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COMPARATIVE STUDIES BETWEEN NITROGIN FIXING METHYLOTROPHIC BACTERIA AND RHIZOBIA OF SOME LEGUME PLANTS

Heba O. Mohamed[(1)], Wedad E.E. Eweda[(2)], Sawsan F. Shehata[(2)] and H.H. Abo Taleb[(1)]

[(1)]Department of Agricultural Microbiology, Soils, Water and Environment Research Institute, Agricultural Research Center, Giza.

[(2)] Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima- Cairo.

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ABSTRACT: *Ninety four isolates of methylotrophs were isolated from green leaves and roots of various legume plants. The ability of various Pink Pigmented Facultative Methylotrophic (PPFM) isolates to grow on Yeast Extract Mannitol (YEM) agar medium with or without 0.05% methanol as a source of carbon, some morphological, physiological characteristics and molecular biology studies, as well as biological nitrogen fixation activity by using ^{15}N isotope technique , were estimated. The obtained results indicated that , PPFM.Lt, PPFM.Sb and PPFM.D methylotrophic bacteria were isolated from Lupine , Soybean and handaqooq legume plants, respectevily, they had the ability to grow on YEM media with or without 0.05% methanol and Congo red dye, as well as were able to induce nodule formation on roots of legume host plants. Morphological and cultural characteristics of the three PPFM isolates, as compared to reference rhizobial strains (USAD 110 and ARC 408), showed that ,all isolates were short rods ,Gram negative and motile. Physiological characteristics of obtained PPFM isolates were catalase positive .All isolates could utilize sucrose and D-glucose, except PPFM.Lt, utilized citrate and methanol as a sole carbon, source except both rhizobial strains (USAD 110 and ARC 408). The three PPFM bacteria were resistant to Ampicillin ,Gentamycin and Colistin, and were susceptible to Kanamycin and Streptomycin, while the two rhizobial strains were resistant to all antibiotics used. The cluster analysis of protein marker data using SDS-PAGE placed the three bacterial PPFM isolates and two rhizobial strains into two main groups, at similarity between them, ranged from 65.0 to 92.5%. Obtained data of the RAPD analysis of DNA from the three PPFMs isolates and the two rhizobial strains , showed that, the similarity between the two main groups PPFMs isolates and rhizobial strains, ranged from 37.38% to92.29% ,depending on used primers .Under greenhouse conditions, application of the three PPFM isolates and / or rational dose of mineral N-fertilizer, as well as the specific rhizobia(USAD 110 for Soybean and ARC 408 for Lupine), scored significant differences in number of nodules , nodule dry weight , nitrogen percentage ,plant N-content, nitrogen derived from air (Ndfa %) and nitrogen amount fixed . Application of rhizobial strains scored the higher amounts of nitrogen content through fixed atmospheric N_2 . From the obtained results it could be concluded that PPFM. Lt isolate may be related to Methylobacterium nodulans .*

***Key words**: Legume plants, Pink-Pigmented Facultatively Methylotrophic (PPFM),Biological Nitrogen Fixation (BNF), Methylobacterium nodulans.*

INTRODUCTION

Legume - *Rhizobium* symibiosis is undoubtedly the most important N_2-fixing process and play a subtle role in providing nitrogen and maintaining/improving soil fertility. Symbiosis between legumes and rhizobia are of a considerable environmental and agricultural importance, since they are responsible for most of the atmospheric nitrogen fixed on land. (Graham and Vance, 2003).

Biological Nitrogen Fixation (BNF) is known to be a key to sustain agriculture and to reduce soil fertility decline. The natural process of BNF that allows micro- organisms to convert atmospheric ($N_{2)}$ to ammonia

(NH_3) assimilable by associated plants. (Sprent, 2008).

The rhizobial species were described so far belonging to three distinct phylogenetic branches within the α-2 subclass of Proteobacteria ; A first large branch comprises of genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Allorhizobium*, the second branch contains the genus *Bradyrhizobium* and the third branch includes the genus *Azorhizobium* (Sy *et al.,* 2001a). Each rhizobial species has a defined host range, varying from very narrow to very broad. A part from rhizobia, very close relatives of rhizobia, such as members of □ subgroup of proteobacteria and few □Proteobacteria are also recognized to form nodules in legumes.

Sy *et al.,* (2001a) and Jourand *et al.,* (2004) described a novel fourth rhizobial branch within α-2 subclass of Proteobacteria belonging to genus *Methylobacterium* for a group of aerobic facultatively methylotrophic, legume root nodule-forming and nitrogen fixing bacteria. The genus *Methylobacterium* includes a variety of pink pigmented facultative methylotrophic bacteria (PPFMs) that are able to grow on C1 compounds, such as formate, formaldehyde and methanol as sole carbon sources, as well as on wide range of multi-carbon growth substances(Green, (1992) and Lidstrom (2002)).Several nodulating *Methylobacteria* were isolated from tropical and sub tropical legumes, such as beans, cowpea, blackgram and soybean with high nitrogenase activity. (Raja *et al* .(2006) and Madhiayan *et al.* (2009)).

The present work aims to isolate bacteria belonging to pink pigmented facultative methylotrophic bacteria(PPFMs) and to study the ability of nodule formation and nitrogen fixation by them with some legume plants , as well as some morphological , physiological and molecular characteristics of PPFMs, and to evaluate the symbiotic relationship between PPFMs isolates and rhizobia on nodulation status and nitrogen fixation with some legume crops (Lupine and Soybean).

MATERIALS AND METHODS

1. Isolation and Purification of PPFM Isolates

1.1. From leaf surfaces of legume plants:

Green leaves obtained from different legumes (Chickpea, Faba bean, Lupine, Fenugreek, Common bean, Peanut, Soybean, Egyptian Clover and Alfalfa) were collected from different locations (Behera, Menia, Kalubia and Giza). The leaves were handled aseptically and were used either directly or rinsed in a sterile water, then they were pressed firmly to the surface of a specific solid medium of Methanol Mineral Salts (MMS) agar medium (Holland and Polacco, 1992), then discarded and plates were closed, sealed with para film and incubated at 28°C for 3-5 days.The growing small pink-pigmented separated colonies were selected and successively sub cultured on the same specific medium several times. A well defined pure colonies were sub cultured on slants of the Met-AMS medium and incubated at 28°C for 3-5 days. The growing pure cultures were kept at 4°C.

1.2.From root nodules of the wild legume plant(Handaqooq):

Root nodules were surface sterilized with 0.1%mercuric chloride and 70%alcohol , serially washed nodules with a sterile distilled water .The suspensions of crushed nodules were placed on MMS agar medium and incubated at 28°C for 3-5 days. The growing small pink-pigmented separated colonies were selected and successively sub cultured on the same specific medium several times. Then, a well defined pure colonies were sub cultured on slants of the Met-AMS medium and incubated at 28°C for 3-5 days. The growing pure cultures were kept at 4°C.

