C. and held for 5 min. The GC interface and source temperatures were set to 250° C. and EI^+ mass spectra were acquired at 70 eV from 0 to 47 min with an acquisition rate of 1 spectra/sec. Chromatographic peaks were identified either from existing mass spectral and retention time data from standards previously analysed at Rothamsted Research LTD (Harpenden Herts, UK) or from the NIST mass spectral database in conjunction with retention data obtained from the literature (30). Determination of the accurate mass, to within 5 ppm, of M^+, $M\text{-}15^+$, $M\text{-}57^+$ was used to verify analyte identifications. The chromatograms obtained for each sample were compared to the derivatization reagent blank.

Data Analysis

Data were subjected to statistical evaluation using one-way analysis of variance (ANOVA) and Tukey's multiple comparison Test.

Results

Regulation of pho Operon Genes.

The author have evaluated, under P-sufficient (13 mM) conditions, the global effects triggered by IAA overproduction in *S. meliloti* cells using a transcriptional profiling approach. The author compared the gene expression patterns of wild type 1021 with those of RD64 and 1021 treated with IAA (1021+IAA). To verify the specificity of IAA effects, the author also compared the expression patterns of 1021 untreated cells with those of four chemically or functionally similar molecules such as indole (1021+Ind), tryptophan (1021+Trp), indole-3-carboxylic acid (1021+ICA) and 2,4-dichlorophenoxyacetic acid (1021+2,4-D) (42) (Table S1 to S6).

TABLE S1

S. meliloti 1021 genes whose relative expression level increases or decreases after treatment with 0.5 mM IAA.

Gene ID	Gene name	Description	M[a]	P Value
SMc01169	ald	PROBABLE ALANINE DEHYDROGENASE OXIDOREDUCTASE PROTEIN	3.024845281	0.000556412
SMc02514	smc02514	PUTATIVE PERIPLASMIC BINDING ABC TRANSPORTER PROTEIN	1.763677824	0.021591241
SMb20893	gguB	probable sugar uptake ABC transporter permease protein	1.753795629	0.000556412
SMb20922	smb20922	HYPOTHETICAL PROTEIN	1.711383754	0.000222661
SMc04087	smc04087	PUTATIVE TRANSMEMBRANE PROTEIN	1.592749709	0.055113061
SMb20895	chvE	probable sugar uptake ABC transporter periplasmic solute binding protein precursor	1.424536893	0.002763939
SMb21183	htpG	probable chaperonine heat shock hsp90 proteins family	1.383579163	0.009776774
SMa1118	hspC2	probable HspC2 heat shock protein	1.295763868	0.054729049
SMc01103	rbsK	PROBABLE RIBOKINASE PROTEIN	1.199865871	0.062047659
SMc03168	smc03168	PUTATIVE MULTIDRUG EFFLUX SYSTEM PROTEIN	1.164524891	0.032740196
SMc03168	smc03168	PUTATIVE MULTIDRUG EFFLUX SYSTEM PROTEIN	1.164524891	0.032740196
SMb21197	oppB	putative oligopeptide uptake ABC transporter permease protein	1.111217702	0.03441727
SMc02786	smc02786	PUTATIVE TRANSLOCASE TRANSMEMBRANE PROTEIN	1.109949597	0.027211213
SMc02729	smc02729	HYPOTHETICAL TRANSMEMBRANE PROTEIN	0.894214516	0.077655861
SMc02475	smc02475	PUTATIVE OUTER MEMBRANE LIPOPROTEIN PRECURSOR	0.733925161	0.054292477
SMc01628	smc01628	PUTATIVE PERIPLASMIC BINDING ABC TRANSPORTER PROTEIN	0.712282328	0.060800942
SMc00364	rplT	PROBABLE 50S RIBOSOMAL PROTEIN L20	−0.714986207	0.026564529
SMc01309	rplC	PROBABLE 50S RIBOSOMAL PROTEIN L3	−0.724654269	0.055113061
SMc00568	rpsF	PUTATIVE 30S RIBOSOMAL PROTEIN S6	−0.761629923	0.060779136
SMb21177	phoC	phosphate uptake ABC transporter ATP binding protein	−0.79391639	0.025150574
SMc01296	rpsN	PROBABLE 30S RIBOSOMAL PROTEIN S14	−0.832571686	0.030646137
SMc01295	rpsH	PROBABLE 30S RIBOSOMAL PROTEIN S8	−0.899568721	0.007947725
SMc00363	rpmI	PROBABLE 50S RIBOSOMAL PROTEIN L35	−0.980671144	0.022316759
SMb21174	phoT	phosphate uptake ABC transporter permease protein	−1.206701338	0.008998087
SMc01291	rpmD	PROBABLE 50S RIBOSOMAL PROTEIN L30	−1.237159143	0.019628185
SMc01301	rpmC	PROBABLE 50S RIBOSOMAL PROTEIN L29	−1.272963317	0.007455448
SMc01319	rplJ	PROBABLE 50S RIBOSOMAL PROTEIN L10 (L8)	−1.438053855	0.011918941
Mc01302	rplP	PROBABLE 50S RIBOSOMAL PROTEIN L16	−1.862233128	0.009776774

