Arab Univ. J. Agric. Sci., Ain Shams Univ., Cairo, 13(3), 717-729, 2005

ISOLATION, PURIFICATION AND IDENTIFICATION OF SOME MICROORGANISMS PRODUCE PLANT GROWTH PROMOTING SUBSTANCES (METHYLOTROPHIC BACTERIA)

[47]

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ABSTRACT

Recently, the potential economical importance of the methylotrophic bacteria encouraged the isolation of this group. In the present study five Egyptian isolates were obtained from green leaves surface of legume plants named PPFM.C (ChickPea), PPFM.Ph (Common bean), PPFM.F (Faba bean), PPFM.P (Peanut) and PPFM.S (Soybean) ,to study their general characters which belonging to methylotrophic bacteria. Morphological studies indicate that all isolates were short rods, gram negative and motile. All Physiological studies to the isolates gave the same results except PPFM.F which could not grow in peptone medium. All isolates were sensitive to Kanamycin but they were resistant to Erythromycin. There was a great range in the ability of the isolates to grow on different sodium chloride concentrations indicating that PPFM.Ph grew well in 5 % sodium chloride, and they were able to excrete and produce cytokinin. Molecular biology studies indicated that there was a great similarity between PPFM.C and PPFM.Ph (99.34%). Identification was carried out to the5 isolates, PPFM.F may be related to *Methylobacterium mesophilicum*, PPFM.P may be related to *M. fujisawaense* and PPFM.Ph, PPFM.C and PPFM.S were related to *M. radiotolerans*.

Key words: Methylotrophs, Pink-Pigmented Facultatively Methylotrophic (PPFM), Green leaves of legume plants, Cytokinin, *Methylobacterium* sp.

INTRODUCTION

The last century, the vast development of industrial chemistry created different agrochemical compounds for use as fertilizers, pesticides, soil conditioners and phytohormons. The extensive use of such chemicals for increasing production of sustainable agriculture led to increase both production cost and environmental pollution. However one of the most important factors to build up soil fertility

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and plant growth is the ability of microorganisms to produce and release various growth promoting substances. Plant Growth Promoting Rhizobacteria (PGPR) can affect plant growth in two different ways indirectly or directly. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deteriorus effect of one or more phytopathogenic organisms. While, the direct promotion of plant growth by PGPR from the most potential either providing the plant with a component that is synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment **(Frankenberger & Arshad, 1995 and Gilick, 1995).**

Many microbes live on phylloplane and feed on materials leached from the leaf. A variety of organic and inorganic substances has been found in leaf leachates including all essential elements, free sugars and alcohols, all amino acids, many organic acids, growth regulating substances, vitamins, alkaloids, phenols, auxins and cytokinins **(Basile *et al* 1985; Leifert *et al* 1991 and Holland & Polacco, 1994).**

The term methylotrophic is used to describe a wide variety of bacteria which can utilize single carbon compounds more reduced than carbon dioxide as a sole carbon source. Bacteria which utilize only such compounds are termed obligate methylotrophs; while those which can utilize more complex organic molecules are termed facultatively methylotrophes **(Whittenbury and Dalton, 1981)**

The most abundant group of methylotrophs isolated from surfaces of green plants were the Pink Pigmented Facultatively Methylotrophs (PPFMs) **(Corpe & Basile, 1982; Corpe, 1985 and Hoon & Kim, 1989).**

In addition to their ability to colonize the phyllosphere, members of *Methylobacterium* were also able to colonize the rhizosphere of the tested plant species **(Omer, 2004).**

The present work is aimed to isolate, purify and identificate some microorganisms produce plant growth promoting substances (PPFMs) from leaf surfaces of some legume plants.

MATERIAL AND METHODS

I. Isolation of Pure Colonies

Ammonium Mineral salts medium (AMS) was used to isolate and enrichment PPFMs bacteria, which content (gL^{-1}): $MgSO_4.7H_2O$ 1.0; $CaCl_2$ 0.2; iron complex 0.004; $(NH4)_2SO_4$ 0.5; Trace elements solution 0.5% (vol/vol); agar 12.5 (if added) and supplemented with methanol 0.05% (vol/vol) (Met-AMS medium) **(Whittenbury *et al* 1970).** Green leaves obtained from different legume plants were collected from different locations (Behera, Giza and Nubaria Research stations, Agricultural Research Center (ARC) and

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used to obtain single colony with Pink Pigmented Facultative Methylotrophs (PPFMs) according to **Corpe, (1985).** Identification was carried out according to **Bergey's manual of determinative bacteriology, 9th edition, (1994).**

II. Morphological and Staining Characteristics (Barrow and Feltham, 1993)

Pure colonies were examined microscopically to determine gram reaction and shape. Whereas motility was tested in liquid culture. Morphology of colonies were determined for each isolate which streaked on solid Met-AMS medium and incubated for 3 days at 28°C, and using a binocular microscope.

