Appendices

Appendix I

Table A1 Table A2 Biochemical test analysis and Gram nature of diazotrophic endophytic bacteria

Sugar Fermentation	M1	M3	M4	M4J	M5	M6	MS1	MS3	W1	W3	WS1	WS4	WS5	S1	SS1	SS2	SS4	SS5	SS8
Glucose	+	-	+	+	+	-	+	+	+	-	-	+	-	+	-	-	+	+	+
Galactose	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylose		-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-
Meso-erythritol	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	+	+	-	-	-	+	+	-	-	+	-	+	-	-	-	-	-
Sucrose	+	-	+	+	-	-	+	+	+	-	-	+	-	+	-	-	-	-	-
Fructose	+	-	+	+	-	-	+	+	+	-	-	+	-	+	-	-	-	-	-
Mannitol	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Characteristics																			
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Hugh leifson Oxidase test	$+^a$	$+^b$	$+^a$	$+^a$	$+^a$	$+^b$	$+^b$	$+^a$	$+^a$	$+^a$	$+^b$	-	$+^b$	$+^a$	$+^a$	$+^b$	-	$+^a$	$+^a$
Methyl Red (MR)	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-
Voges Proskaver (VP)	+	-	+	+	-	-	+	+	+	-	-	+	-	+	-	-	-	-	-
Indole test	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Gram's nature	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve

Sugar Fermentation	R1	R2	R3	RS1	RS2	RS3	RS4	RS5	RS8	P1	PS1	PS3
Glucose	+	+	+	+	-	+	+	-	-	+	+	+
Galactose	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-
Meso-erythritol	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	-	-	+	+	+
Sucrose	+	+	-	-	-	+	-	-	-	+	+	+
Fructose	+	+	+	+	+	+	+	-	-	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-	-	+	-
Characteristics												
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	-	+	+	+	+	+	+	+	+	+
Hugh leifson Oxidase test	+[a]	+[a]	+[a]	+[a]	+[b]	+[a]	+[a]	+[b]	+[b]	+[a]	+[b]	+[b]
Methyl Red (MR)	+	+	+	+	-	+	+	-	-	-	+	+
Voges Proskaver (VP)	+	+	+	+	-	+	+(faint)	-	-	+		+
Indole test	-	+	-	-	-	-	-	-	+	-	-	-
Gram's nature	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve

[a] Fermentative, [b] Oxidative

Table A2 Plant growth promoting traits in diazotrophic endophytic bacterial isolates from *Poaceae* plants

Bacterial Isolate[a]	**PSA**[b]	**IAA(µg/ml)**	**Siderophore (µg/ml) (Type)**[c]	**Enzyme activity (µmole min^{-1}ml^{-1})**				**AFA**[d]
				Cellulase	Pectinase	Protease	Chitinase	
***Ralstonia* sp. M1**	-	362±2.3	0.52±0.03 (H)	-	0.255±0.05	-	-	-
***Arthrobacter* sp. M3**	-	48.13±4.2	0.77±0.03(M)	0.02±0.01	1.270±0.34	0.07±0.0	0.01±0.00	-
***Staphylococcus* sp. M4**	+	323±3.2	0.84±0.00(M)	0.02±0.00	1.245±0.23	-	0.01±0.00	-
***Staphylococcus* sp. M4J**	+	668±7.8	0.20±0.00(H)	-	1.906±0.47	-	UD	-
***Acinetobacter* sp. M5**	+	33.8±4.5	0.38±0.02(M)	0.01±0.00	0.035±0.01	-	0.01±0.00	-
***Arthrobacter* sp. M6**	-	890.66±6.4	0.85±0.00(M)	0.01±0.00	0.856±0.32	0.08±0.01	UD	-
***Ralstonia* sp. MS1**	+	67±4.7	1.16±0.02(M)	0.01±0.00	2.457	-	UD	-
***Pantoea* sp. MS3**	+	395±6.2	0.86±0.02(M)	0.02±0.00	UD	-	0.01±0.00	-
***Arthrobacter* sp.W1**	-	163±1.5	1.53±0.02(M)	0.02±0.00	4.74±0.46	-	0.01±0.00	-
***Rhizobium* sp. W3**	-	186±4.6	2.07±0.04(H)	0.01±0.00	2.43±0.18	-	-	-
***Microbacterium* sp. WS1**	-	148.6±3.5	1.25±0.05(H)	0.01±0.00	2.43±0.24	UD	0.01±0.00	-
***Arthrobacter* sp. WS4**	-	93.6±3.6	0.66±0.06(M)	0.02±0.01	2.04±0.33	0.01±0.00	0.01±0.00	-
***Pseudomonas* sp. WS5**	-	118±4.6	1.48±0.05(M)	0.02±0.00	0.77±0.31	-	0.01±0.00	-
***Aeromicrobium* sp. R1**	+	103.87±3.7	4.37±0.02 (C)	0.05±0.01	1.84±0.15	-	-	-
***Rhizobium* sp. R2**	-	528.66±4	5.82±0.07(M)	0.02±0.00	3.14±0.34	-	-	-
***Brevundimonas* sp. R3**	-	65.6±1.7	0.32±0.02(M)	UD	1.24±0.20	-	-	-
***Achromobacter* sp. RS1**	+	-	0.74±0.06(M)	-	0.48±0.07	-	-	-
***Microbacterium* sp. RS2**	+	50.6±4.3	0.46±0.07(M)	0.03±0.00	0.56±0.30	-	-	-
***Achromobacter* sp. RS3**	+	-	0.60±0.06(H)	UD	2.21±0.30	-	-	-
***Achromobacter* sp. RS4**	+	57.6±5.6	0.43±0.05(M)	UD	1.59±0.55	-	-	-
***Achromobacer* sp. RS5**	+	-	0.29±0.08(M)	UD	2.08±0.26	-	-	-

***Achromobacter* sp. RS8**	+	-	0.85±0.06(M)	UD	2.05±0.44	-	-	-
***Bacillus* sp. S1**	-	58.66±5.06	0.86±0.02(M)	0.01±0.00	0.89±0.33	-	-	-
***Streptomyces* sp. SS1**	+	56.11±5.83	0.24±0.01(M)	0.01±0.00	-	-	-	+
***Rhizobium* sp. SS2**	-	343±6.42	0.22±0.03(M)	0.02±0.00	0.31±0.12	-	-	-
***Streptomyces* sp. SS4**	+	99.46±4.39	0.21±0.02(M)	0.02±0.00	-	-	-	-
***Streptomyces* sp. SS5**	+	67±2.66	1.38±0.05(M)	0.01±0.00	-	-	-	+
***Streptomyces* sp. SS8**	-	-	0.56±0.07(M)	0.02±0.00	0.85±0.27	-	-	+
***Methylobacterium* sp. P1**	-	45.6±4.8	1.00±0.06 (C)	-	-	-	-	-
***Methylobacterium* sp. PS1**	-	35.46±3.4	3.28±0.04 (M)	-	-	-	-	-
***Bacillus* sp. PS3**	-	-	1.53±0.02(H)	-	1.30±0.18	-	-	-