Gene ID, name and description are as reported at http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/

[a]The M value refers to the $\log_2$ of the ratio of intensities of each spots in the two channels (21).

TABLE S2

S. meliloti 1021 genes whose relative expression level increases or decreases after treatment with 0.5 mM Indole.

Gene ID	Gene name	Description	M[a]	P Value
SMc04040	ibpA	PROBABLE HEAT SHOCK PROTEIN	2.4491133	4.02E−06
SMc03253	smc03253	PUTATIVE L PROLINE 3 HYDROXYLASE PROTEIN	1.9505191	2.94E−06
SMc04087	smc04087	PUTATIVE TRANSMEMBRANE PROTEIN	1.9437362	2.61E−05
SMc04141	gst9	PUTATIVE GLUTATHIONE S TRANSFERASE PROTEIN	1.7081209	7.08E−06
SMa1329	sma1329	Putative proline dipeptidase	1.6577909	4.32E−06
SMc01106	smc01106	PROBABLE SMALL HEAT SHOCK PROTEIN	1.51285	2.13E−06
SMc04140	smc04140	PUTATIVE ATP BINDING ABC TRANSPORTER PROTEIN	1.4108408	0.00363899
SMb21177	phoC	phosphate uptake ABC transporter ATP binding protein	1.282009	8.09E−07
SMa1128	degP4	DegP4 protease like protein	1.15192	4.02E−06
SMb21183	htpG	probable chaperonine heat shock hsp90 proteins family	0.9544155	3.16E−05
SMc01312	fusA1	PROBABLE ELONGATION FACTOR G PROTEIN	0.912809	0.00022133
SMc03829	smc03829	PUTATIVE TRANSPORT SYSTEM PERMEASE ABC TRANSPORTER PROTEIN	0.8604338	0.00071899
SMc03167	smc03167	PUTATIVE MULTIDRUG EFFLUX SYSTEM PROTEIN	0.7656327	0.00022133
SMa1077	nex18	Nex18 Symbiotically induced conserved protein	0.7412711	0.00029644
SMc02857	dnaK	HEAT SHOCK PROTEIN 70 (HSP70) CHAPERONE	0.7321448	0.00024744
SMa1087	sma1087	Putative cation transport P type ATPase	0.7198729	0.00707161
SMc01318	rplL	PROBABLE 50S RIBOSOMAL PROTEIN L7/L12 (L8)	−0.703652	0.00024113
SMc00335	rpsA	30S RIBOSOMAL PROTEIN S1	−0.708311	0.00076724
SMc01311	tufA	PROBABLE ELONGATION FACTOR TU PROTEIN	−0.711049	1.03E−05
SMc00383	gst3	PUTATIVE GLUTATHIONE S TRANSFERASE PROTEIN	−0.747804	0.00180021
SMc03159	smc03159	PUTATIVE ATP BINDING ABC TRANSPORTER PROTEIN	−0.938019	0.00040829
SMc00182	smc00182	PUTATIVE TRANSCRIPTION REGULATOR PROTEIN	−0.942917	1.96E−05
SMc01946	livK	PUTATIVE LEUCINE SPECIFIC BINDING PROTEIN PRECURSOR	−1.076561	7.38E−05
SMc01308	rplD	PROBABLE 50S RIBOSOMAL PROTEIN L4	−1.252414	9.97E−06
SMc00364	rplT	PROBABLE 50S RIBOSOMAL PROTEIN L20	−1.373283	4.18E−05
SMc02501	atpD	PROBABLE ATP SYNTHASE BETA CHAIN PROTEIN	−1.40503	8.30E−07
SMc01291	rpmD	PROBABLE 50S RIBOSOMAL PROTEIN L30	−1.484709	1.03E−05
SMc04114	pilA1	PUTATIVE PILIN SUBUNIT PROTEIN	−1.887557	2.94E−06

Gene ID, name and description are as reported at http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/

[a]TheM value refers to the $\log_2$ of the ratio of intensities of each spots in the two channels (21).