III. Physiological Characteristics of PPFM isolates (Jenkins and Jones, 1987)

III.1. Catalase test

Isolates growth for 24 h were emulsified with 20 vol. H2O2 and observed for the production of effervescence for up to 1 min.

III.2. Carbon source utilization

Different carbon sources D- glucose (D-$C_6H_{12}O_6$) and ethanol (CH_3CH_2OH) were used separately to study the ability of PPFM isolates to grow on different carbon sources by adding 0.2% (w / vol) on carbon base in AMS agar medium.

III.3. Citrate utilization

Plates containing Simmon's citrate agar medium **(Simmon, 1926)** were inoculated with 1 ml of each obtained PPFM isolates and incubated for 96 h at28°C then examined for citrate utilization.

III.4. Growth on peptone rich nutrient agar (Oxoid CM55), (Green *et al* 1988)

Peptone rich nutrient agar plates were prepeared and streaked with loopful of each PPFM isolates which obtained and incubated at 28°C for 96 h then examined for growth.

III.5. Starch hydrolysis (Lelliot and Stead, 1987)

Starch was added at 0.2 % (w/vol) before sterilization of Met-AMS. agar medium (121°C for 20 min) ,plates were inoculated with 1 ml of each PPFM isolate and incubated for 96 h at 28°C .Iodine solution was used to flood the incubated plates then examined for starch hydrolysis.

III.6. Acid production from glucose (Jenkins and Jones, 1987)

Met-AMS broth medium with 1%(w/vol) glucose was inoculated by each isolate and incubated at 28°C from 1 to 7 days, Using an equal volume of 0.2 %(w/v) aqueous bromothymol blue

III.7. Staining of metachromatic granules (volutin granules) by using methylene blue (Mahmoud, 1988)

Prepared A good smear of each PPFM isolate and flood its slides with 1% methylene blue solution for 1 min, after removing methylene blue solution adding

a drop of oil immersion and examined these slides for volutin granules.

III.8. Acetylene Reduction Activity (ARA)

The ARA of PPFM cultures were estimated according to **Hardy *et al* (1973)** using DEL-SI-DN 200/250 gas chromatograph.

III.9. 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.

Arab Univ. J. Agric. Sci., Ain Shams Univ., Cairo, 13(3), 717-729, 2005

Table 1. Antibiotics resistance standard range (Inhibition zone diameter, mm)

Antibiotics	Concentrations	Diameter (mm)		
		Resistant	Intermediate	Susceptible
Ampcillin	10µg	≤13	14-16	≥ 17
Bacitracin	10unit	≤ 8	9-12	≥ 13
Erythromycin	15µg	≤15	16-20	≥ 21
Kanamycin	30µg	≤13	14-17	≥ 18
Streptomycin	50µg	≤11	12-14	≥ 15

III.10. Tolerance to salinity

Gradient salt concentrations of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0% NaCl were prepared in Met-AMS broth to evaluate the tolerance of obtained PPFMs isolates to salinity, different concentrations of NaCl were inoculated by 1 ml of new PPFM cultures and incubated at 28°C for 0,24,48,96 and 144 hours and counting the appearance colonies on Petri dishes contain Met-AMS Agar medium at the same time in the same order.

IV. Cytokinin production

Radish (*Raphanus sativus*) cotyledons bioassay was used as qualitative method according to **Lethman (1968)** to study the ability of PPFM isolates to produce and release cytokinin .

