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ISOLATION AND IDENTIFICATION OF SOME PPFMs BACTERIAL ISOLATES AND THEIR POTENTIALITY AS BIOFERTILIZERS AND BIOCONTROL AGENTS TO *Rhizoctonia solani*

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ABSTRACT

A total of 12 isolates of pink-pigmented, facultative methylotrophic bacteria (PPFMs) were isolated on selective AMS medium from clay soil. Samples were collected from two locations (Sidi Salem and Agricultural Farm, Sakha Agri. Res. Station). There after, all the classical recommended methods used for identification of all the obtained isolates were carried out including cultural, morphological, microscopical as well as biochemical characteristics. The results revealed that all the isolates were gram negative, rod shaped, motile, colonies were light pink or pink or orange/pink in colour due to pigmentation. Biochemical characteristics revealed that all the isolates showed positive results for oxidase, catalase, urease and citirate test as well as indole production. None of the isolates showed positive results for casein hydrolysis. The isolates of ML_4, ML_8 and ML_{10} gave a positive reaction for starch hydrolysis test, while the others showed negative results. Regarding, cellulose degradation, the isolates ML_2, ML_7,ML_8 and ML_{12} gave a positive reaction. The obtained results highly recommended that all the obtained isolates could be classified as follows: ML_1 and ML_6 *Methylobacterium radiotolerans;* ML_2 *and* ML_5 *Methylobacterium suomiense;* ML_3 *Methylobacterium aminovorans;* ML_4 and ML_9 *Methylobacterium thiocyanatum;* ML_7, ML_8 and ML_{10} *Methylobacterium rhodesianum*; ML_{11} *Methylobacterium fujisawaens* and ML_{12} *Methylobacterium rhodinum.* Screening the isolates for beneficial growth characters and biocontrol agent to *R. solani.* In general, *Methylobacterium rhodinum.*and *Methylobacterium aminovorans* recorded the highest values of indole acetic acid production, 28.15 and 27.50 µg ml^{-1}, and 70.41 and 67.46 µg ml^{-1} for GA production, and 0.580, 0.560 µ moles of siderophores production, and 1.217 and 1.193 mg N/g for fixed nitrogen respectively. Also, the two species exhibiting obvious antibiosis against *R. solani* as recorded 72.61 and 71.20% for inhibition of *R. solani* on PDA medium, and 69.30 and 66.80% for inhibition of *R. solani* on AMS medium respectively.

Keywords: PPFMs bacteria, *R. solani,* indole acetic acid, gibberellic acid, siderophores.

INTRODUCTION

Bacteria by far the most important a bundant organisms in the soil and they play a key role in nutrient cycling and soil fertility. Various interactions occur between bacteria and plant roots that can beneficial, neutral or harmful. Several rhizobacterial strains have been found to increase plant growth, called plant growth promoting rhizobacteria (PGPR) (Kloepper and schroh, 1980). In the similar ways suppressive of diseaes provoking microorganisms can occur through microbial antagenses in the rhizosphere. Methylotrophic

bacteria is successful example that can achives the two purposes as PGPR with antifungal activity. Many of the microbes living on the phylloplane probably lead a saprophytic lifestyle, feeding on materials leached from the leaf. One such example is *Methylobacterium* sp. a pink pigmented facultative methylotroph (PPFM) which was first identified as covert contaminants from the tissue culture of liverwort, *scapania nemorosa* (Basile *et al.*, 1969). This bacterium provides a useful model for the unappreciated kinds of interactions between plants and bacteria that take place routinely on lab and in culture dishes (Green and Bousifield, 1982).The genus *Methylobacterium* is composed of a variety of pink pigmented methylotroph (PPFM) and non-pigmented facultative methylotroph (NPFM) bacteria which are capable of growing on C1 compounds such as formate, formaldehyde, methanol and methylamine as well as on a wide range of multicarbon growth substates such as C2, C3 and C4 compounds. PPFMs are ubiquitous in nature and frequently reported on various plant species, those are a substantial part of the aerobic, heterotrophic microflora of the surfaces of young leaves. These bacteria are commonly found in soils, as well as on the surfaces of leaves, seeds and in the rhizosphere of a wide variety of plants, with highest numbers on actively growing and meristamatic tissue (Holland, 1997) Methylotrophs have been reported to influence seed germination and seedling growth by producing plant growth regulators like zeatin and related cytokinins and auxins and to alter agronomic traits like branching, seedling vigour, rooting and heat/cold tolerance (Omer *et al.*, 2004).