TABLE S3

S. meliloti 1021 genes whose relative expression level increases or decreases after treatment with 0.5 mM Tryptophan.

Gene ID	Gene name	Description	M[a]	P value
SMa1321	virB1	virB1 type IV secretion protein	4.444843574	5.76E−10
SMc03253	smc03253	PUTATIVE L PROLINE 3 HYDROXYLASE PROTEIN	2.688816083	1.21E−07
SMc04140	smc04140	PUTATIVE ATP BINDING ABC TRANSPORTER PROTEIN	2.032282837	0.000658944
SMb20672	smb20672	putative sugar uptake ABC transporter permease protein	1.815183664	0.000464709
SMc04141	gst9	PUTATIVE GLUTATHIONE S TRANSFERASE PROTEIN	1.580678927	0.002877464
SMa1128	degP4	DegP4 protease like protein	1.449385608	0.002545682
SMc02507	sitC	PUTATIVE IRON TRANSPORT SYSTEM MEMBRANE ABC TRANSPORTER PROTEIN	1.132611055	0.005755651
SMb20585	ggt	putative gamma glutamyltranspeptidase protein	1.089676324	0.018044699
SMa2189	sma2189	putative integrase/recombinase	0.993022383	0.005634243
SMa1007	sma1007	Copper protein putative	0.980054843	0.031998986
SMa2145	sma2145	probable aminomethyltransferase	0.914452315	0.022642532
SMa0707	sma0707	dihydrodipicolinate synthase putative	0.908418132	0.046709835
SMa0711	sma0711	putative ABC transporter permease protein MalFG family	0.886253547	0.041859671
SMa1118	hspC2	probable HspC2 heat shock protein	0.866291958	0.008200888
SMc04147	smc04147	PUTATIVE PERMEASE PROTEIN	0.826220763	0.027089558
SMa1087	sma1087	Putative cation transport P type ATPase	0.751037358	0.048246323
SMa1073	sma1073	TRm23b IS ATP binding protein	0.742254734	0.025726092
SMc01169	ald	PROBABLE ALANINE DEHYDROGENASE OXIDOREDUCTASE PROTEIN	0.737931247	0.023749056
SMc01311	tufA	PROBABLE ELONGATION FACTOR TU PROTEIN	−0.722897465	0.001153449
SMc01314	rpsL	PROBABLE 30S RIBOSOMAL PROTEIN S12	−0.734864111	0.016575446
SMc01313	rpsG	PROBABLE 30S RIBOSOMAL PROTEIN S7	−0.751779496	0.01095587
SMc01293	rplR 3	PROBABLE 50S RIBOSOMAL PROTEIN L18	−0.763652168	0.012690901
SMc04434	rpmH	PROBABLE 50S RIBOSOMAL PROTEIN L34	−0.775772779	0.003459468
SMc01636	smc01636	PUTATIVE TRANSCRIPTION REGULATOR PROTEIN	−0.94728054	0.003589821
SMb21566	groEL5	putative heat shock protein groEL	−1.020953539	0.00012659
SMc00182	smc00182	PUTATIVE TRANSCRIPTION REGULATOR PROTEIN	−1.096524292	0.003162499
SMb20984	nirB 4	putative nitrite reductase [NAD(P)H] large subunit protein	−1.109685898	0.000376277
SMc00155	aroF	PROBABLE DAHP SYNTHETASE PROTEIN	−1.56466062	8.11E−05

Gene ID, name and description are as reported at http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/

[a]The M value refers to the $\log_2$ of the ratio of intensities of each spots in the two channels (21).