V. Molecular biology studies

Studying the similarity among the PPFM isolates using protein pattern and random amplification of DNA as follows:

V.1. Electrophoretic studies of PPFMs protein by sodium dodecyl sulphate polyachrelamide gel electrophoresis (SDS-PAGE)

Protein pattern of PPFM isolates was done according to **Laemmili (1970),** using standard protein marker at three molecular weights (66, 48 and 29 K.d) and data were analyzed by 1. D Advanced program

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V.2. Random Amplified Polymorphism DNA (RAPD) analysis

RAPD analysis of PPFM isolates was done according to **Welliams *et al* (1990).** Protocol was carried out by using three primers: Primer No.1, Primer No.2 and Primer No.5. The different molecular weight of bands were determine against PCR marker promega G317A by un weighted pair-group method based on arithmetic mean (UPGMA).

RESULTS AND DISCUSSION

Five isolates of methylotrophs were obtained from green leaves of legume plants, the isolates and their sources were listed in Table (2), **Corpe and Basile (1982)** reported that the most abundant group of methylotrophs isolated from surfaces of green plants were the pink-pigmented facultatively methylotrophs (PPFMs).

Table 2. Code of PPFM isolates obtained from leaf surfaces of different legume plants

Location sites	Plant leaf samples	Scientific name	Code of isolates
Behera	Chickpea	*Cicer arietinum*	PPFM. C
Giza	Common bean	*Phaseolus vulgaris*	PPFM.Ph
Nubaria	Faba bean	*Vicia faba*	PPFM.F
Nubaria	Peanut	*Arachis*	PPFM.P

Data presented in Table (3) and Fig. (1) show that PPFM isolates nearly have the same morphological and cultural characteristics. Microscopically examination indicates that, all isolates were short rods, gram negative and motile. Morphology of colonies was done using binocular indicating that all isolates have the same cultural properties except PPFM.F colonies which appear bigger than the others.

Data in Table (4) reveal some physiological characteristics of PPFM isolates obtained from green leaves of different legume plants. The five isolates were catalase positive, utilize D-glucose, ethanol and citrate. All isolates grew well on peptone rich nutrient agar except PPFM.F. They were not able to hydrolyze starch, did not produce acid from glucose, contained volutine granules, while Acetylene Reduction Activity (µmole C_2H_4/h) was negative that mean the five isolates could not fix nitrogen. These results are in agreement with those obtained by **Corpe & Basile (1982); Jinkens & Jones (1987) and Hoon & Kim (1989)**.

Resistance of PPFM isolates to antibiotics

For studying antibiotic resistance of PPFM isolates using Table (1) to clear the data in Table (5) which show that, PPFM.Ph was the most susceptible one

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for various antibiotics, PPFM.P had the highest resistance among all tested isolates. Kanamycin had the highest inhibiting effect on bacterial growth if compared with Erythromycin among other antibiotics. These data were in agreement with **Green *et al* (1988).**

Effect of different salt concentrations on PPFM isolates

Data in Table (6) indicate that four isolates can not grow in 5% NaCl, but PPFM.Ph isolate can grow at the same concentration, while there is no growth at 6% NaCl concentration for all PPFM isolates . In case of PPFM.S isolate there is no growth in 4% NaCl concentration **(**after 144h).

Cytokinin production

Figure (2) illustrate that PPFM isolates have The ability to produce and release cytokinin as measured by radish cotyledons bioassay, PPFM.Ph isolate gave the highest amount of releasing

Arab Univ. J. Agric. Sci., Ain Shams Univ., Cairo, 13(3), 717-729, 2005

Table 3. Some morphological and cultural characteristics of PPFM isolates:

Isolates / Characters	PPFM.C	PPFM.Ph	PPFM.F	PPFM.P	PPFM.S
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 (mm)	1.0-2.0	0.5-1.0	2.0-3.0	0.5-1.0	1.0-2.0
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Elevation	Convex	Convex	Convex	Convex	Convex
Edge	Entire	Entire	Entire	Entire	Entire
Color	Pink	Pink	Pink	Pink	Pink

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Fig. (1): General morphological characteristics of PPFM isolates.
(A) Gram staining. (B) Growth on Met-AMS agar plates.
(C) Colonies appearance on Met-AMS agar medium.