2.Identification of the Selected PPFM Isolates as Compared to Rhizobia

Characterization and identification of experiments were carried out as described in Bergey's Manual of Systematic

Bacteriology, 2nd edition (2005). These experiments include the following:

2.1. Morphological and staining characteristics of PPFM isolates:

Pure colonies were examined microscopically according to Barrow and Feltham (1993) to determine Gram reaction and cell shape. Whereas, motility was tested in a liquid culture. Morphology of colonies were observed for each PPFMs isolate on a solid MMS medium and incubated for 3days at 28°C whereas, USAD110 and ARC 408 colonies grown on YEM agar medium (Vincent, 1970) and incubated for1- 3 days at 28°C and were examined using a binocular microscope.

2.2. Physiological characteristics of PPFM isolates:

Physiological characteristics of PPFM isolates were tested, according to Jenkins and Jones (1987), as follows:

2.2.1. Catalase test:

PPFM Isolates and rhizobia grown for 24 h were emulsified with 20 vol. H_2O_2 and were observed for the production of effervescence for up to 1 min.

2.2.2.Carbon sources utilization:

Different carbon sources, namely sucrose , mannitol ,glycerol, D-glucose , citrate, ethanol and methanol were used separately to study the ability of PPFM isolates and rhizobia to grow on different carbon sources by adding 0.2% (w / v) in MMS basal agar medium or YEM agar medium.

3. Antibiotic Resistance of PPFM Isolates (Quinn *et al.* 1994)

Five antibiotics were used as shown in Table (1) to estimate the antibiotic resistance of obtained PPFM isolates and rhizobia . The procedure was undertaken as follows: The antibiotic discs were gently placed on (MMS) agar plates, inoculated by one ml of each three PPFM isolates using a sterile pointed forceps to ensure complete contact with the medium surface and incubated for 96h at 28ºC.Whereas, USAD110 and ARC 408grown on YEM agar plates by the same steps. The degree of sensitivity was estimated by measuring the visible clear zone of inhibition produced by diffusion of the used antibiotic discs into the surrounding medium.

Table (1). Antibiotic resistance standard range of Gram- negative bacteria (Inhibition zone diameter, mm).

Antibiotics	Concentrations	I.Z. Diameter (mm)		
		Resistant	Intermediate	Susceptible
Ampicillin	10µg	≤ 13	14-16	≥ 17
Gentamycin	10µg	≤ 12	13-14	≥ 15
Colistin	10µg	≤ 8	9-10	≥ 11
Kanamycin	30µg	≤ 13	14-17	≥ 18
Streptomycin	50µg	≤ 11	12-14	≥ 15

After Quinn *et al.*, (1994)

4. Molecular Biology Studies:

The similarity among the PPFM isolates and rhizobia was studied using protein pattern and random amplification of DNA, as follows:

4.1. Electrophoretic studies of PPFMs and Bradyrhizobial protein by sodium dodecyl sulphate poly achrelamide gel electrophoresis (SDS-PAGE):

Protein pattern of PPFM isolates and rhizobia was mad according to Laemmili (1970), using a standard protein marker at seven molecular weights (15,25, 35,50,75,100 and 150 K.d) and data were analyzed by 1. D advanced program.

4.2. Random amplified polymorphism DNA (RAPD) analysis:

RAPD analysis of PPFM isolates and rhizobia was carried out, according to Welliams *et al.* (1990). Protocol was performed by using three primers: Primer No.2, Primer No.4 and Primer No.6. The different molecular weights of bands were determined against PCR marker promega G317A by unweighed pair-group method based on arithmetic mean (UPGMA). SDS-PAGE and RAPD analysis were kindly determined at the molecular biology lab, Plant Pathology Research Institute, ARC, Giza.

5. Greenhouse Experiments

5.1.Cross inoculation experiment:

A pot experiment was carried out in a glass greenhouse at Giza Research Station of ARC, to evaluate the ability of the five selected PPFMs bacteria (PPFM.Lt , PPFM.Sb, PPFM.D, PPFM.Tr and PPFM.M)- which grew well on YEM medium- to form nodules on the roots of various legume host plants ; Lupine (*Lupinus termis,* variety Giza 2), Soybean (*Glycine max,* variety Giza 111) ,Egyptian Clover(*Trifolium alexandrinum*, variety Saro 4) and Alfalfa (*Medicago stiva*, variety Ismailia 1), were planted in cleaned pots of 30 cm diameter filled with 5 kg washed and sterilized sandy soil. The soil was pretreated overnight with 1%HCL ,washed several times with tap water to be acid free ,and then with distilled sterilized water. Physico-chemical properties of the used soil are shown in (Table 2). Seeds of the four legume host plants were inoculated with 10 ml (containing $4x10^9$cells ml^{-1}) of liquid media of the five selected PPFM isolates separately for each pot. The inoculated seeds were sown in the sterilized pots and kept in a greenhouse for irrigation with tap water. Plant samples were picked up after 45 days of planting to determine nodules formation on roots of the different legume host plants.

Physico-chemical analysis of the soil used was carried out according to Jackson (1973) at Soil Analysis Lab., Soils, Water and Environmental Research Institute, ARC, Giza.

5.2. Biological nitrogen fixation (BNF) activity by using ^{15}N isotope dilution method:

Two pot experiments were carried out in a glass greenhouse at Giza Research Station of ARC, to evaluate the effect of the most efficient PPFMs bacteria (PPFM.Lt , PPFM.Sb and PPFM.D) on nodulation status, plant growth , plants nitrogen content, as compared with specific rhizobia for lupine and soybean host plants tested and the efficiency of PPFMs bacteria (PPFM.Lt , PPFM.Sb and PPFM.D) to fix atmospheric nitrogen was determined, as well as estimation of the amount of N_2 fixed by using ^{15}N isotope dilution method, according to Chalk (1985)and Danso (1988).

Seeds of Lupine (*Lupinus termis*,variety Giza2) and Soybean (*Glycine max* variety Giza 111) and grains of two reference crops(wheat *Triticum astivum*) variety Sakha 195) and maize(*Zea mays*, variety third hyperdized 310) were planted in cleaned pots of 30 cm diameter filled with 5 kg washed and sterilized sandy soil . The seeds of lupine and soybean were inoculated with 10 ml (containing $4x10^9$ cells ml^{-1}) of liquid medium of the three selected PPFMs isolates (PPFM.Lt , PPFM.Sb and PPFM.D) and the specific rhizobia to both legume plants ARC 408 for lupine and USAD 110 for soybean for each pot.