Gibberellic acid is a group of plant growth regulators, which act by modifying the plant morphology (Atzorn *et al.*, 1988). It induces the uptake of minerals like K and Ca, increase the chlorophyll content, soluble sugars and protein content of the plants. Besides that it enhance better growth and faster elongation rate in shoot due to induction of active hairy root zone (Hamida and Elkomy, 1998). Some of the microorganisms that are reported to produce GA are *Rhizobium leguminosarum* bv. *phaseoli* (Jansen *et al.*, 1992), *Azospirillum brasilense* and *Azospirillum lipoferum* (Piccoli *et al.*, 1996).

Siderophores are low molecular weight, extracellular compounds with a high affinity for ferric iron. They sequester ferric iron, whose concentration is very low in well aerated soils, in a form that cannot be utilized by the pathogen, thereby reducing its number and / or activity. Earlier *Methylobacterium* belongs to α-protobacteria are grouped under *Pseudomonas*. Specific strains of *pseudomonas* produce siderophore which enhanced the plant growth and acted as biocontrol agent (Leong, 1986)

Poorniammal *et al.*, (2009) showed that methylobacterial isolates are significantly reduced the linear mycelial growth of plant pathogens like *Sclerotium rolfsii, Pythium, Fusarium oxysporum, Fusarium udum, Macrpohomina* and *Phytophthora in vitro*.

MATERIALS AND METHODS

1- Media used:

It is worthy to note that every media is used for isolation and study the characteristic of PPFMs bacteria:

a. Isolation of Pink-pigmented facultative methylotrophes by ammonium mineral salt medium (AMS), (Whittenbury *et al.*, 1970).
b. The growth of *R. solani* by potato dextrose agar medium (Okon *et al.*, 1977).
c. Estimation of fixed nitrogen by N-free malate medium (Okon *et al.*, 1977).
d. Estimation of indole production test by tryptone glucose broth medium (Seeley and Vandemark, 1981).
e. Estimation of casein hydrolysis test by skim milk agar medium (Smibert and Kreig, 1981).
f. Estimation of citrate utilization test by simmon's citrate agar medium (Seeley and Vandemark, 1981).
g. Estimation of cellulose degradation test by czapadox mineral salt agar medium (Seeley and Vandemark, 1981).

2- Isolation and purification of methylotrophs (PPFMs):

One gram of sample (soil) was grinded using pestle and mortar, serially diluted up to 10^{-6} dilutions and one ml of the aliquot from 10^{-4}, 10^{-5} and 10^{-6} dilutions was transferred to the sterile petri dishes. After plating with AMS medium, the plates were incubated for 3-5 days at 30°C. colonies of methylotrophs were picked, purified by the streak plate method and the well isolated colonies were preserved in AMS agar slants. Isolates were maintained on AMS slants at 4°C in a refrigerator for further use. All isolates were examined for their cell shape, motility and gram reaction. Also, all isolates were examined for oxidase, urease, catalase tests ; casein hydrolysis; starch hydrolysis, cellulose degradation, citrate utilization and indole production

All the isolates were tested for utilization of some carbon sources *viz.*, D-Glucose, Fructose, Ethanol, Citrate, Acetate, Tartarate ,Arabinose, Thiocyanate, Cyanate, D-Xylose, D-Fucose, L-Glutamate and Nutrient agar. These carbon sources were substituted for methanol in AMS medium at 0.5% (w/v) level. Presence of growth was observed after 15 days of inocubation at 30°C and growth was compared to a negative control containing no added carbon source.