[a] Bacterial strains are denoted by alphabetical designation which denote their origin: M- Maize leaf; MS-Maize stem; W-Wheat leaf; WS- Wheat Stem; R- Rice leaf; RS- Rice Stem; S- Sorghum leaf; SS- Sorghum Stem; P- Pearl millet leaf; PS- Pearl millet stem, [b]Phosphate solubilization ability as seen on Pikovskaya's agar, [c]Siderophore type designated as H-hydroxamate, C-catecholate and M-mixed type, [d]Antifungal activity against *Rhizoctonia solani*, UD- Activity was undetectable in assay but positive on plate , + indicates positive and – indicates negative

Fig. A1 Representative for siderophore detection by different methods

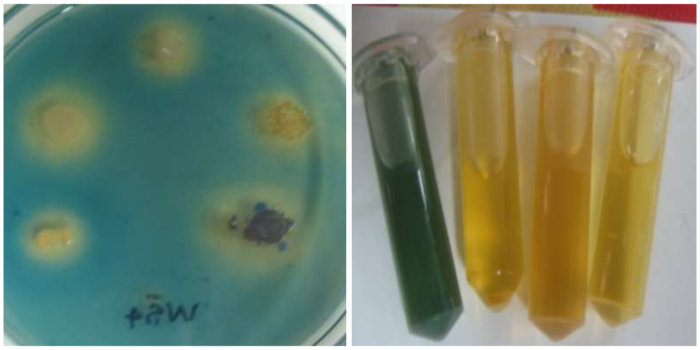


Fig. A2 Protease activity of diazotrophic endophytic bacteria

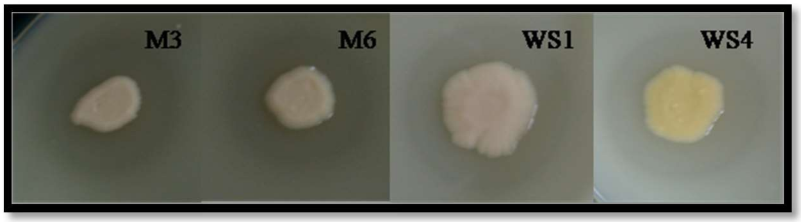


Fig. A3 Pectinase activity in diazotrophic endophytic bacteria

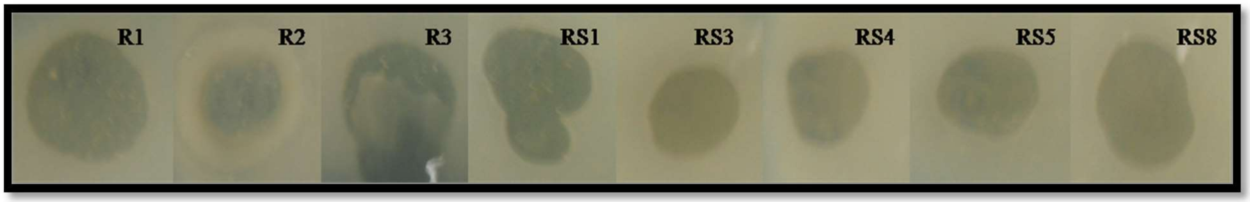


Fig. A4 Cellulase activity determined on plate assay

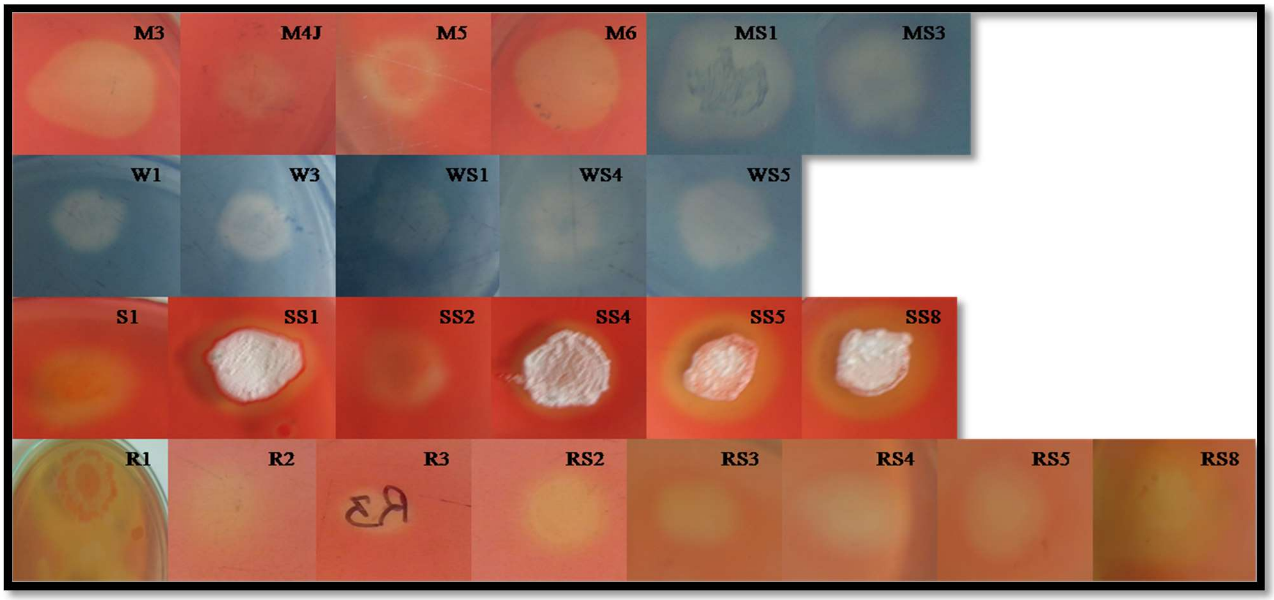


Fig. A5 Chitinase activity in diazotrophic endophytic bacteria

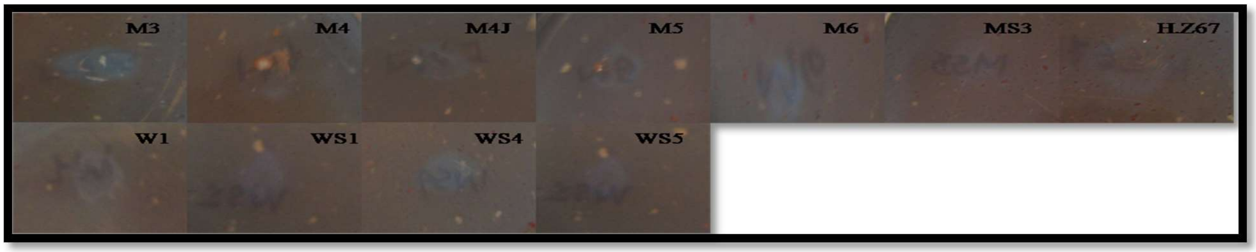
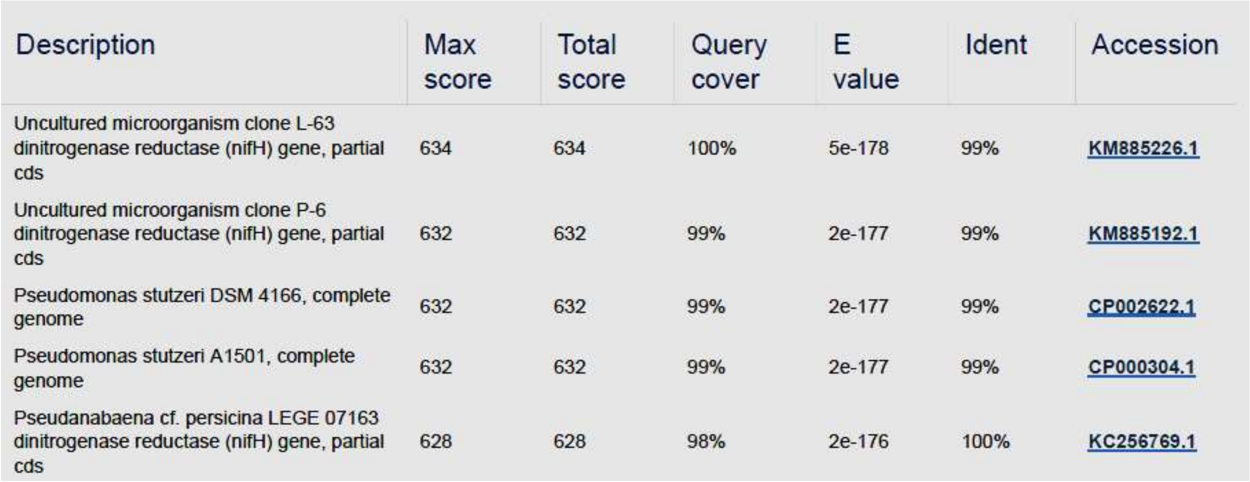