Table (2). Some physico-chemical properties of the used soil.

Property	Values
Mechanical analysis	
Sand %	81.30
Silt %	15.17
Clay%	3.53
Texture grade	Sandy
Chemical analysis	
Water holding capacity %	13.7
Saturation percentage (SP) %	13%
P^H	7.58
E.C. (dSm^{-1})	0.57
Organic matter %	0.4
Total nitrogen %	0.011
Anions ($meql^{-l}$)	
CO_3^{--}	0.0
HCO_3^-	0.62
Cl^-	0.76
SO_4^{--}	1.66
Cations ($meql^{-l}$)	
Ca^{++}	1.58
Mg^{++}	0.82
Na^+	0.64

The layout of the experiments consisted of 12 treatments with 3 replicates, in a completely randomized block design. as follows:

1. Without any addition (control) (C).
2. Without inoculation + 50 kg N fed. $^{-1}$ (recommended dose by the Ministry of Agriculture) (F. N).
3. Inoculation with *Rhizobium* (R. Inoc.).
4. Inoculation with *Rhizobium* + 15-25 kg N fed^{-1} (activation dose in case of rhizobial inoculation by the Ministry of Agriculture) (R. Inoc+ 1/3N).
5. Inoculation with *Rhizobium* + 50-75 kg N fed.$^{-1}$ (R. Inoc+ F.N).
6. Inoculation with PPFM. D+ 15-25 kg N fed. $^{-1}$ (PPFM.D +1/3N).
7. Inoculation with PPFM. Lt+ 15-25 kg N fed. $^{-1}$ (PPFM. Lt +1/3N).
8. Inoculation with PPFM. Sb+ 15-25 kg N fed. $^{-1}$ (PPFM. Sb+1/3N).
9. Inoculation with PPFMs mixed culture of PPFM. D, PPFM. Lt and PPFM. Sb + 15-25 kgN fed. $^{-1}$ (PPFM Mix+1/3N).
10. Reference Field Crop Without any addition (RFC)
11. Reference Field Crop+ 120 kg N fed. $^{-1}$ (RFC+F.N).
12. Reference Field Crop+40 kg N fed. $^{-1}$ (RFC+1/3N).

The recommended doses of phosphorus and potassium (P & K) fertilizers were added, as follows: Super phosphate (1.5 g pot^{-1}) equals 150 kg super phosphate (15.5%P_2O_4) and potassium sulphate (0.5g. pot^{-1}) equals 50 kg potassium sulphate

(48%K_2O) were added before planting. Nitrogen (N) fertilization as ammonium sulphate (20.5 % N) was applied at rates of 0.0, 1.0 and 3.0 g $pot^{-1}$and 0.0,1.25 and 3.75for lupine and soybean, respectively, and the corresponding values for the two reference crops were 0.0,2.0 and 6.0 g pot^{-1} in two equal spilt doses at 3 and 5 weeks after planting and^{15}N labeled fertilizer(as ammonium sulphate 10% atom excess) was added at dilution with10% of amount N-fertelizer applied. The pots were kept in the greenhouse and irrigated with water. Plant samples were picked up after 60 of planting plant to determine nodules number (No. $plant^{-1}$), dry weight of nodules (mg $plant^{-1}$), plant dry weight (g $plant^{-1}$) and plant nitrogen content (mg $plant^{-1}$) . To evaluate the amount of N_2 fixed, ^{15}N dilution method was used, according to Chalk (1985)and Danso (1988).

The percentage of N derived from the atmosphere (%Ndfa) by the legume was calculated by the following equations:

$$1)\ \%Ndfa = \left(1 - \frac{\text{atom\%}^{15}\text{N excess } N_2\text{-fixing plant}}{\text{atom\%}^{15}\text{ N excess reference plant}}\right) \times 100$$

2) N_2 fixed (mg $plant^{-1}$) = % Ndfa x Total Nitrogen (T.N)

6. Statistical analysis

Results were statistically analyzed by the least significant difference test (LSD) at P <0.05, by using (MSTAT) Microcomputer Statistical Program (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

I. Isolation and Some Morphological and Physiological Characteristics of PPFM Ninety four isolates of methylotrophs were isolated from green leaves and roots of legume plants, the isolates and their sources are listed in Table (3). Our results agreed with that of Corpe and Basile (1982) and Holland (1997). They reported that; the most abundant group of methylotrophs were isolated from surfaces of green plants and that of Samba *et al* (1999) whom reported that, the bacterial isolates from root nodules of wild legumes plants at tropical and sub-topical regions belonged to the methylotrophs group .

Data in Table (4) showed that, ability of the obtained PPFM isolates to grow on Yeast Extract Mannitol (YEM) agar medium with or without 0.05% methanol, as a source of carbon. PPFM.Lt, PPFM.Sb, PPFM.Tr, PPFM.M and PPFM.D isolates belonging to Lupine , Soybean , Egyptian clover, Alfalfa and Handaqooq legume plants had the ability to grow on YEM media with or without 0.05% methanol .But only PPFM.Lt, PPFM.Sb and PPFM.D succeeded to grow on all tested YEM media with or without 0.05% methanol and / or presence of Congo red dye ,giving cultural load up to($4x10^9$cfu.ml^{-1}).

Cross inoculation test on legume plants revealed that ,the PPFM.Lt, PPFM.Sb and PPFM.D were able to induce legume root nodules for all tested plants (Lupine , Soybean , Egyptian clover and Alfalfa), as compared to PPFM.Tr and PPFM.M, which failed to induce any legume root nodule for the same tested plants (Table 5). As consequence of the above mentioned results , Sy *et al.* (2001b) concluded that ,the group of strains and isolates made up of facultatively methylotrophic root- nodule forming and nitrogen fixing bacteria may be regarded as a novel *Methylobacterium* species.

Data presented in Table (6) show that , PPFM isolates nearly had the same morphological and cultural characteristics, as compared with the reference rhizobial strains used. As well as , microscopic examination indicated that all isolates were short rods ,Gram negative and motile. Morphology of colonies was made using binocular indicated that all isolates had the same cultural properties, compared to the reference rhizobial strains used.These results were in agreement with Green *et al.* (1988), Jaftha *et al.* (2002) and Orf, Heba *et al .* (2005).

Table (3). Code and Number of PPFM isolates isolated from leaf surfaces of some legume crops growing in different Egyptian soil types.