TABLE S4

S. meliloti 1021 genes whose relative expression level increases or decreases after treatment with 0.5 mM ICA.

Gene ID	Gene name	Description	M[a]	P Value
SMc01169	ald	PROBABLE ALANINE DEHYDROGENASE OXIDOREDUCTASE PROTEIN	1.709603089	0.00835282
SMc02603	smc02603	PUTATIVE TRANSPORT TRANSMEMBRANE PROTEIN	1.484428133	0.01927884
SMc04040	ibpA	PROBABLE HEAT SHOCK PROTEIN	1.473713787	1.97E−05
SMa1128	degP4	DegP4 protease like protein	1.148294032	0.005172893
SMc03253	smc03253	PUTATIVE L PROLINE 3 HYDROXYLASE PROTEIN	1.14006745	0.00220191
SMc04140	smc04140	PUTATIVE ATP BINDING ABC TRANSPORTER PROTEIN	1.126173329	0.027805968
SMa1118	hspC2	probable HspC2 heat shock protein	1.111188207	0.020857534
SMc04307	cyaD2	PUTATIVE ADENYLATE/GUANYLATE CYCLASE TRANSMEMBRANE PROTEIN	1.011985231	0.00097178
SMc03168	smc03168	PUTATIVE MULTIDRUG EFFLUX SYSTEM PROTEIN	1.005018738	0.007398989
SMa1077	nex18	Nex18 Symbiotically induced conserved protein	0.993757572	0.00097178
SMc00537	smc00537	PUTATIVE TRANSPORT PROTEIN	0.958342276	0.045926511
SMa1306	virB9	VirB9 type IV secretion protein	0.940431647	0.029383575
SMc01534	smc01534	PUTATIVE OMEGA AMINO ACID PYRUVATE AMINOTRANSFERASE PROTEIN	0.927685887	0.007398989
SMc03829	smc03829	PUTATIVE TRANSPORT SYSTEM PERMEASE ABC TRANSPORTER PROTEIN	0.906503799	0.010242834
SMc00514	smc00514	PUTATIVE MONOOXYGENASE PROTEIN	0.872057618	0.028207167
SMb21183	htpG	probable chaperonine heat shock hsp90 proteins family	0.7619012	0.030552462
SMc03037	flaA	FLAGELLIN A PROTEIN	−0.71144865	0.008867601
SMc00323	rpsO	PROBABLE 30S RIBOSOMAL PROTEIN S15	−0.72195502	0.008965819
SMc04114	pilA1	PUTATIVE PILIN SUBUNIT PROTEIN	−0.72744163	0.018953535
SMc02498	atpH	PUTATIVE ATP SYNTHASE DELTA CHAIN PROTEIN	−0.72807496	0.003920621
SMc03030	flgG	FLAGELLAR BASAL BODY ROD PROTEIN	−0.75006865	0.036232645
SMc01309	rplC	PROBABLE 50S RIBOSOMAL PROTEIN L3	−0.75579918	0.008143182
SMc01793	smc01793	PUTATIVE GLYCOSYLTRANSFERASE PROTEIN	−0.76252145	0.005300736
SMc00335	rpsA	30S RIBOSOMAL PROTEIN S1	−0.776312	0.018932734
SMc00868	atpF	PROBABLE ATP SYNTHASE B CHAIN TRANSMEMBRANE PROTEIN	−0.83061618	0.011065196
SMc00913	groEL1	60 KD CHAPERONIN A PROTEIN	−0.83494684	0.039308482
SMc01326	tufB	PROBABLE ELONGATION FACTOR TU PROTEIN	−0.87682266	0.012773579
SMc00912	groES1	10 KD CHAPERONIN A PROTEIN	−1.49142677	0.004572783

Gene ID, name and description are as reported at http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/

[a]The M value refers to the $\log_2$ of the ratio of intensities of each spots in the two channels (21).