3- Screening the efficiency of PPFMs isolates based on the production of some beneficial growth properties:

1. Indole acetic acid production:

The IAA production by PPFM isolates under *in vitro* condition was determined following the method of Ivanova *et al.* (2001). One hundred ml quantities of AMS liquid medium were dispensed in 250 ml Erlenmeyer flasks and sterilized at 1.5 atmospher for 15 min. Freshly prepared, filter sterilized solution of L-tryptophan was added to a final concentration of 100 mg l^{-1}, one ml of the standard inoculum (10^{-9} cells ml^{-1}) of PPFM isolate was added to each flask and incubated at 30°C. in a shaker. In order to avoid photo

inactivation of the biologically active compounds, the flasks were wrapped with black paper during incubation. After 7 days of incubation period, 25 ml of the sample was withdrawn and the cells were centrifuged at 5000 rpm for 15 min for quantitative estimation of IAA. Quantitative estimation of IAA production in PPFM cell free extract were done by spectrophotometric method (Sy *et al.*, 2001)

A quantity of 0.5 ml of the sample was taken in a test tube and 1.5 ml of distilled water was added followed by a 4 ml of Salper's reagent (1.0 ml of 0.5 M $FeCl_3$ and 50 ml of 35% $HClO_4$) and incubated in darkness for 1 hr at 28°C. The intensity of the pink colour developed was read in UV/Visible Spectrophotometer (Model 6705) at 540 nm. By referring to a standard graph prepared with chemical grade indole-3-acetic acid, the quantity of IAA in the sample was determined and expressed as µg $ml^{-1}$of culture filtrate.

2. Gibberellic acid (GA) production:

Extraction of gibberellins was done as described by Tien *et al.*, 1979. Three to four days old PPFM culture of AMS liquid medium was centrifuged for 15 min at 10,000 rpm and the supernatant was taken. The cell pellet was re-extracted with phosphate buffer (pH 8.0) and again centrifuged. Both supernatants were pooled, acidified at pH 2.5 using 5 N hydrochloric acid and partitioned (1:5) of ethyl acetate then was dried at 32°C and the residue redissolved in 2 ml of distilled water containing 0.05 percent of Tween 80.

Fifteen ml of ethyl acetate fraction was taken and 2 ml of zinc acetate solution was added (21.9 g of zinc acetate was dissolved in 80 ml of distilled water then one ml of glacial acetic acid was added and the volume made up to 100 ml with distilled water) After 2 min, 2 ml of potassium ferrocyanide solution was added (1.6 g of potassium ferrocyanide solution in 100 ml of distilled water) and the mixture was centrifuged at 10,000 rpm for 10 min. Five ml of supernatant was added to 5 ml of 30 percent hydrochloric acid and the mixture was incubated at 20°C for 75 minutes. The blank was prepared with 50 percent hydrochloric acid. The absorbance was measured at 254 nm in UV/Visible Spectrophotometer (Model 6705). From the standard graph using standard gibberellic acid solution the amount of GA produced by the PPFM isolates was calculated and expressed as µg ml^{-1} broth (Mahadevan and Sridhar, 1982).

3. Nitrogen estimation (microKjeldahl method):

To 250 ml conical flask 100 ml of the N-free malate medium was dispensed and autoclaved. Later one ml of 24 h old culture inoculum was added to each flask. The flasks were incubated at 37°C for seven days. After 7 days of incubation the culture was homogenized and 10 ml was digested with 5 ml of concentrated H_2SO_4 along with 0.2 g digestion catalyst mixture K_2SO_4 : $CuSO_4$: selenium (100:10:1). After cooling, volume was made up to 10 ml with distilled water. Later ten ml of aliquot were transferred to microkjeldhal distillation unit. The sample was mixed with 20 ml of 40 % NaOH Ammonia evolved was trapped in four percent boric acid mixed indicator (bromocresol green, 0.066 g and methyl red, 0.033 g in 100 ml methanol) till the solution turned from pink to green. It was titrated against

0.05 N H_2SO_4 and total nitrogen content of the culture was determined and results were expressed as mg N fixed per g of malate.