Code of isolates	No. of isolates	Cultivation site	Soil type	Plant leaf samples	Scientific name
1. PPFM.A	4 4	Behera Menia	Silty loam	Chickpea	*Cicer arietinum*
2. PPFM.V	3 3	Behera Menia	Silty loam	Feba bean	*Vicia faba*
3.PPFM.Lt	4 11	Kalubia Menia	Clay loam	Lupine	*Lupinus termis*
4. PPFM.G	6	Menia	Clayey	Fenugreek	*Trigonella foenum*
5. PPFM.Ph	7 3	Giza Kalubia	Clayey	Common bean	*Phaseolus vulgaris*
6. PPFM.H	12	Behera	Calcourius	Peanut	*Arachis hypogaea*
7. PPFM.Sb	18	Menia	Loamy	Soy bean	*Glycine max*
8. PPFM.Tr	6 3	Kalubia Giza	Clayey	Egyptian clover	*Trifolium alexandrinum*
9. PPFM.M	4 3	Giza Menia	Clayey	Alfalfa	*Medicago stiva*
10. PPFM.D	٣	Behera	Sandy	Handaqooq	*Melilotus officinalis*
Total	94	4	٦	١٠	----------

Table (4). Ability of PPFM isolates to grow on Yeast Extract Mannitol (YEM) agar medium.

PPFM isolates	Growth on media (37°C / 72h)					
	No. of isolates	YEM	YEM +0.05% methanol	YEM+0.05% methanol without mannitol	YEM + Congo red	YEM +0.05% methanol + Congo red
1. PPFM.A	4 4	- -	- -	- -	- -	- -
2. PPFM.V	3 3	- -	- -	- -	- -	- -
3.PPFM.LT	4 11	+ +	++ ++	++ ++	+ +	+ +
4. PPFM.G	6	-	-	-	-	-
5. PPFM.Ph	7 3	- -	- -	- -	- -	- -
6. PPFM.H	12	-	-	-	-	-
7. PPFM.Sb	18	+	++	++	+	+
8. PPFM.Tr	6 3	+ +	++ ++	+ +	- -	++ ++
9. PPFM.M	4 3	++ ++	++ ++	- -	- -	- -
10.PPFM.D	3	+	++	++	+	+

(-) No growth (+) 10^{6}-10^{7} cfu ml^{-1} (++) 10^{8}-10^{9} cfu ml^{-1}

Table (5). Cross inoculation experiment to show the formed nodules on roots of some legume plants by PPFM isolates.

host plant / Isolates	Lupine	Soybean	Egyptian clover	Alfalfa
1-PPFM.Lt	++	+	-	-
2-PPFM.Sb	+	++	-	-
3-PPFM.D	+	+	++	++
4-PPFM.Tr	-	-	-	-
5-PPFM.M	-	-	-	-

(-) No nodule formation (+) Nodule formation (≤ 12nod. Plant^{-1})
(++)Nodule formation (> 12nod. Plant^{-1})

Table (6). Some morphological and cultural characteristics of PPFM isolates as compared with rhizobial strains.

Isolates / Characters	PPFM.Lt	PPFM.Sb	PPFM.D	USAD 110	ARC 408
Morphological characters (smear):					
- Cell shape	Short rod	Short rod	Short rod	Short rod	Short rod
-Gram Reaction	G^{-ve}	G^{-ve}	G^{-ve}	G^{-ve}	G^{-ve}
-Motility	Motile	Motile	Motile	Motile	Motile
Colony morphology (solid medium):					
-Shape	Circular	Circular	Circular	Circular	Circular
-Diameter(m.m)	0.5-1.0	1.0-2.0	1.0-2.0	1.0-2.0	0.5-1.0
-Opacity	opaque	Opaque	opaque	translucent	translucent
-Elevation	Convex	Convex	Convex	Convex	Convex
-Edge	Entire	Entire	Entire	Entire	Entire
-Color	Pale Pink	Pink	Pink	White	White

Data in Table (7) reveal some physiological characteristics of the obtained PPFM isolates, as compared with the reference rhizobial strains. The three isolates (PPFM.Lt, PPFM.Sb and PPFM.D) and two reference rhizobial strains (USAD 110 and ARC 408) were catalase positive . Utilization of different carbon sources showed that , all isolates and strains can utilize sucrose and D-glucose, except PPFM.Lt, and utilized citrate and methanol, except both rhizobial strains (USAD 110 and ARC 408). These results are in agreement with those obtained by Sy *et al.* (2001a), Jaftha *et al.* (2002) and Orf, Heba *et al* .(2005).

For studying antibiotic resistance of the three PPFM isolates and two rhizobial strains, five antibiotics were used as shown in Table (2) to clear up the obtained results in Table (8), which showed that, the three PPFM bacteria were resistant to Ampicillin ,Gentamycin and Colistin antibiotics and were susceptible to Kanamycin and Streptomycin antibiotics, while the two rhizobial strains were resistant to all antibiotics tested .These results was in agreement with Jourand *et al.*(2004) and Orf, Heba *et al* .(2005).

Table (7). Some physiological characteristics of PPFM isolates as compared with Rhizobial strains.

Isolates / Characters	PPFM.Lt	PPFM.Sb	PPFM.D	USDA 110	ARC 408
Catalase	+	+	+	+	+
Carbon source utilization:					
-Sucrose	-	+2	+3	+1	+2
-Mannitol	+2	+3	+3	+3	+3
-Glycerol	+2	+2	+3	+3	+3
-D-glucose	-	+2	+3	+2	+2
-Ethanol	+1	+2	+2	+2	+2
-Citrate	+1	+2	+3	-	-
-Methanol	+3	+3	+3	-	-

(-) No growth (+1) 10^{3}-10^{5} cfu ml^{-1}
(+2) 10^{6}-10^{7} cfuml^{-1} (+3) > 10^{8}cfu ml^{-1}

Table (8). Effect of different antibiotics on PPFM isolates and the reference rhizobial strains growth as measured by diameter of Inhibition zone (mm).

Antibiotics	concentrations	Inhibition Zone Diameter (mm)				
		PPFM.D	PPFM.Lt	PPFM.Sb	USAD110	ARC 408
Ampicillin	10µg	0.0 (R)	0.0 (R)	10.0 (R)	10.0 (R)	8.0 (R)
Gentamycin	10µg	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)
Colistin	10µg	0.0 (R)	0.0 (R)	20.0 (S)	20.0 (S)	18.0 (S)
Kanamycin	30 µg	0.0 (R)	0.0 (R)	15.0 (S)	16.0 (S)	7.0 (R)
Streptomycin	50µg	0.0 (R)	0.0 (R)	0.0 (R)	3.0 (R)	7.0 (R)

(R): Resistant (S): Susceptible

II. Molecular Biology Studies

To study the similarity among PPFM isolates and rhizobial strains , Protein pattern and Random Amplification of the DNA tests were carried out.

Protein pattern of PPFM isolates and rhizobial strains :

Cluster analysis of protein marker data placed the bacterial PPFM isolates and rhizobial strains into two main groups (Fig .1).The similarity between the three PPFM isolates and the two rhizobial strains ranged from 65.01 to 92.5%.