Table 4. Some physiological characteristics of PPFM isolates obtained from green leaves of different legume plants

Characters \ Isolate	PPFM.C	PPFM.Ph	PPFM.F	PPFM.P	PPFM.S
Catalase	+	+	+	+	+
Carbon source utilization D-Glucose	+	+	+	+	+
Ethanol	+	+	+	+	+
Citrate	+	+	+	+	+
Growth on peptone rich nutrient agar (Oxiod CM 55)	+	+	-	+	+
Starch hydrolysis	-	-	-	-	-
Acid production from glucose	-	-	-	-	-
Volutine granules	+	+	+	+	+
Acetylene reduction activity (ARA)	-	-	-	-	-

Table 5. Effect of different antibiotics on PPFM isolates growth as measured by diameter of Inhibition zone (mm)

Zone Diameter mean (mm)						
PPFM.S	PPFM.P	PPFM.F	PPFM.Ph	PPFM.C	Concentration	Antibiotics
15.0	8.3	10.6	9.6	13.0	10µg	Ampcillin

12.0	8.3	07.6	9.6	12.0	10u	Bacitracin
11.0	8.3	10.6	12.0	11.0	15µg	Erythromycin
18.0	17.0	18.0	20.0	16.0	30µg	Kanamycin
09.0	15.3	12.6	19.3	15.0	50µg	Streptomycin

Table 6. Effect of different NaCl concentrations on the growth of PPFM isolates on Met-AMS agar medium (after 144h).

NaCl concent. / Isolates	0.0	0.5	1.0	2.0	3.0	4.0	5.0	6.0
PPFM.C	+++	+++	++	++	++	+	-	-
PPFM.Ph	+++	+++	+++	+++	+++	++	+	-
PPFM.F	+++	+++	+++	+++	++	+	-	-
PPFM.P	+++	+++	+++	++	++	+	-	-
PPFM.S	+++	+++	++	+	+	-	-	-

(+++) > 10^{6}-10^{7} cfu (++) ≥ 10^{4}-10^{5} cfu (+) < 10^{4}cfu (-) no growth

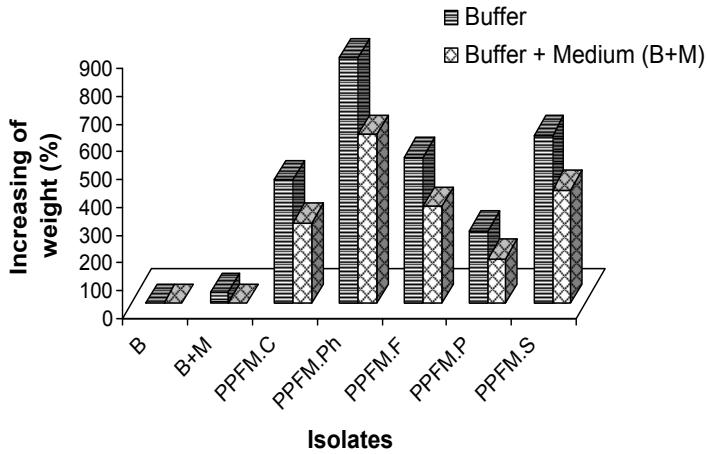

Fig. 2. Increases percentage of radish cotyledons weight as affected by cytokinin release from different PPFM isolates

Arab Univ. J. Agric. Sci., Ain Shams Univ., Cairo, 13(3), 717-729, 2005

cytokinin as compared to other different tested PPFM isolates. Generally, all obtained PPFM isolates had positive effect on producing and releasing cytokinin by following order: PPFM.Ph>PPFM.S> PPFM.F> PPFM.C > PPFM.P. This result was in agreement with **Holland (1997)** who reported that, cytokinins are produced by the microbial symbionts of plants not by plants them selves. Also **Omar (2004)** reported that several *Methylobacterium* spp produce cytokinins, e.g. zeatin riboside.

Molecular biology studies

To study the similarity among PPFM isolates, protein pattern and Random Amplification of the DNA were carried out. It is clear from Fig. (3), that the similarity of protein profiles for the five PPFM isolates ranged from 34.22 to 65.99 where PPFM.F came in a separated group within 34.22 similarities with the other four isolates. Among the bacterial PPFM isolates the highest similarity (65.99) was between PPFM.Ph and PPFM.C, while the similarity between PPFM.S and the group containing PPFM.Ph and PPFM.C was 58.32. The lowest similarity (34.22) was between PPFM.F and any of the other isolates.

Data in Fig. (4) show the Random Amplification of the DNA from the five PPFM isolates, there were different degrees of polymorphism according to the used primers. There was high similarity between PPFM.C and PPFM.Ph (99.34) when primer No.1 was used.