$$\text{Percent N} = \frac{\text{Titre value} \times 0.014 \times \text{N of } H_2SO_4 \times \text{Vol made}}{\text{Volume of sample used}} \times 100$$

4. Siderophores production

The PPFM isolates were grown in AMS liquid medium for 7 days in shaking inocubator at 250 rpm at 28°C. The cultures were centrifuged at 12000 rpm for 30 min and 20 ml of the supernatant filtrate was extracted twice with equal amount of ethyl acetate after adjusting the pH to 2.0 with 0.1 N HCl then evaporated to dryness and dissolved in 5 ml distilled water. Five ml of Hathway reagent (added 1 ml of 0.1 M ferric chloride and 1 ml of 0.1 N HCl to 100 ml of distilled water followed by 1 ml of 0.1 M potassium ferricyanide) were added to the assay solution and allowed to stand for the colour to develop. To estimate of siderophores, the absorbance was read in UV/Visible Spectrophotometer (Model 6705) at 700 nm with 2, 3-dihydroxy benzoic acid as standard according to Reeves *et al.*, 1983.

4- Antagonist effect of isolates against *R. solani* (*in vitro*):

To investigate the role of methylotrophs (PPFMs) as antagonistic agents against damping-off caused by *R. solani,* potato dextrose agar (PDA) and ammonium mineral salts (AMS) medium were performed in Petri-dishes (9 cm diam.) by applying a dual culture technique (Sadfi *et al.,* 2001). All isolates were streaked in one side of the plate and the host fungi *R. solani* (disc 5 mm in diameter cut from the edge of a 7 day-old culture were placed at the other side of the antagonist with 3 replicates. Plates were incubated at 30°C for 5 days. Reduction percent of *R. solani* after 5 days was calculated by the formula of Whipps (1987).

$$\text{Reduction \%} = \frac{R1 - R2}{R1} \times 100$$

Where:
R1 = growth in control plates (without antagonism)
R2 = growth in the presence of the bioagent

RESULTS AND DISCUSSION

1- Isolation and identification of methylotrophs (PPFMs):

Pink pigmented facultative methylotrophs (PPFMs) were isolated from clay soil samples collected which designated as ML_1 to ML_{12}. The results of the morphological characteristics of PPFM isolates are presented in Table 1. All the isolates were gram negative, rod shaped, motile and colonies coulor were light pink or pink or orange/pink pigmentation.The same observations was found by Green and Bousifield (1982) rod shape for young culture (72 h) but pleomorphic shape for old culture (7 d). Also, they added the motility of all

isolates may be by a single polar, subpolar or lateral flagellum, while, some isolates are not vigorously motile and all isolates are gram negative.

Table 1:Some morphological characters of the methylotrophs isolates.

Isolates	Cell shape	Gram reaction	Pigmentation	Motility
ML_1	Rod	Negative	Orange/pink	Motile
ML_2	Rod	Negative	Dark pink	Motile
ML_3	Rod	Negative	Dark pink	Motile
ML_4	Rod	Negative	Light pink	Motile
ML_5	Rod	Negative	pink	Motile
ML_6	Rod	Negative	Orange/pink	Motile
ML_7	Rod	Negative	Orange/pink	Motile
ML_8	Rod	Negative	pink	Motile
ML_9	Rod	Negative	pink	Motile
ML_{10}	Rod	Negative	pink	Motile
ML_{11}	Rod	Negative	pink	Motile
ML_{12}	Rod	Negative	Dark pink	Motile

Data presented in Table 2 show the results of some biochemical tests for the studied PPFM isolates. All the isolates showed positive reactions of oxidase, catalase, urease, citrate utilization and indole production, but these isolates showed negative results for casein hydrolysis test. In respected to starch hydrolysis, all isolates showed negative reaction except that of ML_4, ML_8 and ML_{10}. Regarding, cellulose degradation, the isolates ML_2, ML_7,ML_8 and ML_{12} gave a positive reaction.