TABLE S5

S. meliloti 1021 genes whose relative expression level increases or decreases after treatment with 0.5 m1\4 2,4-D.

Gene ID	Gene name	Description	M[a]	P Value
SMa1077	nex18	Nex18 Symbiotically induced conserved protein	2.58085875	0.03163763
SMa1128	degP4	DegP4 protease like protein	1.55249531	0.01374723
SMb21221	smb21221	putative sugar uptake ABC transporter periplasmic solute binding protein precursor	1.02658325	0.03928731
SMc03805	tesB	PROBABLE ACYL COA THIOESTERASE II PROTEIN	0.99913786	0.05718535
SMc00591	smc00591	HYPOTHETICAL/UNKNOWN SIGNAL PEPTIDE PROTEIN	0.86290789	0.03042775
SMc03859	rpsP	PROBABLE 30S RIBOSOMAL PROTEIN S16	−0.7103949	0.04708837
SMc00335	rpsA	30S RIBOSOMAL PROTEIN S1	−0.7145391	0.04366812
SMc02905	dnaX	PUTATIVE DNA POLYMERASE III SUBUNIT TAU PROTEIN	−0.726406	0.03042775
SMc00363	rpm1	PROBABLE 50S RIBOSOMAL PROTEIN L35	−0.8021142	0.04684434
SMc02717	leuA1	2 ISOPROPYLMALATE SYNTHASE PROTEIN	−0.8087469	0.00997117
SMc02692	rplY	PUTATIVE 50S RIBOSOMAL PROTEIN L25	−0.8486084	0.00716822
SMc00151	gph1	PROBABLE PHOSPHOGLYCOLATE PHOSPHATASE PROTEIN	−0.8712822	0.00723807
SMc01883	smc01883	HYPOTHETICAL TRANSMEMBRANE PROTEIN	−0.8788488	0.03163763
SMc01300	rpsQ	PROBABLE 30S RIBOSOMAL PROTEIN S17	−0.9523795	0.00226024
SMc01291	rpmD	PROBABLE 50S RIBOSOMAL PROTEIN L30	−0.9964348	0.03465032
SMc01214	smc01214	PUTATIVE ZINC CONTAINING ALCOHOL DEHYDROGENASE PROTEIN	−1.0392929	0.03653619
SMc03979	gap	PROBABLE GLYCERALDEHYDE 3 PHOSPHATE DEHYDROGENASE PROTEIN	−1.0652513	0.05688975
SMc01858	smc01858	CONSERVED HYPOTHETICAL PROTEIN	−1.0825725	0.01522216
SMc02498	atpH	PUTATIVE ATP SYNTHASE DELTA CHAIN PROTEIN	−1.2509153	0.01508863
SMc01298	rplX	PROBABLE 50S RIBOSOMAL PROTEIN L24	−1.2601536	0.01930926
SMc00912	groES1	10 KD CHAPERONIN A PROTEIN	−1.2715839	0.04708837
SMc00869	atpF2	PROBABLE ATP SYNTHASE SUBUNIT B' TRANSMEMBRANE PROTEIN	−1.3178757	0.00726489
SMc04003	rpmJ	PROBABLE 50S RIBOSOMAL PROTEIN L36	−1.331155	0.00346374
SMc02340	smc02340	PUTATIVE TRANSCRIPTION REGULATOR PROTEIN	−1.4082815	0.01027773
SMc01299	rplN	PROBABLE 50S RIBOSOMAL PROTEIN L14	−1.5873658	0.00366764
SMc01326	tufB	PROBABLE ELONGATION FACTOR TU PROTEIN	−1.6636138	0.00282898

TABLE S5-continued

S. meliloti 1021 genes whose relative expression level increases or decreases after treatment with 0.5 m1\4 2,4-D.

Gene ID	Gene name	Description	M[a]	P Value
SMc01319	rplJ	PROBABLE 50S RIBOSOMAL PROTEIN L10 (L8)	−1.7881878	1.40E−06
SMc01301	rpmC	PROBABLE 50S RIBOSOMAL PROTEIN L29	−2.0066958	0.00366764

Gene ID, name and description are as reported at http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/

[a]The M value refers to the $\log_2$ of the ratio of intensities of each spots in the two channels (21).

