



## Studies on the Effects of PGPR Consortium against Mulberry Root Rot Infected Plants

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### ABSTRACT

Mulberry (*Morus alba* L.) is grown as monocrop to produce leaf for silkworm rearing. Root rot disease is a major problem due to severe yield and economic losses in mulberry cultivation. In the present study, the PGPR isolates (*Pseudomonas fluorescens*, *Bacillus velezensis*, *Azotobacter* sp. and *Azospirillum* sp.) have treated to check its antagonistic activity against root rot pathogens of *M. phaseolina*, *F. oxysporum*, *F. solani* in vitro condition. Among the four PGPR strains tested *P. fluorescens* recorded maximum PI % and zone of inhibition (74.41 % and 26 mm), (72.41%, 28mm), (73.56%, 26mm) followed by *Bacillus velezensis* (71.26 %, 24mm), (70.11%, 26mm), (68.96%, 25mm), *Azotobacter* sp. (60.91%, 21mm), (63.21%, 24mm), (64.36%, 22mm) and *Azospirillum* sp. (58.62%, 20mm), (60.91%, 23mm), (63.21%, 21mm) were recorded the minimum antagonistic activity against all the three pathogens. Further to find the potential of PGPR isolates, the PGPR strains were made into consortium of various concentrations and applied to the mulberry plants. Which increases, the number of leaves, number of branches and shoot length of mulberry (*Morus alba* L.) plant against inoculated pathogen control. In vivo condition result showed that the 30 % PGPR consortium formulation had a significant effect on leaves, branches and shoot length. The number of leaves per plant recorded maximum in T3 (30 % PGPR consortium) 37.3, when compared to the inoculated control 20.6 and the number of branches increase to 6.2, when compared to the inoculated control 1.7 and the highest shoot length increase to 106.2 cm and inoculated control 72.3 cm and reduction of less yellowing symptoms. With these results, it is clear that the PGPR consortium in maximum plant growth and reduces the diseases incidences.

**Key words:** Antagonistic activity, PGPR consortium, root rot disease, mulberry.

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### INTRODUCTION

The mulberry, *Morus indica* L., is a commercial crop that may be grown anywhere in the world, from temperate to tropical climates. Mulberry leaves are feed to silkworms (*Bombyx mori* L.), is regarded as a necessary component of the sericulture industry [1]. Around 0.216 million acres of land are used for mulberry cultivation in India [2]. Mulberry has developed a greater susceptibility to different illnesses, including root rot infection. Due to its epidemic nature and tendency to kill, this disease has become more concerning. This disease has become more worrisome as a result potential to fully destroy the plant. Due to repeated harvesting of leaf the soil nutrients gets depleted and makes the plant susceptible to soil-borne diseases [3]. Root rot is the most serious disease owing to its epidemic nature and its potentiality to kill the plants and poses a severe problem during mulberry cultivation in the sericulture practicing countries. After introducing high yielding varieties followed by intensive cultivation practices, mulberry became vulnerable to root rot disease [4].

Many PGPR have secretion systems that enable them to create antimicrobial substances such antibiotics, volatile organic compounds, and lytic enzymes that allow them to limit the growth of potentially phytopathogenic bacteria [6],[7] highlight the fact that PGPRs not only work together with the root to have positive impacts on plant development but also have positive effects on estimating phytopathogenic bacteria. Potential plant growth-promoting strains have reported several antimicrobial substances, surfactants, and plant growth promoters colonising plant roots, and bacterial secretions control the interaction between plants and PGPR resulting to different plant growth promotion special effects[8],[9],[10]. A class of bacteria known as rhizobacteria inhabits the rhizosphere saprophytically. According to some of them can operate as plant growth promoters and as biocontrol agents against diseases to increase crop production [11].

In order to develop environmentally friendly management strategies for mulberry root rot disease, the current study was conducted to assess the effect of native isolates of the PGPR strains and its consortium for protecting mulberries against root rot pathogens (*Macrophomina phaseolina*, *Fusarium oxysporum*, and *Fusarium solani*).

## MATERIAL AND METHODS

### **Inoculum preparation:**

In this investigation, three isolates of the root rot pathogen *Macrophomina phaseolina*, *Fusarium oxysporum* and *Fusarium solani* were employed. They were raised for three days at 25°C in the dark on PDA. Mycelia discs (5 mm in diameter) were placed in a 500 ml flask with 100 cc potato dextrose broth and shaken at 120 rpm for 7 days at 25°C. After being extracted, the fungus suspension was diluted with sterile distilled water. For use as inoculum in subsequent trials, it was adjusted to  $5 \times 10^{-6}$  cfu/ml [11].