Fig. A6 BLASTn results of *NifH* gene sequences of selected diazotrophic endophytic bacterial isolates

Isolate*: Arthrobacter* sp.W1 *nifH* gene, partial sequence (346 bp)

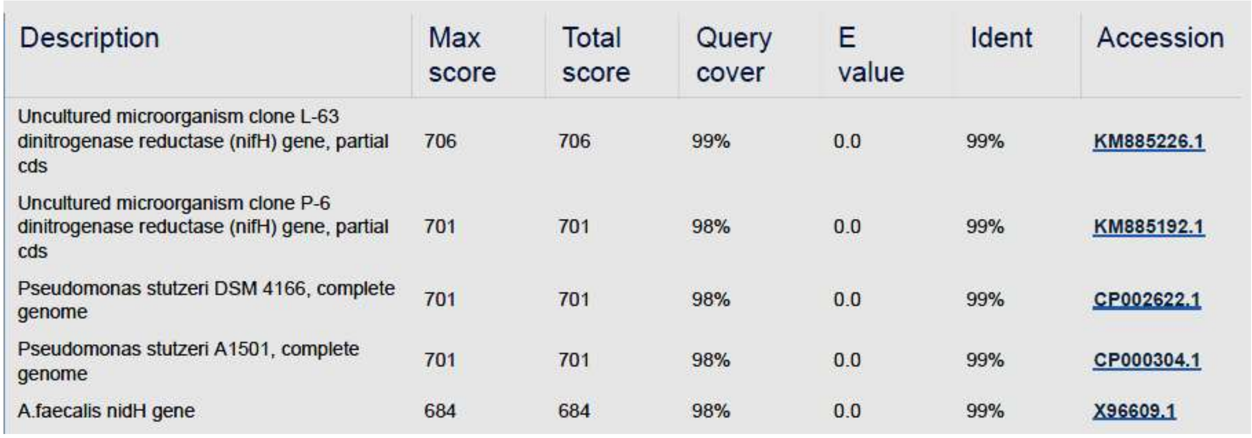
CACTCGCCTGATCCTGCACTCCAAGGCGCAGAACACCATCATGGAAATGGCCGCCGA
GGCCGGTACCGTGGAAGACCTGGAACTCGAGGACGTGCTCAAGACCGGCTACGGCG
ACATCAAGTGCGTCGAGTCGGGCGGTCCGGAGCCGGGCGTGGGCTGCGCCGGTCGC
GGCGTGATCACCGCGATCAACTTCCTCGAAGAGGAAGGCGCCTACGAGGATGACCTG
GACTTCGTCTTCTACGACGTGCTCGGCGACGTGGTCTGTGGCGGCTTCGCCATGCCCA
TCCGCGAGAACAAGGCCCAGGAGATCTACGTGGTCTGCTCCGGCGAGATGATGGCCA
TGTAC



Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured microorganism clone L-63 dinitrogenase reductase (nifH) gene, partial cds	634	634	100%	5e-178	99%	KM885226.1
Uncultured microorganism clone P-6 dinitrogenase reductase (nifH) gene, partial cds	632	632	99%	2e-177	99%	KM885192.1
Pseudomonas stutzeri DSM 4166, complete genome	632	632	99%	2e-177	99%	CP002622.1
Pseudomonas stutzeri A1501, complete genome	632	632	99%	2e-177	99%	CP000304.1
Pseudanabaena cf. persicina LEGE 07163 dinitrogenase reductase (nifH) gene, partial cds	628	628	98%	2e-176	100%	KC256769.1

- **Isolate***: Bacillus* sp. S1 *nifH* gene, partial sequence (387 bp)