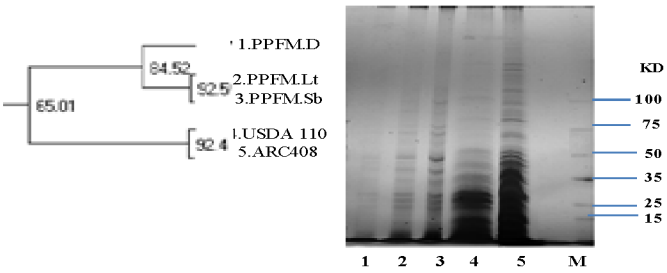

Fig. (1): Protein pattern of PPFM isolates and rhizobial strains using Sodium Dodecyle Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE).

The first major cluster included PPFM isolates was divided into two minor clusters (a and b). The minor cluster(a) included the isolate PPFM.D at a similarity of 84.52% of the minor cluster (b). The highest similarity (92.5%) came between PPFM. Lt and PPFM.Sb (cluster b), whereas the similarity between USDA 110 and ARC 408 was 92.4%. There had been comparative taxonomical studies of methylotrophic bacteria and rhazobia. These results are on line with those obtained by Urakami *et al.* (1985), Jenkins and Jones (1987) and Hood *et al*. (1988).

RAPD analysis of DNA:

Data in Figs. (2 ,3 and 4) show that the random amplification of the DNA placed the three PPFM isolates and the two rhizobial strains into two major groups, which were divided into minor clusters, giving different degrees of polymorphism according to the used primers. The similarity; 1) at cause of using primer No. (2) ranged from 51.4% to 83.87%. 2) At cause of using primer No. (4) Similarity between the two main groups (PPFMs isolates and rhizobial strains) ranged from 54.57% to 85.85% .3) At cause of using primer No. (6) the similarity between the two main groups ranged from 37.38% to 92.29%.

In general, the highest polymorphism between PPFMs isolates and rhizobial strains in the protein profile and random amplification of DNA could be attributed to difference in metabolism or secretion of some different metabolites, and there were genetic diversity and phylogeny between them.The high similarity between PPFMs isolates and rhizobial strains gave an indication to a high genetically relatedness among them in nodule formation and nitrogen fixation (Sy *et al.*,2001b and Endalkachew,2005).

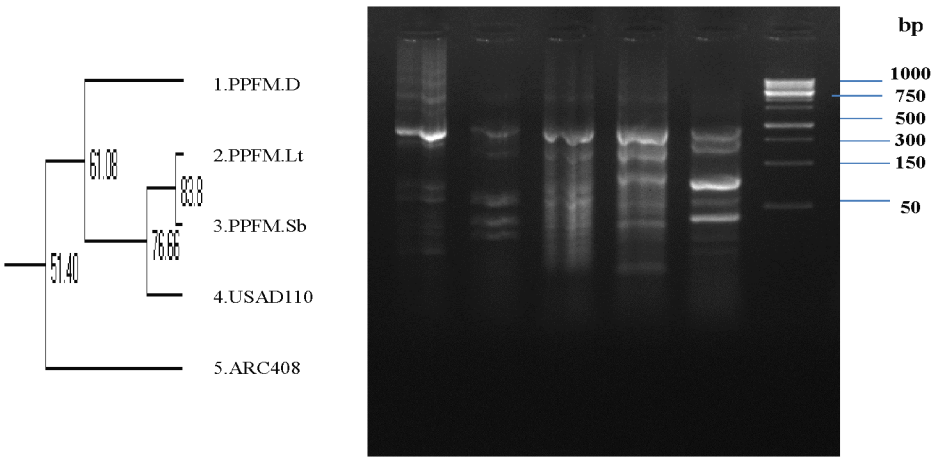

Fig. (2): Random Amplified Polymorphism DNA (RAPD) analysis using Primer(2).

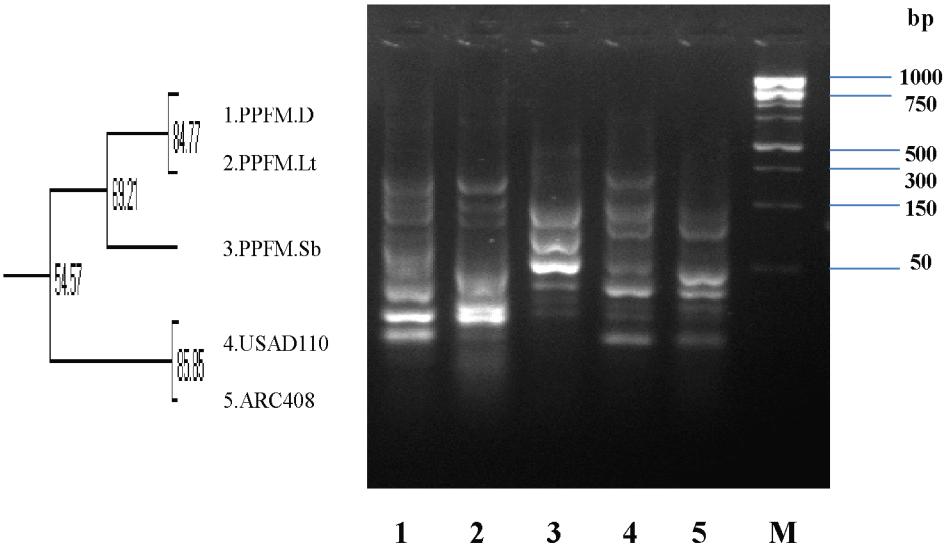

Fig. (3): Random Amplified Polymorphism DNA (RAPD) analysis using Primer(4).

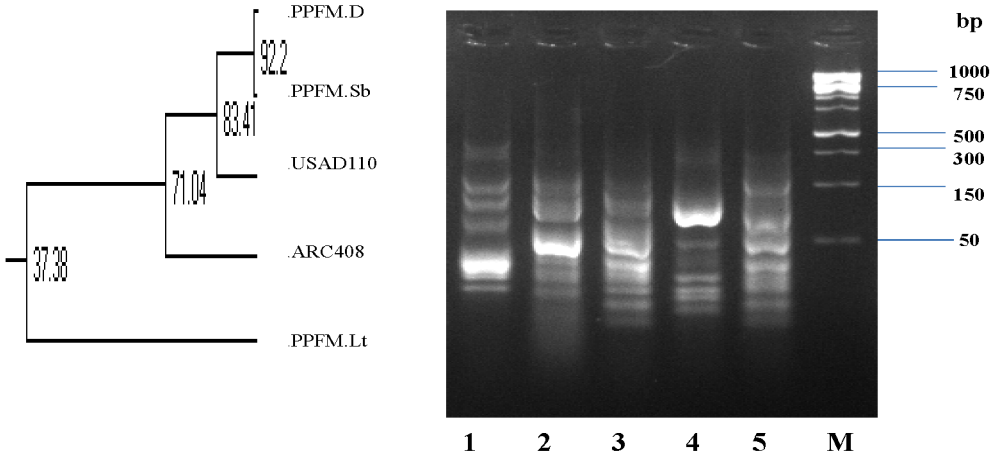

Fig. (4): Random Amplified Polymorphism DNA (RAPD) analysis using Primer(6).