Similar findings were obtained also by Thangamani (2005) He mentioned that starch hydrolysis test was positive for strains of NPFM-OS-03, NPFM-OS-04, NPFM-Co-01 and *Methylobacterium nodulans* ORS-2060. Cellulose degradation was positive for NPFM-OS-05. PPFM are capable of growing of C1 compounds as well as on a wide range of multicarbon substrates (Green and Bousifield, 1982 and Green, 1992).

Table 2: Some biochemical characteristics of the methylotrophs isolates.

Tests / Isolates	Oxidase test	Catalase test	Urease test	Citirate Utilization	Indole Production	Casein Hydrolys's	Starch Hydrolysis	Cellulose Degradation
ML_1	+	+	+	+	+	-	-	-
ML_2	+	+	+	+	+	-	-	+
ML_3	+	+	+	+	+	-	-	-
ML_4	+	+	+	+	+	-	+	-
ML_5	+	+	+	+	+	-	-	-
ML_6	+	+	+	+	+	-	-	-
ML_7	+	+	+	+	+	-	-	+
ML_8	+	+	+	+	+	-	+	+
ML_9	+	+	+	+	+	-	-	-
ML_{10}	+	+	+	+	+	-	+	-
ML_{11}	+	+	+	+	+	-	-	-
ML_{12}	+	+	+	+	+	-	-	+

Carbon utilization test:

The results clearly were compared to those cited in Bergeys Manual of Systematic Bacteriology (Green and Bousifield, 1982; Madhaiyan *et al.*, 2007 and Raja *et al.*, 2008). The obtained results highly recommended that all the obtained isolates could be classified as follows: ML_1 and ML_6 *Methylobacterium radiotolerans;* ML_2 *and* ML_5 *Methylobacterium suomiense;* ML_3 *Methylobacterium aminovorans;* ML_4 and ML_9 *Methylobacterium thiocyanatum;* ML_7, ML_8 and ML_{10} *Methylobacterium rhodesianum*; ML_{11} *Methylobacterium fujisawaens* and ML_{12} *Methylobacterium rhodinum.*

Anthony (1982) demonstrated that facultative methylotrophs could utilize different carbon sources and assimilate C1 compound via serine pathway. However, when they are grown on complex organic substrates they have complete (Tri carboxlic acid cycle, TCA). These results were as similar as our observations.

Table 3: Utilization of some carbon sources by PPFMs isolates.

Carbon sources	Isolates											
	ML_1	ML_2	ML_3	ML_4	ML_5	ML_6	ML_7	ML_8	ML_9	ML_{10}	ML_{11}	ML_{12}
D-Glucose	+	+	-	+	+	+	-	-	+	-	+	+
Fructose	-	+	+	+	+	-	+	+	+	+	+	+
Ethanol	W	+	-	-	+	W	-	-	-	-	+	+
Citrate	+	-	-	+	-	+	-	-	+	-	+	+
Acetate	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	-	-	+	-	+	-	-	+	-	+	-
Tartarate	-	-	-	+	-	-	-	-	+	-	+	-
Thiocyanate	-	-	-	+	-	-	-	-	+	-	-	-
Cyanate	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	+	-	-	+	-	+	-	-	+	-	+	+
D-Fucose	+	W	-	+	W	+	-	-	+	-	+	-
L-Glutamate	+	-	+	+	-	+	W	W	+	W	+	+
Nutrient agar	+	+	+	+	+	+	+	+	+	+	+	+

The carbon substrates were substituted for methanol in AMS medium at 0.5%(w/v)
+, Growth; -, no growth; W, weak growth; Mean of three replicates.

3. Screening of methylotrophs isolates for production of some growth promoting substances and other beneficial characters:

1. Indole-acetic acid (IAA) production:

All the isolated isolates were found to produce indole acetic acid (Table 4) but with varying degree. *Methylobacterium rhodinum* was superior in producing IAA as it recorded (28.15 µg ml^{-1}) followed by *Methylobacterium aminovorans* (27.50 µg ml^{-1}). While *Methylobacterium fujisawaens* was recorded the least in this respect (9.04 µg ml^{-1}).