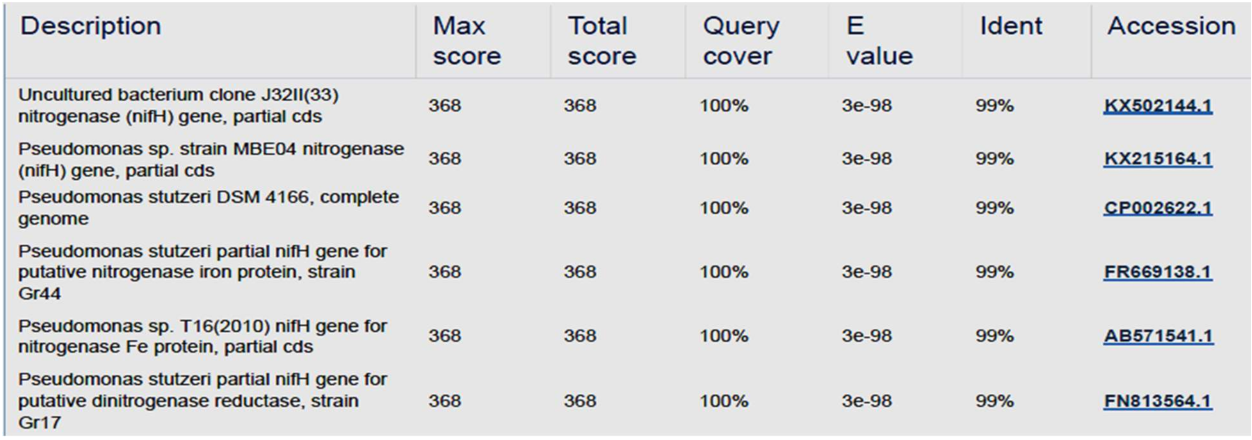
AGAGGTCATGATCGTCGGCTGCGACCCCAAGGCCGACTCCACTCGCCTGATCCTGCA
CTCCAAGGCGCAGAACACCATCATGGAAATGGCCGCCGAGGCCGGTACCGTGGAAG
ACCTGGAACTCGAGGACGTGCTCAAGACCGGCTACGGCGACATCAAGTGCGTCGAGT
CGGGCGGTCCGGAGCCGGGCGTGGGCTGCGCCGGTCGCGGCGTGATCACCGCGATC
AACTTCCTCGAAGAGGAAGGCGCCTACGAGGATGACCTGGACTTCGTCTTCTACGAC
GTGCTCGGCGACGTGGTCTGTGGCGGCTTCGCCATGCCCATCCGCGAGAACAAGGCC
CAGGAGATCTACGTGGTCTGCTCCGGCGAGATGATGGCCATGTACGC



Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured microorganism clone L-63 dinitrogenase reductase (nifH) gene, partial cds	706	706	99%	0.0	99%	KM885226.1
Uncultured microorganism clone P-6 dinitrogenase reductase (nifH) gene, partial cds	701	701	98%	0.0	99%	KM885192.1
Pseudomonas stutzeri DSM 4166, complete genome	701	701	98%	0.0	99%	CP002622.1
Pseudomonas stutzeri A1501, complete genome	701	701	98%	0.0	99%	CP000304.1
A.faecalis nidH gene	684	684	98%	0.0	99%	X96609.1

- **Isolate***: Rhizobium* sp. R2 *nifH* gene, partial sequence (208 bp)

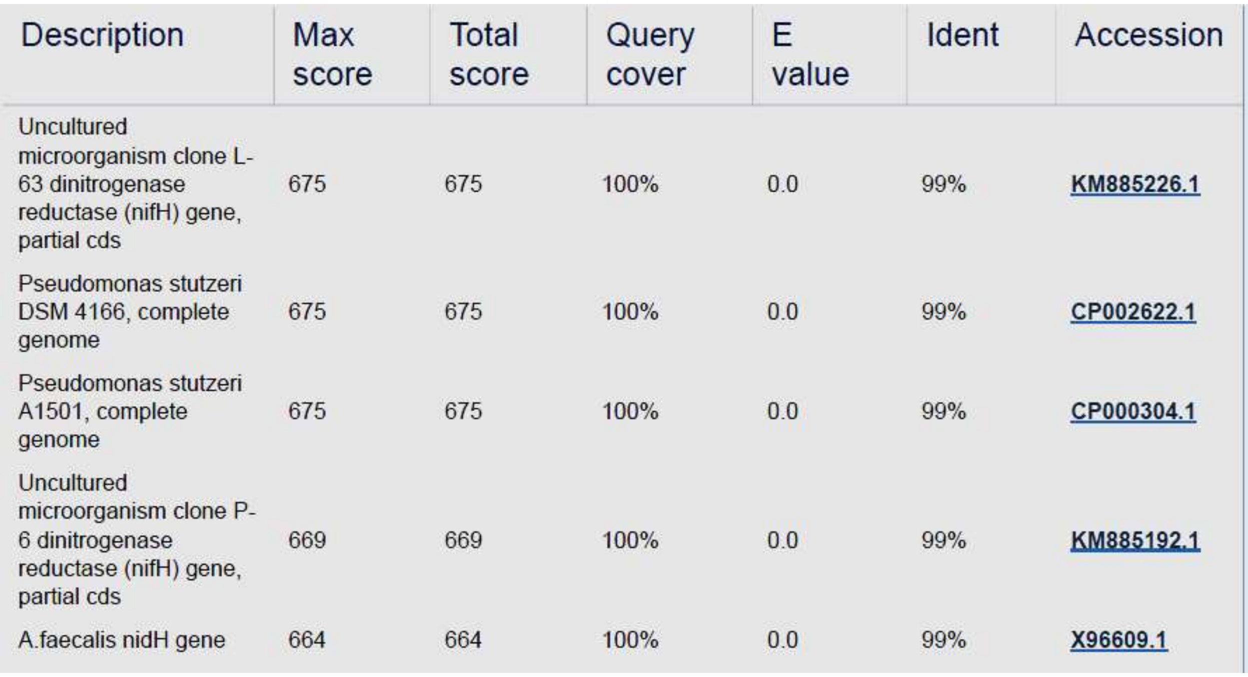
ACACCATCATGGAAATGGCCGCCGAGGCCGGTACCGTGGAAGACCTGGAACTCGAG GACGTGCTCAAGACCGGCTACGGCGACATCAAGTGCGTCGAGTCGGGCGGTCCGGA GCCGGGCGTGGGCTGCGCCGGTCGCGAAGTGATCACCGCGATCAACTTCCTCGAAGA GGAAGGCGCCTACAAGGATGACCTGGACTTCGTCTTCTA



Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured bacterium clone J32II(33) nitrogenase (nifH) gene, partial cds	368	368	100%	3e-98	99%	KX502144.1
Pseudomonas sp. strain MBE04 nitrogenase (nifH) gene, partial cds	368	368	100%	3e-98	99%	KX215164.1
Pseudomonas stutzeri DSM 4166, complete genome	368	368	100%	3e-98	99%	CP002622.1
Pseudomonas stutzeri partial nifH gene for putative nitrogenase iron protein, strain Gr44	368	368	100%	3e-98	99%	FR669138.1
Pseudomonas sp. T16(2010) nifH gene for nitrogenase Fe protein, partial cds	368	368	100%	3e-98	99%	AB571541.1
Pseudomonas stutzeri partial nifH gene for putative dinitrogenase reductase, strain Gr17	368	368	100%	3e-98	99%	FN813564.1

