In Vitro Biocontrol Activity of *Methylobacterium Extorquens* Against Fungal Pathogens

R. POORNIAMMAL, S.P. SUNDARAM AND K. KUMUTHA

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See end of the article for authors' affiliations

Correspondence to :
S.P. SUNDARAM
Department of Agricultural Microbiology,
Tamil Nadu Agricultural University,
COIMBATORE (T.N.) INDIA

SUMMARY

Four *Methylobacterial* isolates were tested for biocontrol potential against fungal pathogens. The isolates significantly reduced the linear mycelial growth of *Fusarium udum, F. oxysporum, Pythium aphanidermatum* and *Sclerotium rolfsii.* The effect of volatile antibiotics on the mycelial growth of *F. oxysporum a*nd *F. udum* was studied. AM1 and CO-47 produced highly effective antibiotic compared to others. All the *Methylobacterium* strains tested showed negative reaction to HCN production test. The isolate CO-47 produced maximum IAA (9.228 µg ml^{-1}). The isolate AM1 recorded higher salicylic acid production (0.218µg ml^{-1}). The four *Methylobacterium* strains tested for phosphate solubilization, no one produced a clearing zone in Pikovskyas medium. The growth of *Methylobacterium* in fungicides amended medium revealed that all are compatible with Blue copper in 0.1% and 0.2% but Mancozeb was compatible in 0.1% and not compatible in 0.2%. Biocontrol potential of *Methylobacterium* sp. for fungal pathogens was reported under *in vitro* condition.

Key words : *Methylobacterium*, Biocontrol, Fungal pathogens

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Biological control is an environmental-friendly strategy to reduce crop damage caused by plant pathogens. Increasing concerns about pesticide use by the general public and governmental agencies are severely limiting the availability and use of many important pesticides. Fungicides that have been used as standards in many disease control programme have been increasingly regulated. Research efforts with pathogens have indicated that most of the biocontrol organisms either did not control the pathogen or they did not perform as well as selected fungicides. Pink pigmented facultative methylotroph (PPFM) belonging to the genus *Methylobacterium* are a physiologically interesting group of bacteria that preferentially utilize methanol and other reduced one carbon compounds such as formate and formaldehyde as sole source of carbon and energy *via* serine pathway (Green, 1992).These are ubiquitous in nature and are thus found in a variety of habitats (Green and Bousifield, 1983), including soil, dust, fresh water, leaf surfaces and nodules. PPFMs isolated from the liverwort stimulated growth and development of the liverwort (*Scapania nemorosa)* and *Streptocarpus prolixus* (flowering plant) in tissue cultures, a positive commensal interaction was proposed (Corpe and Basile, 1982).The growth enhancing effects of PPFM on plants in a tissue culture system where they produced vitamin B_{12} and stimulated growth, they also produced growth hormones *viz.*, cytokinins (Koeing *et al.*, 2002) and auxins. *Methylobacterium* have been shown to stimulate seed germination, seedling establishment and increasing productivity of a plant by spraying PPFM bacteria on a plant (Holland, 1997). In the present investigation, biocontrol potential of *Methylobacterium* sp. has been tested against fungal pathogens under *in vitro* conditions.

MATERIALS AND METHODS

Bacterial and fungal collections:

Methylobacterium sp. strains Co-47, MV-10, LE-1 and AM-1 were obtained from the Department of Agricultural Microbiology, Agricultural College and Research Institute, Coimbatore, Tamil Nadu.The plant pathogens like *Sclerotium rolfsii, Pythium aphanidermatum, Fusarium oxysporum, F.udum, Macrpohomina* and *Phytophthora* were obtained from the Department of Plant pathology, TNAU,Coimbatore.

Cultivation condition:

Methylobacterium were grown for 72 h on Ammonium mineral salt (AMS) medium (pH 6.8) supplemented with methanol 0.5% and cycloheximide (30µg ml^{-1}) (Whittenbury *et al.*, 1970). All the fungal pathogens were

maintained and grown on Potato dextrose agar (PDA) before use.

In vitro inhibition of plant pathogens by Methylobacterium sp.:

Methylobacterial isolates were tested for their ability to inhibit plant pathogens like *Sclerotium rolfsii, Pythium, Fusarium oxysporum, F.udum, Macrpohomina* and *Phytophthora in vitro*, on agar plates.