Costacurta and Vanderleyden (1995) Mentioned more detailes about the biosynthesis of indole acetic acid. They concluded that several IAA biothynesis with the serine pathway of C1 metabolism (*Methylobacterium mesophilicum* and *Aminobacter aminovorans*), the ribulose monophosphate pathway (*Methylobacterium mays*) and the ribulose biphosphate pathway (*Paracoccus kondratievae*) synthesize IAA, ILA and IPA respectively. The serine pathway bacteria, indole-3 acetamide was also detected IAA is synthesized from tryptophan through tryptamine in three steps of which only

the first step (the decarboxylation of tryptophan) is specific to this biosynthetic pathway. It is surprised that tryptophan decarboxylase and tryptophan sidechain oxidase (TSO) have not yet been found in *Methylobacterium* sp. (Ivanova *et al.*, 2001, Madhaiyan (2002), Senthilkumar (2003) and Thangamani and Sundaram (2005b) have also reported that the presence of tryptophan would increase the IAA production under *in vitro* conditions.

Many epiphytic and soil microorganisms are able to synthesize and secrete auxin, primarily IAA therefore, they influence the growth of plants either beneficially or adversely (Fett *et al.*, 1987). Such microorganisms like *Azospirillum*, *Rhizobium* and *Pseudomonas* may exert beneficial effects on plants while, phytopathogenic *Pseudomonas, Agrobacterium* and *Xanthomonas* bacteria exert adverse effects (Fett *et al.*, 1987). The synthesis of indole compounds in methylobacteria was found to be strongly inhibited by ammonium ions, the substitution of KNO_3 or $(NH_4)_2$ SO_4 in the cultivation medium augmented the amount of synthesized indole by 2 to 15 times, depending on the strain. The addition of tryptophan to the cultivation medium enhanced the synthesis of indole compound or production of auxin by Methylobacteria (Ivanova *et al.*, 2001).

2. Giberellic acid (GA) production:

All of the isolates showed positive results for gibberellic acid production (Table 4). *Methylobacterium rhodinum* still showed the superiority in producing GA production (70.41 µg ml^{-1}) followed by *Methylobacterium aminovorans* (67.46 µg ml^{-1} of). While *Methylobacterium fujisawaens* was inferior in produce (27.12 µg ml^{-1}) only.

Katznelson and Cole (1965) reported that *Pseudomonas fluorescence* produced 1-14 µg ml^{-1} GA of culture filtrate. Also, Anu Rajan (2003) documented the production of giberellic acid by some of PPFM isolates and the GA production varies from 10.9 µg ml^{-1} to 106.97 µg ml^{-1}.

3. Siderophores production:

Siderophores production by the different isolates are presented in Table 4.

The maximum siderophors level was recorded by *Methylobacterium rhodinum* (0.580 µ moles of a-2,3,dihydroxy benzoic acid) followed by *Methylobacterium aminovorans* (0.560 µ moles of a-2,3, dihydroxy benzoic acid). While the minimum siderophores production was recorded by *Methylobacterium fujisawaens* (0.240 µ moles of a-2,3, dihydroxy benzoic acid). Holland (1997) has examined the role of PPFM in iron nutrition in *Vicia faba*. Under iron-limited conditions, *Methylobacterium mesophilicum* was found to produce siderophores with either methanol or galactose as the carbon source. The role of PPFM-produced siderophores by *Methylobacterium mesophilicum* have also been discussed. In the present study, results of the production of siderophores revealed that all the *Methylobacterium* isolates produced siderophores ranging from 0.240 µ moles of DHBA (*Methylobacterium fujisawaens)* to 0.580 µ moles of DHBA (*Methylobacterium rhodinum*). Similarly, Anu Rajan (2003) and Senthilkumar (2003) observed catechol type siderophore producted by facultative methylotrophs.

Table 4: Production of IAA (µg/ml), GA (µg/ml), Siderophors (µ moles) and fixed nitrogen (mg/g of malate) by *Methylobacterium* isolates.