- **Isolate**: *Pantoea* sp. MS3 *nifH* gene, partial sequence (368 bp)

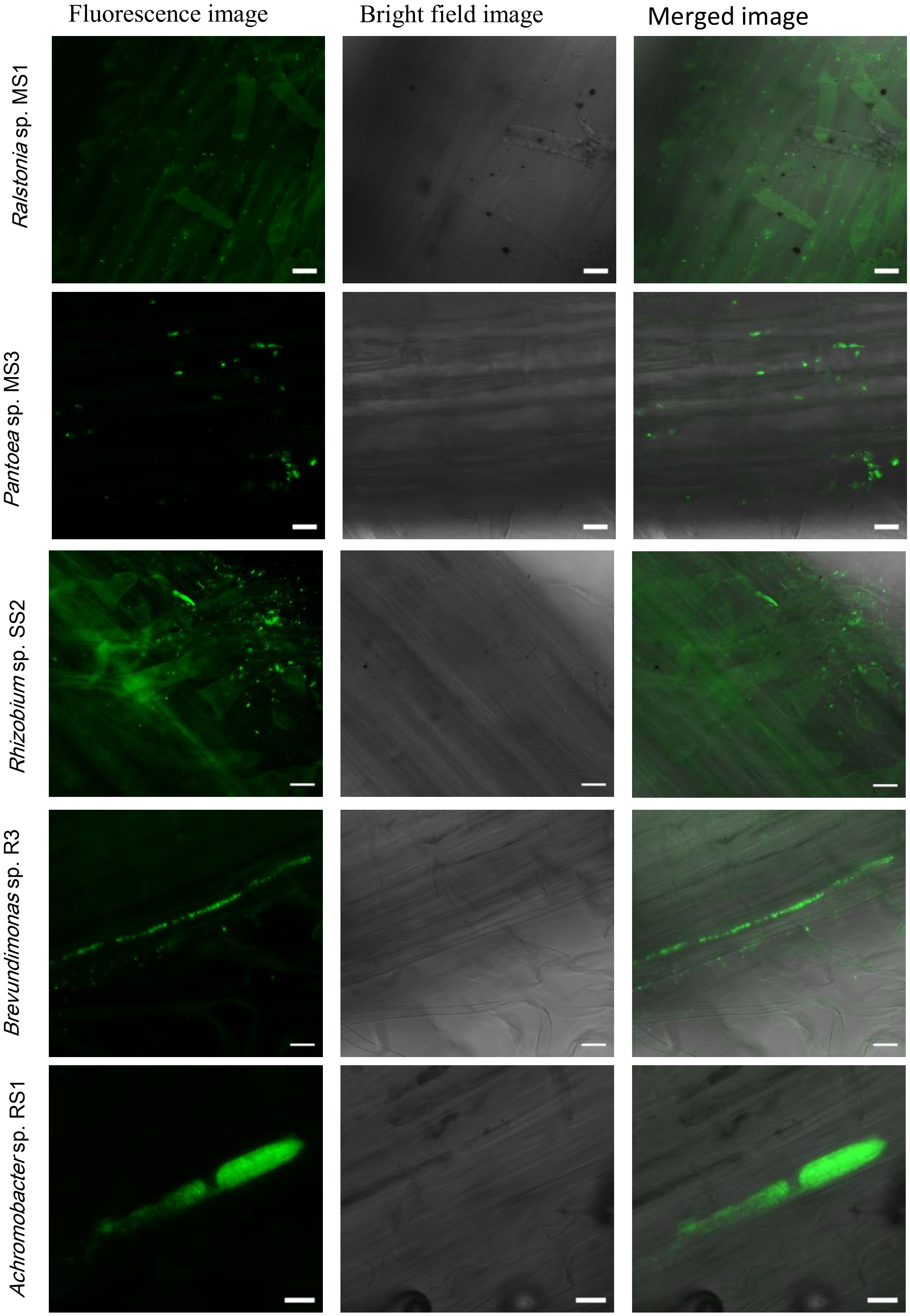
GATCGTCGGCTGCGACCCCAAGGCCGACTCCACTCGCCTGATCCTGCACTCCAAGGC
GCAGAACACCATCATGGAAATGGCCGCCGAGGCCGGTACCGTGGAAGACCTGGAAC
TCGAGGACGTGCTCAAGACCGGCTACGGCGACATCAAGTGCGTCGAGTCGGGCGGTC
CGGAGCCGGGCGTGGGCTGCGCCGGTCGCGGCGTGATCACCGCGATCAACTTCCTCG
AAGAGGAAGGCGCCTACGAGGATGACCTGGACTTCGTCTTCTACGACGTGCTCGGCG
ACGTGTCTGTGGCGGCTTCGCCATGCCCATCCGCGAGAACAAGGCCCAGGAGATCTA
CGTGGTCTGCTCCGGCGAGATGATGGC