III. Nodulation and Biological Nitrogen Fixation Activity of PPFMs

The symbiotic interrelationship and plant growth traits of the three methylotrophic isolates were studied under greenhouse conditions.The soil used was free of native rhizobia, but able to form noduls on plant roots of both soybean and lupine legume plants Fig. (5, a to c). Application of PPFM isolates (PPFM.D, PPFM.Lt and PPFM.Sb), as such or mixed with a rational amount of mineral N-fertilizer as well as the specific rhizobia(USAD 110 for soybean and ARC 408 for lupine),led to scored significant differences in the number of noduls , nodule dry weight , nitrogen percentage ,plant content of nitrogen derived from air (Ndfa %) and amount of fixed nitrogen . PPFM.D isolate recorded the lowest nodule number per plant (28 nodules plant^{-1}), as compared with PPFM.Lt , PPFM.Sb and the mixturs and these numbers were 56-50 , 67-36 and 53-33 for soybean and lupine, respectevily. Moreover , PPFM.Sb and PPFM.Lt recorded high nodule numbers and nodule dry weights, compared to USAD 110 and ARC 408 rhizobial strains, as well as led to scored significant increases , which were 81 ,40 % and 49 ,41 %for the nodule numbers and nodule dry weights of soybean and lupine respectively. There were no significant differences for shoot dry weights (g plant^{-1}) between application of PPFM.Sb and PPFM.Lt, as compared with both USAD 110 and ARC 408 rhizobial strains. Application of USAD 110 had a higher value of plant N-content but no significant difference was found when compared to the applied of PPFM.Sb.

Results in Fig.(5) clearly show that ,the values of nitrogen derived from air (Ndfa %)and N_2-fixed ranged from 84.82 to 93.75 % and 86.95 to 93.38% for soybean and lupine plants, respectively. Application of rhizobial strains led to scored the higher amounts of nitrogen content by fixed atmospheric N_2 ,which attained 151.80 and 81.6 mg plant^{-1} for soybean and lupine plants and scored higher percentage (12.4 and 7.0 %) , compared to apply PPFM.Sb and PPFM.Lt, respectively. These results are in agreement with that of Abotaleb *et al.* (2003) , Thabet and Galal (2003) and Jourand *et al.*(2004), as they reported that legume plants could uptake more than 88% of their N-requirements from symbiosis relationship and fixed atmospheric nitrogen.

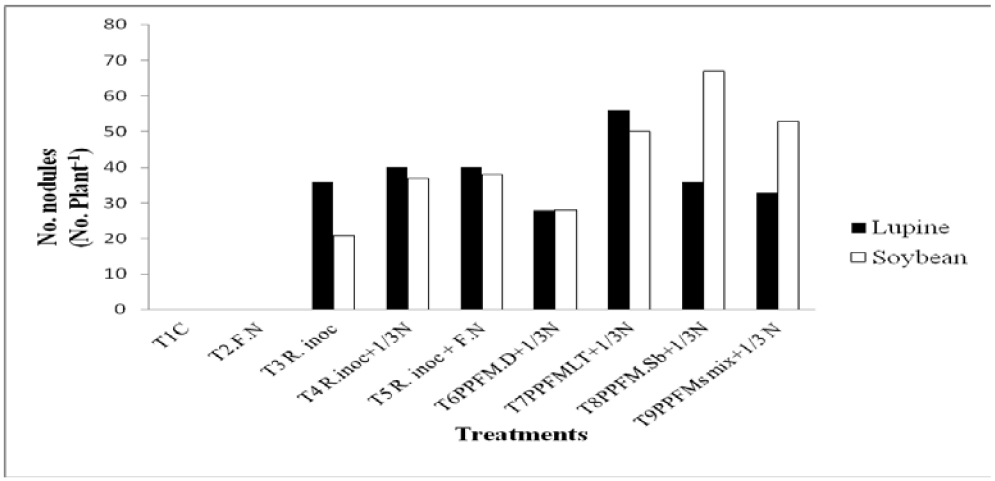

Fig.(5a)Nodules number.

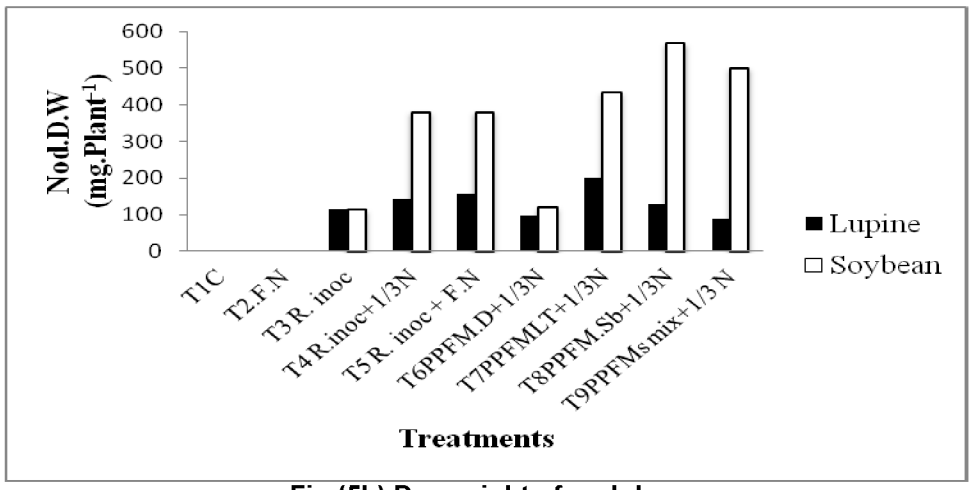

Fig.(5b) Dry weight of nodules.