Per cent inhibition (PI):

Per cent inhibition of test pathogen by the antagonistic strains was evaluated by dual culture technique (Dennis and Webster, 1971). The radial growth of mycelium in mm of antagonist and pathogens were measured and per cent inhibition (PI) was calculated as under –

$$\mathbf{P} = \frac{\mathbf{C} - \mathbf{T}}{\mathbf{C}} \times \mathbf{100}$$

where, C is the growth of test pathogen (mm) in the absence of the antagonist strain; T is the growth of test pathogen (mm) in the presence of the antagonist strain.

Production of volatile antibiotics:

The bioantagonistic bacterial suspension(PPFM) ($5x10^9$ cfu mL^{-1}) were streaked at the centre of one half Petri dish containing PDA medium and a 5-mm disk of a four days old pure cultures of fungal pathogens was placed at the centre of another Petri dish containing PDA. Both half plates were placed face to face preventing any physical contact between the pathogen and the bacterial suspension and were sealed to isolate the inside atmosphere and to prevent loss of volatiles formed. Plates were incubated at 28±2ºC for 48-96 hrs and the growth of the pathogen was measured and compared with controls developed in the absence of the bioantagonist.

Production of IAA and HCN:

Methylobacterial isolates were checked for the ability to produce hydrocyanic acid (HCN) and IAA. HCN production was assayed by the method of Wei *et al*.,1996. Production of IAA was determined according to the method of Gorden and Paleg (1957).

Testing of antagonist for the production of salicylic acid:

The strains were grown in the standard succinate medium at 28±2ºC for 48h. Cells were collected by centrifugation at 6000g for 5min. Four ml of cell free culture filtrate was acidified with 1N HCl to pH 2.0 and salicylic acid was extracted with equal volume of chloroform.

Testing of antagonist bacteria for phosphate solubilization:

The plates containing Pikovskyas agar were inoculated by Methylobacterial strains and incubated at room temperature for 5 days. Appearance of clear zone around the colonies indicated phosphate solubilization.

Compatibility of antagonist with fungicides:

Ammonium mineral salt medium and the respective broth were amended with the fungicides such as Mancozeb and Blue copper at 0.1% and 0.2% concentrations. Then the antagonistic *Methylobacterial* isolates were inoculated in the poisoned media and in the broth. The plates were incubated under room temperature for 48 h and the growth of bacteria was recorded visually and scored either as highly compatible or moderately compatible or not compatible.

RESULTS AND DISCUSSION

The Methylobacterial isolate MV-10 significantly reduced the linear mycelial growth of *F.udum* to an extent of 58.8% over control with an inhibition zone of 1.9 cm

Table 1: Screening of *Methylobacterium* sp. against plant pathogens

Sr. No.	*Methylobacterium* sp.	Mycelial growth (mm)	Inhibition zone (IZ) cm	Per cent inhibition of mycelial growth over control
Macrophomina phaseolina	AM1	60	0.0	33.3
	MV-10	40	0.9	55.5
	CO-47	65	2.5	27.7
	LE-1	42	2.3	53.3
Phytophthora infestans	AM1	55	0.5	38.8
	MV-10	60	0.5	33.3
	CO-47	62	0.0	31.1
	LE-1	60	0.5	33.3
Fusarium oxysporum	AM1	48	1.6	46.0
	MV-10	40	2.5	49.0
	CO-47	45	2.3	50.0
	LE-1	48	1.5	46.6
Fusarium udum	AM1	45	2.1	50.0
	MV-10	37	1.9	58.8
	CO-47	39	1.8	56.6
	LE-1	40	1.6	55.5
Pythium aphanidermatum	AM1	45	1.5	50.0
	MV-10	50	1.9	44.4
	CO-47	52	1.7	42.2
	LE-1	37	2.3	58.8
Sclerotium rolfsii	AM1	50	1.3	44.4
	MV-10	70	0.0	22.2
	CO-47	63	0.5	30.0
	LE-1	60	0.7	33.3

under *in vitro* it was followed by CO-47 and LE-1 with per cent inhibition of 56.6 and 55.5 and an inhibition zone of 1.8 and 1.6 cm, respectively. The *in vitro* assay against *S.rolfsii,* the strain AM1 recorded 44.4% inhibition and inhibition zone of 1.3 cm followed by LE-1 (Table 1). It is recognized that *in vitro* assay for antagonistic potential has inherent limitations. Although many studies have utilized the agar plate assay method to determine biocontrol potential.