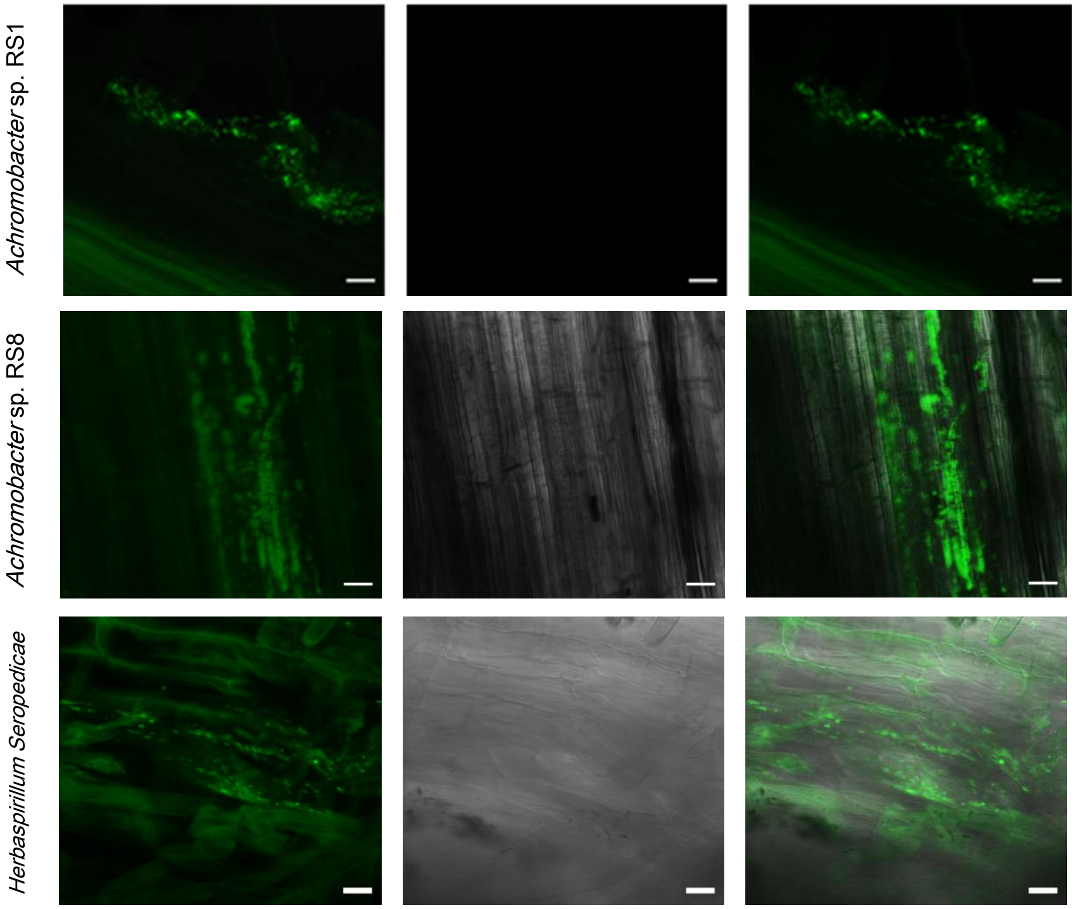


Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured microorganism clone L-63 dinitrogenase reductase (nifH) gene, partial cds	675	675	100%	0.0	99%	KM885226.1
Pseudomonas stutzeri DSM 4166, complete genome	675	675	100%	0.0	99%	CP002622.1
Pseudomonas stutzeri A1501, complete genome	675	675	100%	0.0	99%	CP000304.1
Uncultured microorganism clone P-6 dinitrogenase reductase (nifH) gene, partial cds	669	669	100%	0.0	99%	KM885192.1
A.faecalis nidH gene	664	664	100%	0.0	99%	X96609.1

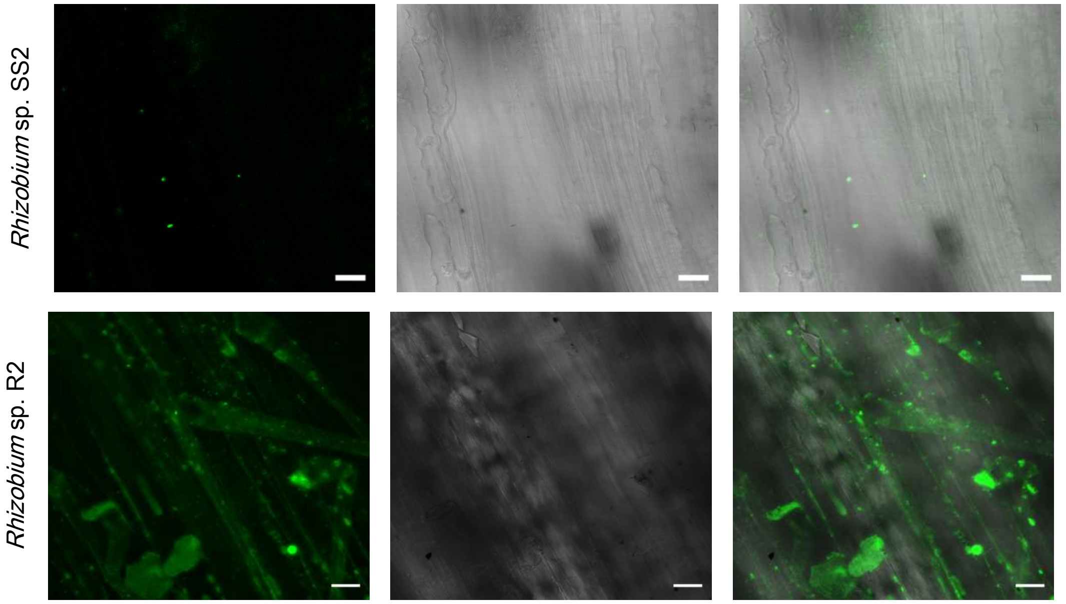
Fig. A7 Confocal laser scanning microscopic images of *gfp* tagged endophytic bacterial isolate inside the plants. Scale bar represent the 20 µm