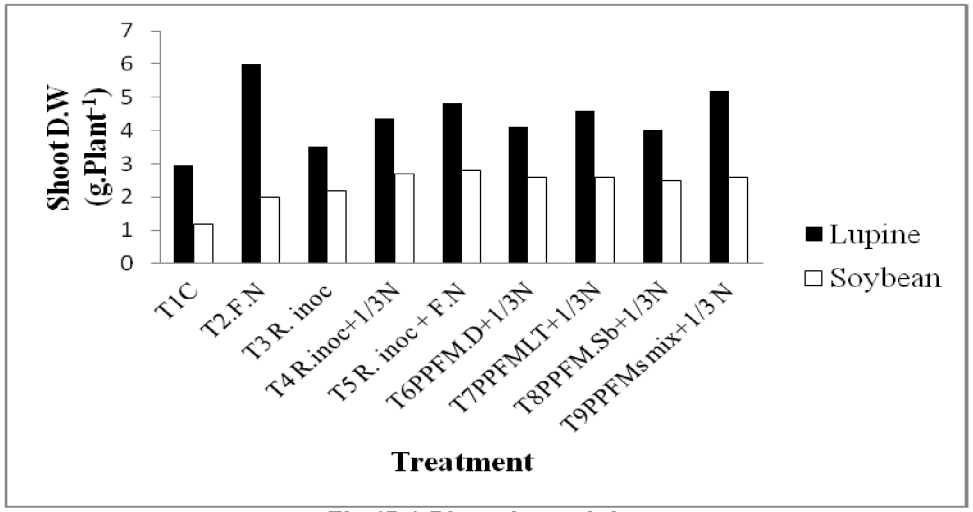

Fig.(5c) Plant dry weight.

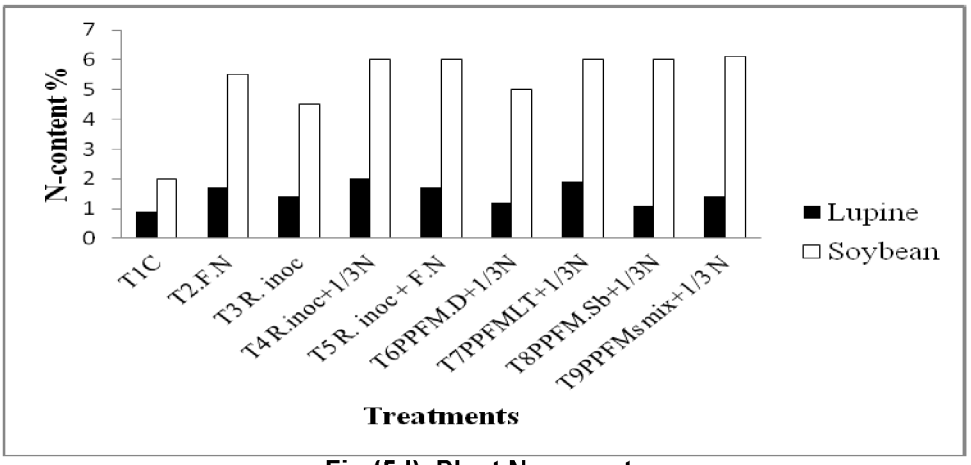

Fig.(5d) Plant N-percent.

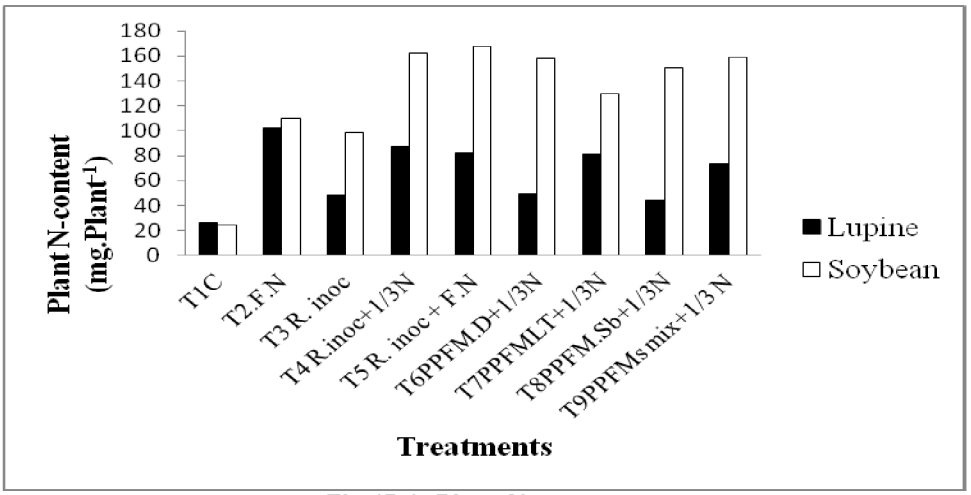

Fig.(5e) Plant N-content.

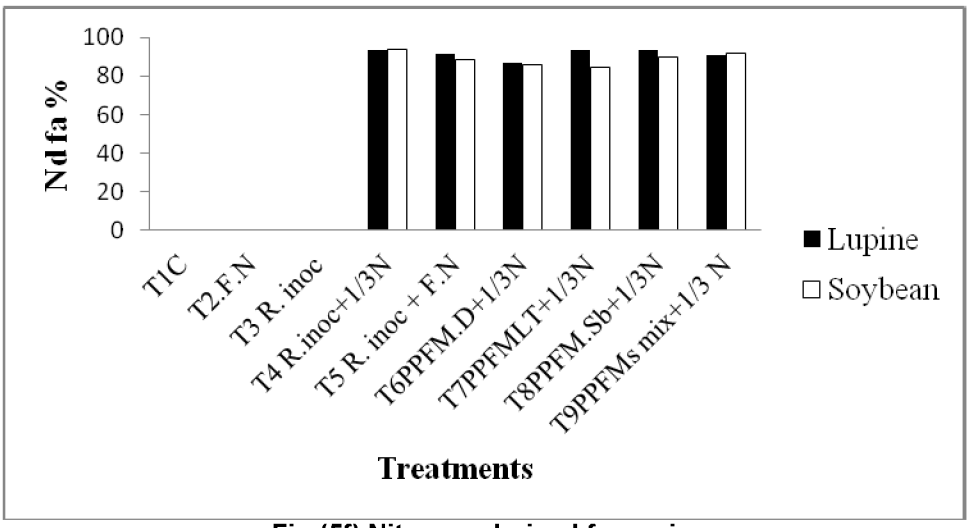

Fig.(5f) Nitrogen derived from air.