Organism	IAA (µg/ml)	GA (µg/ml)	Siderophores (µ moles)	Fixed nitrogen (mg/g of malate)
*M. radiotolerans*1	24.16	57.81	0.510	0.933
*M. suomiense*1	25.99	63.64	0.533	1.187
M. aminovorans	27.50	67.46	0.560	1.193
*M thiocyanatum*1	20.40	52.81	0.472	0.710
*M suomiense*2	18.34	46.60	0.446	0.570
*M radiotolerans*2	14.44	41.80	0.360	0. 383
*M rhodesianum*1	18.92	47.30	0.467	0.633
*M rhodesianum*2	21.24	54.42	0.493	0.883
*M thiocyanatum*2	24.93	58.04	0.517	1.113
*M rhodesianum*3	20.41	52.94	0.479	0.713
M fujisawaens	9.04	27.12	0.240	0.433
M rhodinum	28.15	70.41	0.580	1.217

4. Amounts of fixed nitrogen:

The results presented in Table 4. show that the highest amount of fixed nitrogen was observed for *Methylobacterium rhodinum* (1.217 mg N/g of malate medium) followed by *Methylobacterium aminovorans* (1.193 mg N/g of malate medium), while the lowest amount of fixed nitrogen was recorded for *Methylobacterium radiotolerans* (0.383 mg N/g of malate medium).

Sy *et al.* (2001) reported that *M. nodulans*, a facultative methylotrophic species forming nodules on the legume plant, *Crotalaria* spp., the only species of that genus able to form nodules and fix nitrogen. On the other hand, *M. nodulans* type strain ORS2060, is able to form nitrogen fixing nodules in *Crotalaria glaucoides, Crotalaria perrottetii* and *Crotalaria podocarpa*. Similarly, another legume, *Lotonois bainesii* was also reported to be a host for nodulation and nitrogen fixation by pink pigmented *Methylobacterium* (Jaftha *et al.,* 2002).Also, Raja *et al.* (2006) isolated nodulating and non-nodulating *Methylobacterium* sp. from legumes with high nitrogenase activity. Similarly, Madhaiyan *et al.* (2006) isolated several nodulating *Methylobacterium* from tropical legumes such as field beans, cowpea, black gram, soybean, *Sesbania* with high nitrogenase activity. These isolates are able to form effective nodules in *Crotolaria juncea.*

4- Antagonestic effect(*in vitro*):

Data presented in Table (5) show the influence of the antagonistic efficiency of the different isolates PPFM bacteria against *R. solani* pathogen. Five days after inocubation, all microorganisms under study showed differences in their ability to inhibit mycelial growth of *R. solani* on PDA and AMS media at 30 °C..

The maximum mycelial growth inhibition percentage of *Methylobacterium* isolates which *Methylobacterium rhodinum* recorded 72.61 % and 69.30 %, while the minimum mycelial growth inhibition percentage was recorded for *Methylobacterium suomiense* (17.07 % and 16.40 %) on PDA and AMS media respectively.

El-Katatny *et al.* (2001) explained that inhibition zone in dual cultures is formed due to the production of volatile and non-volatile metabolites as well

as the production of extracellular hydrolytic enzymes. It has been reported that antibiotics and hydrolytic enzymes are not only produced together but act synergistically in mycoparasitic antagonism (Di Pietro *et al.*, 1993 and Schirmböck *et al.*, 1994). Reduction of fungal growth *in vitro* by some *PPFMs* isolates and formation of inhibition zones were presumably due to the metabolites released by the bacteria into the culture medium.

Poorniammal *et al.* (2009) reported that members of the genus *Methylobacterium* are well-known growth regulator producers and also having *in vitro* biocontrol ability of against the phytopathogen, *R. solani*. Where the isolate CO 47
significantly reduced the linear mycelial growth of *R. solani* to an extent of 52.2 % over control with an inhibition zone of 1.4 cm under *in vitro* conditions.

It is worthy to note that results obtained of the present study provide sufficient evidence to recommend the use of isolates *Methylobacterium rhodinum* and *Methylobacterium aminovorans* of (PPFMs) bacteria for future investigation as a good example of plant growth promoting rhizobacteria with successful biocontrol agents.

Table 5: Percent of mycelial growth inhibition rates of *R. solani* on PDA and AMS media by PPFMs bacteria at 5 days after inoculation.