TABLE S6

S. meliloti RD64 genes whose relative expression level increases or decreases as compared to wild-type strain.

Gene ID	Gene name	Description	M[a]	P value
SMc04087	smc04087	Putative transmembrane protein	1.99884592	9.19E−05
SMc01095	mexF1	Probable multidrug efflux system transmembrane protein	1.53758894	1.42E−06
SMc03245	smc03245	Putative amidase protein	1.25311085	0.000236703
SMc01106	smc01106	Probable small heat shock protein	1.18271524	0.000425479
SMc03208	hmgA	Homogentisate 12 dioxygenase protein	1.12512665	2.30E−05
SMc03805	tesB	Probable acyl CoA thioesterase II protein	0.98314209	3.25E-05
SMb21216	smb21216	Putative sugar uptake ABC transporter ATP binding protein	0.89780301	0.043969901
SMb21183	htpG	Probable chaperonine heat shock hsp90 proteins family	0.89755767	0.002792969
SMc02610	glxB	Putative amidotransferase protein	0.85161643	4.73E−06
SMb21295	smb21295	Putative small heat shock protein hsp20 family	0.84263999	3.98E-05
SMb21221	smb21221	Putative sugar uptake ABC transporter periplasmic solute binding protein precursor	0.81363362	0.002008173
SMc04128	smc04128	Putative heavy metal transporting atpase protein	0.78120181	0.001972893
SMc02576	smc02576	Hypothetical acetyltransferase protein	0.72720949	7.11E−05
SMc01326	tufB	Probable elongation factor tu protein	−0.7141322	0.000873039
SMc00868	atpF	Probable atp synthase b chain transmembrane protein	−0.7170929	2.43E-08
SMc00335	rpsA	30S ribosomal protein S1	−0.7502647	0.000335846
SMc00871	atpB	Probable ATP synthase A chain transmembrane protein	−0.8088227	0.000995828
SMb21177	phoC	Phosphate uptake ABC transporter ATP binding protein	−0.8306733	2.95E−06
SMc01309	rplC	Probable 50s ribosomal protein L3	−0.8348891	1.76E−09
SMc00870	atpE	Probable ATP synthase subunit C transmembrane protein	−0.863293	4.11E−05
SMc02498	atpH	Putative ATP synthase delta chain protein	−1.006709	3.98E−05
SMc01301	rpmC	Probable 50S ribosomal protein L29	−1.0467565	4.33E−07
SMc01291	rpmD	Probable 50S ribosomal protein L30	−1.1945951	1.42E−06
SMc01285	rpoA	Probable DNA directed RNA polymerase alpha chain protein	−1.2449298	5.71E−06
SMc01319	rp1J	Probable 50S ribosomal protein L10 (L8)	−1.2628004	2.95E−06
SMc01302	rplP	Probable 50S ribosomal protein L16	−1.3323341	2.94E−06
SMc01804	rplM	Probable 50S ribosomal protein L13	−1.3616399	0.000193529
SMc01830	ureG	Probable urease accessory protein	−1.3746456	1.35E−05

Gene ID, name and description are as reported at http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/

[a]The M value refers to the $\log_2$ of the ratio of intensities of each spots in the two channels (21).

For the genes of the phoCDET operon that code for the high-affinity phosphate uptake system (40) the author found that the phoC was repressed in RD64 as compared to the untreated wild type 1021 cells (Tables S1). The treatment of 1021 with IAA led to a down-regulation of both phoC and phoT genes (Table S6). In contrast, when 1021 cells were treated with Ind, an up-regulation of the phoC gene was observed as compared to the untreated 1021 cells (Table S2). Concerning the addition of Trp, ICA and 2,4-D the author did not find any pho genes among those significantly affected (Tables S3 to S5). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis confirmed these data for RD64, 1021+IAA and 1021+Ind cells (FIG. S1) and moreover, showed that the phoB regulatory gene (4) was down-regulated in RD64 and 1021+IAA cells whereas it was unaffected in 1021+Trp, 1021+ICA and 1021+2,4-D cells, when compared to the untreated 1021 cells.

When Tip was added to RD64 cells, the expression of pho genes was unaffected or slightly reduced as compared to untreated RD64 cells (data not shown). These results suggest that the IAA overproduction in RD64 cells do not lead to Trp starvation.

RT-PCR was, then, employed to analyze the differential expression patterns of pho operon genes under P-starved conditions. The expression level of all five pho genes was highly induced in RD64 and 1021+IAA cells, whereas it was only slightly induced or unchanged in 1021+Ind and 1021+Trp cells when compared to control (FIG. 1).

Phosphatase Enzymes Activity.

Since P limitation in *Rhizobium* can result in the induction of phosphatase enzymes that are directly involved in the mineralization of inorganic phosphorus compound in different types of soils, and in higher P transport rates (1, 28), the transcriptional study was combined with analyses of phosphatase enzyme activity. Phosphate starvation induced an increase in acid phosphatase activity in both RD64 and 1021+IAA cells as compared to 1021 cells (FIG. 2A). Similar results were obtained when alkaline phosphatase was assayed, although the effect was less prominent (FIG. 2B). On the other hand, the activity of the two phosphatase enzymes in 1021+Ind and 1021+Trp cells was unaffected, or only slightly increased compared control (FIG. 2).

Organic Acids Production and P Solubilization.

To evaluate the ability of RD64 strain to solubilize inorganic P when PR is used as the sole P source, the amount of P released into culture media, was measured. The author shows that the soluble P concentration continuously increased for six days, reaching the highest value at day 9 and remained

relatively constant thereafter (FIG. **3**). Interestingly, the P concentration measured in the growth media of RD64 and 1021+IAA was up to 80% higher than that found for control cells. In contrast, the concentration of soluble P was negligible in bacteria free media with or without the addition of IAA.