<u>Root part of the wheat plant</u>

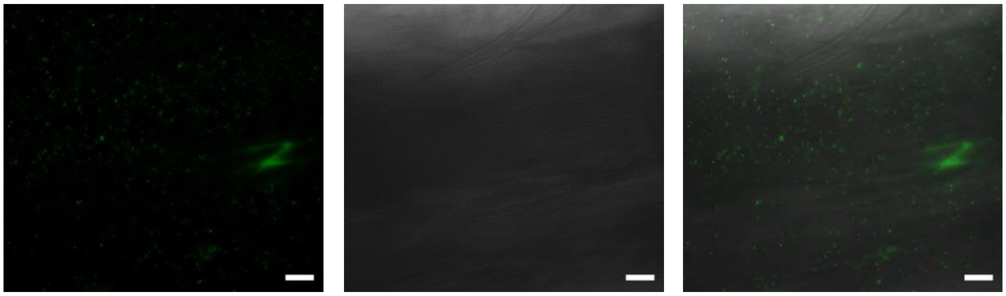




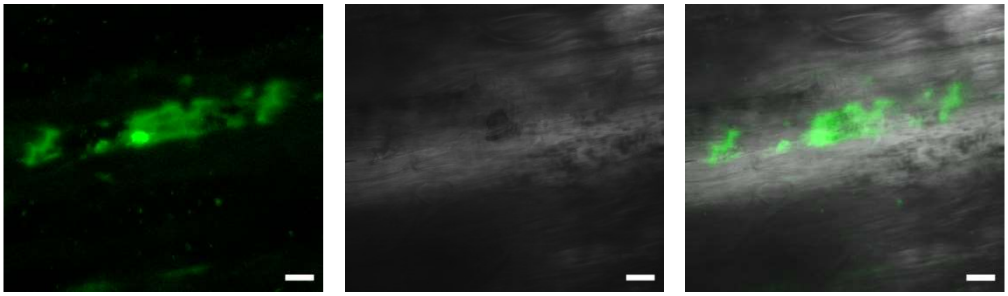
Stem part of the plant



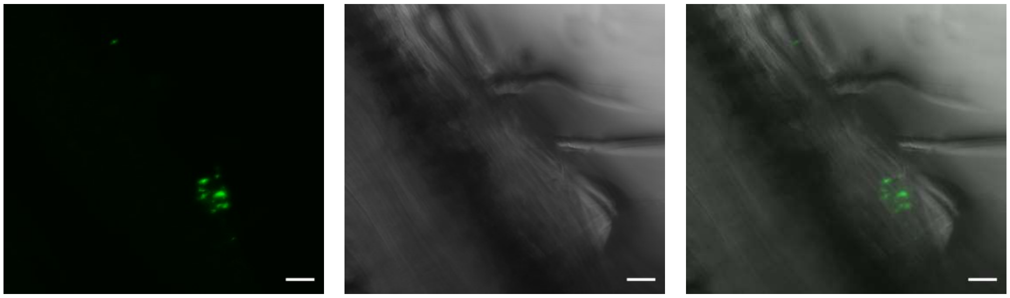
Brevundimonas sp. R3



Brevundimonas sp. R3



Achromobacter sp. RS3



Leaf part of the wheat plant

Achromobacter sp. RS4

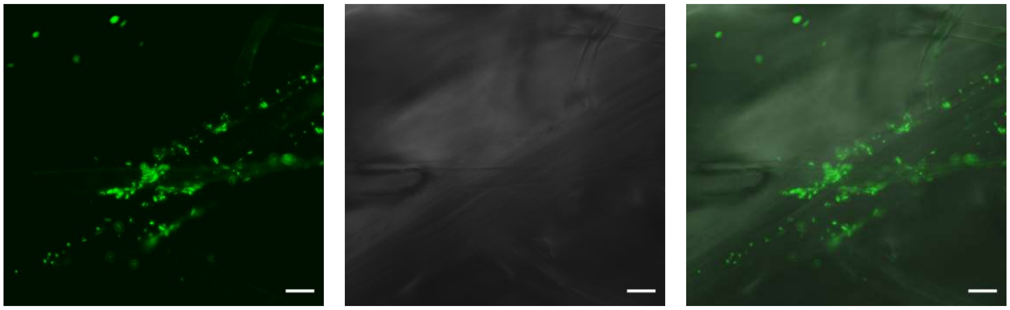
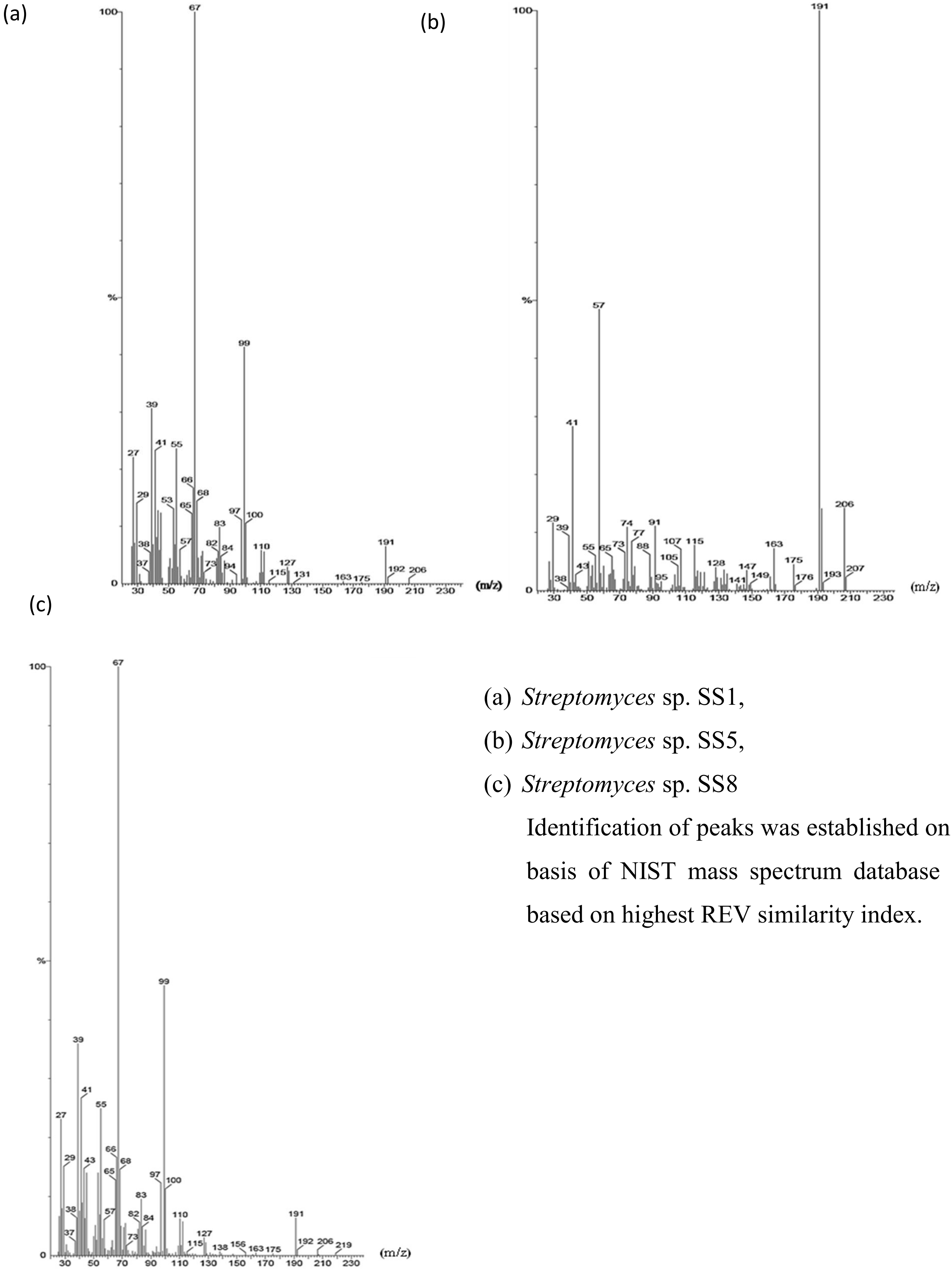


Fig. A8 GC-MS spectra of antifungal extracts from different strains of diazotrophic endophytic *Streptomyces* spp.



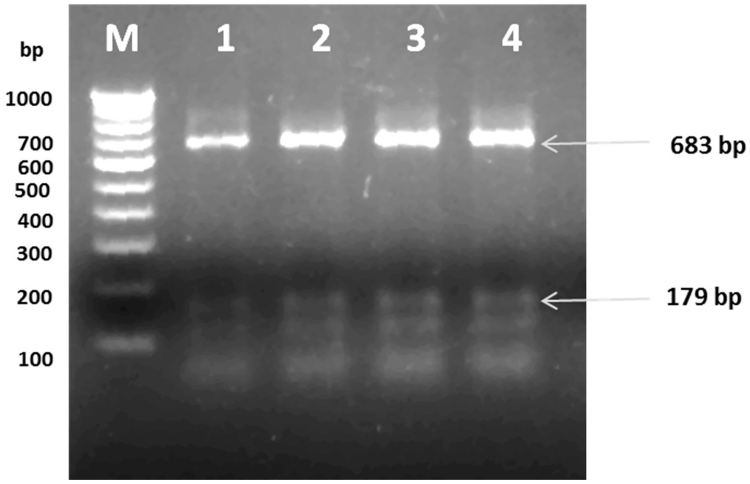
(a) *Streptomyces* sp. SS1,

(b) *Streptomyces* sp. SS5,

(c) *Streptomyces* sp. SS8

Identification of peaks was established on the basis of NIST mass spectrum database and based on highest REV similarity index.