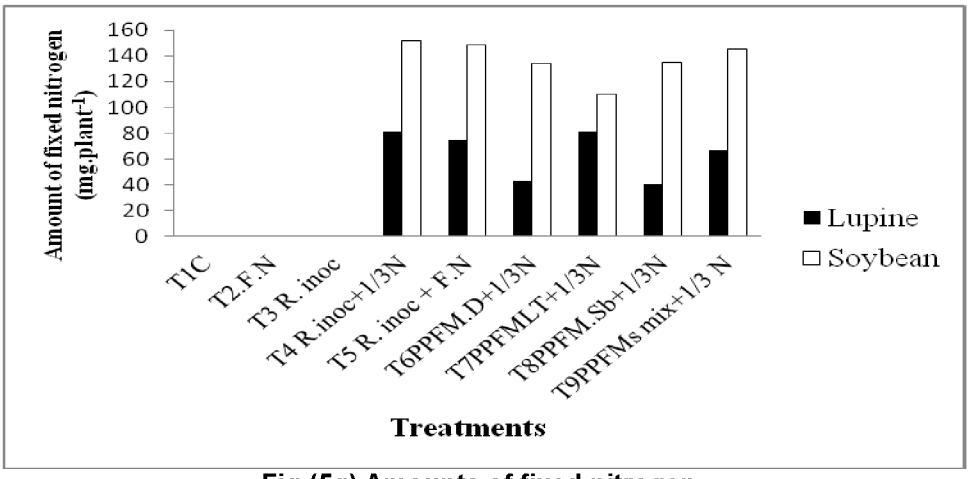

Fig.(5g) Amounts of fixed nitrogen.

Fig. (5): Nodule numbers, dry weight of nodules, plant dry weight, plant N-content, nitrogen derived from air (Ndfa) and amount fixed of lupine and soybean plants as affected by inoculation with various PPFM isolates with or without specific rhizobia.

The experimental data of morphological ,physiological ,molecular biology and biological nitrogen fixation studies were used for identification of the most efficient PPFM isolate (PPFM.Lt) according to Bergey's Manual of Systematic Bacteriology 2nd Ed (2005) and Madhiayan *et al.* (2009),who reported that ,general characteristics of *Methylobacterium nodulans* were:

1-Short rods ,gram negative and motile .
2-Catalase positive.
3-Utilize methanol ,citrate ,ethanol,glycerol and mannitol as sole sources of carbon but unable to utilize sucrose and D-gloucose as sole carbon sources .
4-Resistant to ampiciilin and gentamycin and susceptible to kanamycin and streptomycin.
5-Induce root nodule formation and fix atmospheric nitrogen with various legume host plants.

CONCLUSION

From the above mentioned results, it could be concluded that, the novel isolate(PPFM.Lt), which belongs to nitrogen fixing methylotrphic bacteria, was successfully isolated from legume plants (Lupine) for the first time under Egyptian conditions, and proved the ability to form nodules on roots of the host legume plant (Lupine) and successfully fix atmospheric nitrogen.This strain could be used later as a biofertilizer for nitrogen requirement of legumes.

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دراسات مقارنة بين البكتيرياميثيلية التغذية المثبتة للنيتروجين والريزوبيا لبعض النباتات البقولية

هبه عرف محمد [١]، وداد التهامي السيد عويضة[٢] ، سوسن فوزي شحاتة[٢] ,

حاتم حسين يوسف أبو طالب[١]

[١] قسم الميكروبيولوجيا الزراعية– معهد بحوث الأراضي والمياه والبيئة – مركز البحوث الزراعية –الجيزة.

[٢] قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة عين شمس– شبرا الخيمة– القاهرة.

الملخص العربى

تم عزل ٩٤عزلة تابعة للبكتريا ميثيلية التغذية (PPFMs) من سطح أوراق وجذور العديد من النباتات البقولية،وذلك لإجراء بعض الدراسات الخاصة بقدرة عزلات PPFMs على النموعلى بيئة YEM فى وجود أو عدم وجود ٠.٠٥% ميثانول كمصدر للكربون . كما أجريت بعض الدراسات المورفولوجية والفسيولوجية والبيولوجيا الجزيئية وكذلك تقدير نيتروجين الهواء الجوى المثبت حيويا باستخدام النظير المشع ^{15}N. وأضحت النتائج أن كل من عزلات PPFM.Lt , PPFM.Sb , PPFM.D والتى تم عزلها من العوائل البقولية (الترمس وفول الصويا

والحندقوق على الترتيب)لها القدرة على النمو على بيئة YEM فى وجود أو عدم وجود ٠.٠٥% ميثانول كمصدر للكربون أو أضافة صبغة أحمر الكونغو. وكذلك لها القدرة على التحفيز وتكوين العقد الجذرية على جذور العوائل البقولية .ووجد أن عزلات PPFMs تتشابه مع سلالات الريزوبيا ARC 408, USAD 110 من الناحية المورفولوجية ,حيث أنها عصويات قصيرة سالبة لجرام ومتحركة .أظهرت الأختبارات الفسيولوجية أن عزلات PPFMs موجبة لأختبار الكتاليز. كما ان جميع العزلات لها القدرة على أستخدام السكروز والجلوكوز فيما عدا PPFM.Lt ، والتى لم تستطع أستخدامه كمصدر للكربون . و كذلك فإن سلالات الريزوبيا لم تستطع أستخدام الميثانول والسترات كمصدر للكربون . ووجد ان جميع عزلات PPFMs مقاومة للأمبسلين والكولستين والجنتاميسين وحساسة للكاناميسين والأستربتوميسين بينما أظهرت سلالات الريزوبيا مقاومة لجميع المضادات الحيوية المستخدمة .وأوضحت نتائج أستخدام التفريد الكهربى للبروتين أن كلا من عزلات PPFMs و سلالات الريزوبيا تقع فى مجموعتين رئيسيتين ذات تشابه وراثى يتراوح بين ٦٥.٠١% الى ٩٢.٥%. وعند أستخدام البصمة الوراثية للحمض النووى لعزلات PPFMs وسلالات الريزوبيا المتخصصةأن كلاهما تقعان فى مجموعتين رئيسيتين ذات تشابه وراثى يتراوح بين ٥٣٧.٣٨% الى ٩٢.٢٩% حسب نوع البادئات المستخدمة.أدى أستخدام العزلات التابعة للبكتريا ميثيلية التغذية ، سواء كانت كل مزرعة على حدة أو فى صورة مختطلة فى وجود الجرعة السمادية المرشدة من النيتروجين والتلقيح بالريزوبيا المتخصصة (USAD 110 لمحصول فول الصويا و ARC 408 لمحصول الترمس) وذلك تحت ظروف الصوبة –الى فروق معنوية فى عدد العقد ووزنها الجاف ونسبة النيتروجين والمحتوى النيتروجينى والنسبة المئوية لنيتروجين الهواء الجوى المثبت حيويا (% Ndfa) وكمية النيتروجين المثبتة. وأدى تطبيق أستخدام سلالات الريزوبيا المتخصصة الى الحصول على قيم أعلى بالنسبة للمحتوى النيتروجينى خلال عملية التثبيت الحيوى للنيتروجين الجوى.. ومن النتائج المتحصل عليها يمكن القول بان عزلة PPFM.Lt المعزولة من نباتات الترمس قد تنتمى الى*Methylobacterium nodulans* .