In order to study the relationship between phosphate solubilization and the production of organic acids, culture supernatants were filtered and analysed by high-pressure liquid chromatography (HPLC). Three of the major peaks (FIG. S**2**A) were identified as malic, succinic and fumaric acids, respectively. The identification of these organic acids was confirmed by gas chromatography-mass spectrometry (GC-MS). The concentration of these acids was higher in both RD64 and 1021+IAA compared to control cells, with the highest increment observed for succinic acid (Table 7).

TABLE 7

Organic acids exuded by *S. meliloti* 1021, 1021 + IAA and RD64 cells grown on minimal medium containing 5% PR as the sole phosphate source.

	Organic acid content (mg/L)		
Sample	Malate	Succinate	Fumarate
1021	50 ± 5	105 ± 7	0.14 ± 0.01
1021 + IAA	86 ± 10	864 ± 79	1.7 ± 0.1
RD64	84 ± 8	311 ± 31	0.56 ± 0.06

The IAA concentration added in 1021+IAA was 0.5 mM. All strains were grown in 1% mannitol RMM media (see Materials and methods section). The values reported in the Table are the averages±standard deviation of at least five independent biological experiments ($p<0.001$).

The author compared the amount of soluble P released into bacteria free medium upon external addition of malic, succinic and fumaric acids. To simulate the growth of *S. meliloti* cells the organic acids were added, into bacterial-free media, at the same relative ratios found in bacterial cultures. The author found that P solubilization was more effective when the levels of added organic acids were comparable to those measured during the growth of RD64 and 1021+IAA cells (FIG. **4**A). However, the P released under these conditions (purified acid addition to the liquid medium) was lower than that released from bacterial cultures.

Mt-1021 and Mt-RD64 Plants Growth.

To evaluate the ability of *S. meliloti* to support plant growth under P-limiting conditions, Mt-1021 and Mt-RD64 plants were grown under low (0.02% PR as P source) and high P conditions, and fresh- and dry-weight were evaluated after four weeks of growth. When P-sufficient (8 mM K-phosphate) conditions were used, a significant improvement of Mt-RD64 plants growth was observed as compared to the Mt-1021 plants. The author show that for these plants an enhanced biomass production of the aerial part and of the whole root apparatus was observed (FIG. **5**).

In P-starved conditions (PR as P source), the author observed a reduction of shoot and root fresh weight in—Mt-1021 plants, while for Mt-RD64 plants the differences was statistically significant only for the shoot fresh weight (FIG. **5**). However, if the author compare P-limiting Mt-RD64 plants with Mt-1021 plants grown under P-sufficient conditions the absolute value of their shoot and root fresh weights was not statistically different (FIGS. **5**C and D).

Under P-limiting conditions Mt-RD64 plants also showed a more highly branched root system with abundant lateral roots, but without significant changes in the primary roots length compared to control (FIG. **5**B).

To assess whether the carboxylates released from the roots of Mt-RD64 plants was related to the ability of these plants to be more effective in the acquisition of P from the sparingly soluble PR, the organic acids exuded from the roots was analysed by HPLC (FIG. S**2**B) and GC-MS.

Mt-1021 and Mt-RD64 plants released the same carboxylates but to different levels. Indeed, Mt-RD64 plants released up to 130% more (1.5 ± 0.1 mg g^{-1} root fresh wt, n=5) 2-hydroxyglutaric acid, which is a derivative of the TCA cycle intermediate 2-ketoglutaric acid, than Mt-1021 plants (0.65 ± 0.10 mg g^{-1} root fresh wt, n=5, $p<0.003$).

The amount of soluble P released into the sterilized medium was also measured upon exogenous addition of different amounts of 2-hydroxyglutaric acid. Interestingly, P solubilization was the highest when the added organic acid level was comparable to that released during Mt-RD64 plant growth (FIG. **4**B).

Discussion

It has been previously shown that RD64 cells: a) release higher amounts of IAA, increase nitrogen fixation and triggers the accumulation of storage compounds as PHB (poly-beta-hydroxybutyrate) and starch (21); b) exhibit improved resistance against stress conditions (5); c) show enhanced long-term cell survival (13).

Moreover, Mt-RD64 plants show an improved root nodules development (21) and are able to attenuate the local IAA imbalance by increasing the transcription of cytokinin signaling genes (6), when compared to Mt-1021 plants. These results reinforce the suggestion that modulating auxin and cytokinin levels is a key step in nodule formation (31-32, 34). Mt-RD64 plants with such properties exhibit higher salt-tolerance (5) as compared to Mt-1021 plants.