AM1 strain showed inhibition ranging from 60.2 to 70.2% against *F. oxysporum* and *F. udum.* The LE-1 and MV-10 strain did not produce volatile compounds. *Methylobacterium* CO-47 produced more volatile, which significantly inhibited the mycelial growth of *F. oxysporum* compared to other isolates (Table 2). There was a linear relationship with biocontrol agents in cultures, greater the amount of volatile compounds and consequently less the radial growth and more inhibition percentage. Howell and Stipanovic (1983) reported that *Gliocladium virens* produces different metabolites like gliotoxin, dimethyl glytoxin, gliovirin, hepledidic acid etc., which are inhibitory to several plant pathogenic fungi. No change in colour of filter paper after 4-5 days of incubation at room temperature showed the incapability of strains to produce HCN *in vitro.* The cyanide production was not considered to be a significant factor in the inhibition of fungal growth (Nielson *et al.,* 1998).

Table 2: Effect of volatile antibiotics of *Methylobacterium* sp. against plant pathogens

Sr. No.	*Methylobacterium* sp.	Production of volatile antibiotics	Mycelial reduction over control
F. oxysporum	AM1	+	60.2
	CO-47	+	65.5
F. udum	AM1	+	70.2
	CO-47	+	64.7

All the four strains produced salicylic acid. The strain AM1 (0.218 µg ml^{-1}) produced more. Salicylic acid appears to be essential for ISR to be elucidated. Systemic salicylic acid transport from roots to leaves is one possibility but bacterial salicylic acid could also induce signals for systemic resistance at the root level.

However, all the four strains produced IAA. Production of IAA is reported to be more common in *Methylobacterium.* Doronina *et al.* (2002) reported that aerobic methylobacteria of the genera *Methylobacterium* and *Methylomicrobium* can also synthesize auxins from exogenous L-tryptophan. (Table 3).

Among them, the four strains of *Methylobacterium* sp. tested for phosphate solubilization, no one produced a clearing zone in the Pikovskya medium.The biological process of conversion of unavailable/fixed form of inorganic phosphorus into primary orthophosphate (H_2PO_4) and secondary orthophosphate (HPO_4^{-2}) has been termed as MPS (Baker and Cook, 1974).

Table 3: Salicylic acid, Hydrogen cyanide production, IAA production and Phosphate solubilization by *Methylobacterium* spp

Sr. No.	*Methylobacterium* strains	Hydrogen cyanide production	Salicylic acid (µg/ml)	IAA production (µg ml^{-1})	Phosphate solubilization
1.	AM1	0.0	0.218	6.27	0.0
2.	MV-10	0.0	0.054	7.25	0.0
3.	CO-47	0.0	0.067	9.228	0.0
4.	LE-1	0.0	0.135	6.73	0.0

The present study indicated that Blue copper in 0.1 and 0.2% was highly compatible with *Methylobacterium.* But Mancozeb recorded moderately compatibility in 0.1% and not compatible with 0.2% ,both visual and colony count method (Table 4). The present finding reported that *Methylobacterium* having *in vitro* biocontrol activity against fungal pathogens showed encouraging results. However, further studies are required for testing their efficacy under field conditions.

Table 4.Compatability of *Methylobacterium* sp. with fungicides

Sr. No.	Fungicide	*Methylobacterium* sp.	
		10^6 cfu/ml	Visual observation
1.	Mancozeb		
	0.1%	13.5	+ +
	0.2%	0.0	-
2.	Blue copper		
	0.1%	46	+ + +
	0.2%	25	+ + +
3.	Control	54	+ + +

- : Not compatible, + : Sligtly compatible,
+ + : Moderatly compatible, + + + : Highly compatible

Authors' affiliations:
R. POORNIAMMAL AND K. KUMUTHA, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, COIMBATORE (KARNATAKA) INDIA

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