Fig. A9 Amplified ribosomal DNA restriction analysis of DAPG transformant and wild type strain of *Pseudomonas* sp. WS5



Pseudomonas sp. WS5 strains confirmation harboring pAJK1.2 plasmid using 16S rRNA with HaeIII. Lane M-100 bp marker, Lane 1- *Pseudomonas* sp. WS5, Lane 2- *Pseudomonas* sp. WS5 VC, Lane 3- *Pseudomonas* sp. WS5 (pAJK1.2a), Lane 4- *Pseudomonas* sp. WS5 (pAJK1.2b)

Appendix II Media compositions

Nitrogen free (NFb)-medium per liter

L- malic acid -	5.0 g
K_2HPO_4	0.5 g
$MgSO_4$ $7H_2O$	0.2 g
NaCl	0.1 g
$CaCl_2$	0.02 g
Bromthymol blue (0.5%)	2 ml
Vitamin solution	1 ml
Micronutrient solution	2 ml
Fe EDTA solution (1.64%)	4 ml
KOH	4.5 g
pH	6.8
agar	1.9 g

Vitamin solution (100 mL):

Biotin	10 mg
Pyridoxol-HCl	20 mg

Micronutrient	**(g/l)**
$CuSO_4$	0.4
$ZnSO_47H_2O$	0.12
H_2BO_3	1.4
Na_2MoO_4 $2H_2O$	1.0
$MnSO_4H_2O$	1.5

JNFb consisted of the same components as NFb-medium except following different ingredients and amounts per liter:

K_2HPO_4	0.6 g
KH_2PO4	1.8 g
pH	5.8

Medium preared as mentioned here, only the micronutrient, Vitamin solution, $MgSO_4$ $7H_2O$ and $CaCl_2$ added separately after autoclaving.

Biochemical test media composition

Carbohydrate fermentation test

Ingredients	100 ml
Protease peptone	1 g
Carbohydrate	1 g
Andrade indicator	10 ml

MR-VP test

(a) Methyl Red

This test used to identify the mixed acid production by organisms via butanediol fermentation pathway.

(b) Voges-Proskauer

Sugar fermentation in some organism produced the acetoin via butanediol fermentation pathway. This product reacts with KOH and 1-napthol and turns medium to pink color.

Ingredients	g/l
Peptone	7.00
K_2HPO_4	5.00
Dextrose	5.00

Methyl red reagent

Methyl red	0.1 g
Ethanol (95%)	300 ml
Distilled water	Make up 500 ml

Voges-Proskauer reagent

Solution A: 1-napthol (5% (w/v))

Solution B: KOH (40% (w/v))

Indol test

Indole test is used to detect the formation of indole which produced from tryptophan. This tryptophan is hydrolyzed by tryptophanase to produce is indole.

Ingredients	
Tryptone	10 g
Sodium chloride	5 g

Distilled water	1000 ml

Kovac's reagent

p-dimethylamino benzaldehyde	10 g
iso-amyl alcohol	150 ml
Concentrated HCl	50 ml

Catalase

All cultures were inoculated in LB broth and incubated overnight. Few drops of H_2O_2 was added into the broth and production effervesces indicates the positive result.

Hugh –Leifson test

Organisms can utilize carbohydrates either aerobically or anaerobically and some metabolize using both the process. Under anaerobic condition organism utilizes carbohydrate in the fermentative method. The acid produced under an anaerobic condition which bromothymol blue to yellow color.

Ingredients	g/l
Peptone	2
Sodium chloride	5
Bromothymol blue	0.03
Carbohydrate	10
K_2HPO_4	0.3
Agar	3.0
pH	7.1

Media was prepared as mentioned above and inoculated in duplicate. In one tube covered with mineral oil to create the anaerobic condition. Sugar fermentation can be detected by both the tube aerobic and anaerobic turns yellow.

Colloidal chitin Media

The 10 g of chitin was taken and added the 100 ml of 85% phosphoric acid. Further it kept in refrigerator 5 °C for 24 h and wash with 2 l water. Filter the gelatinous material and wash the till the pH reach to 7.0.

Media preparation

Ingredients	g/l
Colloidal chitin	5.00
$(NH_4)_2SO_4$	1.00
Yeast Extract	0.5
$MgSO_4$	0.3
KH_2PO_4	1.36
Agar	20

Luria-Bertani Broth composition

Ingredients	g/l
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	10.00

Pikovskayas Agar

Ingredients	g/l
Yeast extract	0.500
Dextrose	10.000
Calcium phosphate	5.000
Ammonium sulphate	0.500
Potassium chloride	0.200
Magnesium sulphate	0.100
Manganese sulphate	0.0001
Ferrous sulphate	0.0001
Agar	15.000

Rock Phosphate Medium

Ingredients	g/100 ml
Tris-Cl (1 M stock)	50 ml
NH_4Cl (1 M stock)	1.33 g
$CaCl_2$ (0.1 M stock)	0.3 ml
$MgSO_4$ (0.1 M stock)	0.3 ml
KH_2PO_4 (1 M stock)	0.009 ml

Stocks of NH_4Cl, $CaCl_2$, $MgSO_4$, Tris-Cl, glucose were autoclaved separately and required amount was added to the autoclaved water along with rock phosphate and agar.

Mannitol–Soybean meal medium

Ingredients	g/l
Soyabean meal	20
Mannitol	20

Murashige-skoog Medium

Ingredients	mg/l
Macroelements	
Ammonium nitrate	825.000
Calcium chloride	332.200
Magnesium sulphate	180.690
Potassium nitrate	3040.000
Potassium phosphate monobasic	170.000
Microelements	
Boric acid	6.200
Cobalt chloride hexahydrate	0.025
Copper sulphate pentahydrate	0.025
EDTA disodium salt dihydrate	37.300
Ferrous sulphate heptahydrate	27.800
Manganese sulphate monohydrate	16.900
Molybdic acid (sodium salt)	0.213
Potassium Iodide	0.830
Zinc sulphate heptahydrate	8.600

Potato dextrose broth

Ingredients	g/l
Potatoes, infusion from	200
Dextrose	20

Oat-Meal agar

Ingredients	g/l
Oat Meal	60.000
Agar	12.500