The author shows in the present invention that, under P-stress conditions RD64 strain improves mineral phosphate solubilization when compared to the wild type 1021 strain. The expression level of the phoB activator gene and all genes in the phoCDET regulon were induced in RD64 as compared to 1021, thus suggesting that the P transport rate increased via the high-affinity transport system in this strain. Similar results were obtained when 1021 cells were treated with IAA. This effect seems to be specific since the treatment with structurally or functionally similar molecules did not lead to significant alterations in gene expression. Considering that in *S. meliloti* 1021 a moderate level of activated PhoB is present even under phosphate sufficiency, the author thinks that the degree of induction observed for these genes might be far more relevant in other *S. meliloti* strains lacking the pstC1021 mutation.

Moreover, RD64 cells showed higher levels of acid phosphatase enzymes, which facilitate the hydrolysis of organic P esters. Such cells released higher amount of organic acids, known to be highly effective in mobilizing P from insoluble sources (17, 23), when compared to the untreated 1021 cells. Similar results were obtained when 1021 cells were treated with IAA. The author suggests that the excretion of higher amounts of malic, succinic and fumaric acids, three intermediates of citric acid cycle (TCA), observed for RD64 cells is connected to the enhanced activity of TCA cycle enzymes measured for these cells (21).

Under P-starved conditions, a higher biomass accumulation was observed for Mt-RD64 plants as compared to Mt-1021 plants. It is believed that this effect is linked to the release of higher amount of 2-hydroxyglutaric acid exuded from the roots of Mt-RD64 plants and to the modifications of important root architecture traits, such as root branching,

observed for these plants. Indeed, it has been already reported that, the ability of plants to use insoluble P compounds can be significantly enhanced by engineering plants to produce more organic acids (27) and that IAA plays an important role in root system architecture adjustment during P-deprivation in *Arabidopsis* and other plant species (25, 26, 29, 33). In the present invention the author speculate that the TCA-cycle enzymes up-regulation occurring in RD64 cells (21) leads to a higher carboxylates production, resulting in a positive effect both in P-sufficient and P-starved conditions.

Under P-sufficient conditions, the higher carboxylates synthesis might increase the P soluble levels triggering a more efficient repression of the high-affinity P transport system. Indeed, under P-sufficient conditions, microarray analysis surprisingly shows a further down-regulation of pho genes in RD64 as compared to 1021 cells. On the contrary, under-P-starved conditions, carboxylates overproduction might guarantee the availability of minimal soluble P levels necessary to stimulate the induction of pho operon genes.

The enhanced metabolic activity and the correlated production of more carboxylates in RD64 cells might also occur in bacteroids inside root nodules. Carboxylates accumulated inside nodules of Mt-RD64 plants might be exuded from the root into the rhizosphere (as the author really found for the 2-hydroxyglutaric acid, a derivative of the TCA cycle intermediate 2-ketoglutaric acid) increasing the availability of P to plants.

The present invention demonstrates that both free-living *rhizobia* and nodulated plants with such properties are able to better overcome different stressful environmental conditions, including P-starvation. It is then believed that these abilities might be transferred into *rhizobia* nodulating legume (as *vigna* or chickpea) in order to increase plant yield. Such an application would be particularly interesting in regions where high salinity is a substantial constraint to crop production, PR deposits are widespread, but soluble P is too low, and the use of chemical N fertilizers, which strongly inhibited the symbiotic relationship between *Rhizobia* and legumes, is limited due to their cost. Finally, the combination of higher IAA release, P-solubilization and improved N-fixation could make the Mt-RD64 system a good candidate for legume-cereal intercropping.

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The invention claimed is:

1. A method to provide solubilized phosphorus to a plant able to nodulate and/or to a soil surrounding a growth of said plant comprising:

introducing a bacterium and a phosphate rock into said soil containing said plant; and

inducing the nodulation of said plant with said bacterium,

wherein said bacterium is *Sinorhizobium meliloti* RD64 strain (*S. meliloti* transformed with *iaaM* gene from *Pseudomonas syringae* pv. *savastanoi* and the *tms*2 gene from *Agrobacterium tumefaciens*), and

wherein said RD64 strain has a high indole-3-acetic acid (IAA) content.

2. The method according to claim 1 wherein said bacterium is able to produce the indole-3-acetic acid (IAA) phytohormone.

3. The method according to claim 1 wherein said bacterium is contained within leguminous plant nodules.

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