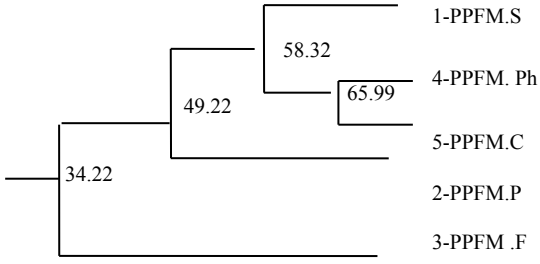

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Fig. 3. Protein pattern of PPFM isolates using Sodium Dodecyle Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

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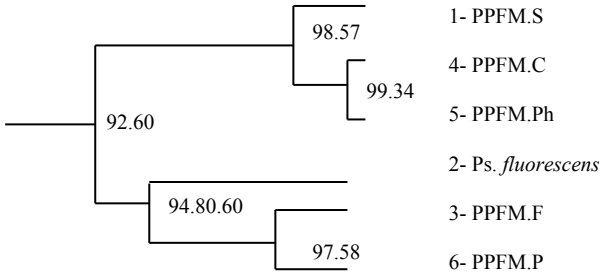

Fig. 4. Random Amplified Polymorphism DNA (RAPD) analysis

Primer No.2 showed the highest polymorphism as the bacterial isolates fell in two main groups with 66.28 similarity between them. Among the PPFM isolates and when primer No. 2 used, the highest similarity (88.04) was between PPFM.C and PPFM.Ph while the lowest similarity (66.28) was between PPFM.Ph or PPFM.C and any of the other isolates .On the other hand, when primer No.1 and No. 5 were used, high similarity was observed with ranges from 92.60 to 98.57 for primer No.1and 99.18 for primer No.5. In general, the low similarity in protein profiles among the used bacterial isolates could be attributed to difference in metabolism or secretion of some different metabolites .The high similarity among the bacterial isolates is an indication to the highly genetically relateness among them. This high similarity could be considered when classifying these isolates. There have been some comparative of methylotrophic bacteria according to **Urakami *et al* (1985) and Holland (1997).**

In conclusion, according to **Bergey's Manual of Determinative Bacteriology, 9th Edition (1994)** all PPFM bacterial

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isolates tested and identified in the present study may be related to one genus Methylobacterium but belong to different species where PPFM.F may be related to *Methylobacterium mesophilicum,* PPFM.P may be related to *Methylobacterium fujisawaense* and PPFM.Ph, PPFM.C and PPFM.S may be related to *Methylobacterium radiotolerans.*

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عزل وتنقية وتعريف بعض الكائنات الحية الدقيقة المنتجة للمواد المحفزة لنمو النباتات (البكتيريا المتغذية على الميثانول)

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أدت الأهمية الاقتصادية للبكتيريا المتغذية على الميثانول إلى تشجيع عزل هذه المجموعة. وقد تم الحصول على 5 عزلات مصرية من أسطح أوراق النباتات البقولية وهي: PPFM.G من الحمص، PPFM.F من الفول البلدي، PPFM.Ph من الفاصوليا، PPFM.S من فول الصويا و PPFM.P من الفول السوداني وذلك لدراسة الصفات العامة لهذه المجموعة. أظهرت الدراسات المورفولوجية جميع العزلات عصوية قصيرة سالبة لجرام ومتحركة. كذلك نتائج الدراسات الفسيولوجية تشابه جميع العزلات في كل الاختبارات ما عدا العزلة PPFM.F لم تستطع النمو على بيئة بها الفركتوز. جميع العزلات حساسة للكانامايسين ومقاومة للإريثروميسين. أيضاً تفاوتت بوضوح قدرة العزلات على النمو في تركيزات مختلفة من كلوريد الصوديوم وقدرة العزلة PPFM.Ph على النمو في 5% كلوريد صوديوم. وكانت جميعها لديها القدرة على إفراز هرمونات السيتوكينين أيضاً. تبين النتائج باستخدام البيولوجيا الجزيئية تشابهاً بين العزلتين PPFM.C و PPFM.Ph بنسبة 99.34%. وطبقاً للنتائج المتحصل عليها

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من جميع الاختبارات السابقة تم التعرف على خمس العزلات PPFM.Ph و PPFM.C و PPFM.S
العزلات حيث كانت PPFM.F تابعة للسلالة تابعة للسلالة *M. radiotolerans*.
Methylobacterium mesophilicum
و PPFM.P تابعة للسلالة *M. fujisawaense*.

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