Organism / Media		Mycelial growth inhibition (%)			
		PDA	GI	AMS	GI
Species of *Methylobacterium*	*M. radiotolerans1*	31.50	2	25.80	2
	M. suomiense1	17.07	1	16.40	1
	M. aminovorans	71.20	3	66.80	3
	M. thiocyanatum1	41.46	2	37.60	2
	M. suomiense2	19.90	1	16.10	1
	M. radiotolerans2	35.36	2	28.60	2
	M. rhodesianum1	66.80	3	28.90	2
	M. rhodesianum2	67.07	3	30.80	2
	M. thiocyanatum2	44.90	2	43.80	2
	M. rhodesianum3	65.80	3	33.90	2
	M. fujisawaens	50.24	3	46.80	2
	M. rhodinum	72.61	3	69.30	3

PDA : Potato dextrose agar., AMS : Ammonium mineral salts
GI : Growth inhibition, was measured on a scale from 0 to 3 (Korsten *et al.*, 1995), where 0 = no growth inhibition, 1 = 1 to 25% growth inhibition, 2 = 26 to 50% growth inhibition and 3 = 51 to 75% growth inhibition.

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عزل و تعريف لبعض عزلات PPFMs البكتيرية وكفاءتها كمسمدات حيوية ومضادات حيوية لفطر الريزوكتونيا سولاني

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تم عزل عدد ١٢ عزلة من بكتيريا PPFMs علي بيئة AMS من التربة الطينية حيث جمعت العينات من موقعين (سيدي سالم – المزرعة البحثية بمحطة البحوث الزراعية بسخا) وتم دراسة الخصائص المورفولوجية، البيوكيميائية والتعريف لهذه العزلات.

وتبين من النتائج أن هذه العزلات البكتيرية سالبة لجرام- عصوية الشكل- ومستعمراتها علي البيئة الصلبة وردية اللون أو الوردي الفاتح أو الوردي/ البرتقالي وذلك راجع لإنتاج الصبغة. ومن الخصائص البيوكيميائية تبين أن هذه العزلات أعطت نتائج إيجابية لإختبارات الأكسوديز- الكاتاليز- اليوريز- السترات وإنتاج الأندول كما أنها غير موجبة لإختبار تحليل الكازين.

العزلات ML_4, ML_8 and ML_{10} أعطت نتائج موجبة لإختبار تحليل النشا بينما باقي العزلات كانت سالبة لهذا الإختبار أما فيما يتعلق باختبار تحليل السليلوز أظهرت العزلات ML_2, ML_7,ML_8 and ML_{12} نتائج موجبة. كما عرفت هذه العزلات علي أنها ML_1 and ML_6 *Methylobacterium radiotolerans;* ML_2 *and* ML_5 *Methylobacterium suomiense;* ML_3 *Methylobacterium aminovorans;* ML_4 and ML_9 *Methylobacterium thiocyanatum;* ML_7, ML_8 and ML_{10} *Methylobacterium rhodesianum*; ML_{11} *Methylobacterium fujisawaens* and ML_{12} *Methylobacterium rhodinum*.

أما من حيث دراسة هذه الأنواع علي إنتاج منظمات النمو فقد حققت *Methylobacterium rhodinum* and *Methylobacterium aminovorans* أعلي القيم حيث سجلت ٢٨,١٥ و ٢٧,٥٠ ميكروجرام/مل أندول أسيتك أسيد و ٧٠,٤١ و ٦٧,٤٦ ميكروجرام/مل حامض الجبريليك و ٠,٥٨٠ و ٠,٥٦٠ ميكرومول من السيدروفورز و ١,٢١٧ و ١,١٩٣ مليجرام/جرام نتروجين مثبت علي التوالي. كذلك نفس النوعين حققوا أعلي نسبة مقاومة لتثبيط نمو فطر *R. solani* بلغت ٧٢,٦١ و ٧١,٢٠% علي بيئة PDA و ٦٩,٣٠ و ٦٦,٣٠% علي بيئة AMS علي التوالي.

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