### **Sample Collection and isolation of different PGPR isolates:**

Mulberry rhizosphere soils samples were collected from B2 CSR, Central Silk Board, Krishnagiri district, Tamil Nadu. Microbial strains were isolated by the serial dilution method. One gram of dried soil was weighed and added to 10 ml of double distilled water in a sterile test tube and shaken well using vortex mixer; this stock solution was then diluted serially up to the dilution of  $10^{-6}$  and 0.1 mL of diluted sample was inoculated on surface of selective King's B agar, Nutrient Agar, Waksman Base agar medium-77 and NFB medium incubated at 30°C for 2 days [12]. The purified colonies were preserved using standard preservation methods.

### **Preparation of PGPR isolates extract**

All the PGPR isolates were inoculated in nutrient broth separately and incubated at 37°C for duration of two days. After incubation adding a 100 ml of ethyl acetate to each conical flask, allowed for mixing by using magnetic stirrer at 1 hr and finally filtered with using of cotton. The residual extract of PGPR is collected in a beaker and the solvent was allowed to evaporate at room temperature. The PGPR extract then stored at 4°C till further use. The resultant residue was then made up to required volume (1%) using dissolved in minimum quantity of DMSO and also distilled water for further studies.

### **Antagonistic activity of PGPR isolates extracts**

The agar well diffusion assay, used to determine the antagonistic activity of PGPR [13]. The PDA medium (20 ml) was poured into each sterile Petri plate, followed by placement of mycelial disc (5 mm in diameter) of the tested PGPR isolates extracts at the center of pathogen the plates. A well (7 mm in diameter) was made by punching the agar with a sterile cork borer on the corner of the plate in four places with equal distance. Then purified extracts of PGPR extracts of *P. fluorescens*, *Bacillus velezensis*, *Azotobacter* sp. and *Azospirillum* sp. were poured into the wells at the rate of 40 µl per well separately and incubated for 72 h at  $28 \pm 2^\circ\text{C}$ . The inhibitory activity of each concentration was expressed as the percent growth inhibition, compared to the control (distilled water only used in the wells). And used this formula,

$$PI = [DC - DT] / DC \times 100$$

Here, DC is radial growth of control (mm), DT is Radial growth in treatment (mm) was measured and multiple comparisons were subjected of ANOVA.

### **Compatibility of PGPR isolates**

PGPR isolates were tested for their compatibility among each other by following the method described [14]. The compatibility was determined for *P. fluorescens* and *B. velezensis*, *Azotobacter* sp., and *Azospirillum* sp., isolates by using NA medium. The bacterial *P. fluorescens* and *Azotobacter* sp., isolate was streaked horizontally on NA medium and the *B. velezensis* and *Azospirillum* sp., isolates were streaked vertically from the streak of the first isolate and incubated at 16°C. Compatibility was tested by overgrowth or by inhibition of *P. fluorescens* and *B. velezensis*, *Azotobacter* sp., and *Azospirillum* sp., isolates by incubating at room temperature and by making observations over a period of 72 h.

### **Pathogenicity test**

The pathogenicity test was conducted under *In vivo* condition. The fungus *Macrophomina phaseolina*, *Fusarium oxysporum* and *Fusarium solani* was mass multiplied in potato dextrose broth medium. Sand and maize powder were mixed at 9:1 ratio (w/w), moistened to 50 per cent moisture content, filled in polypropylene bags at  $\frac{3}{4}$  level and sterilized three times on consecutive days. After sterilization, nine mm mycelial disc of pathogen was inoculated in sand maize medium and incubated at laboratory conditions for fifteen days.

The pathogenicity of fungus was tested on mulberry plant. One month old plants maintained in earthenware pot containing sterilized pot mixture (two feet diameter) at the rate of one sapling / pot were inoculated with pathogen multiplied in sand maize medium @ 5 per cent (w/w) around collar region. The symptoms were recorded on 45 days after inoculation

**Preparation/mass multiplication and bio-formulations of effective strains**

The PGPR strains were selected and mass multiplied with suitable carrier materials. A loopful of isolates of *Pseudomonas fluorescens*, *Bacillus velezensis*, *Azotobacter* sp. and *Azospirillum* sp. were inoculated into the sterilized King's B and Nutrient Agar broth, respectively, and incubated in a rotary shaker at 150 rpm for 72 hrs at room temperature ( $28\pm 2^\circ\text{C}$ ), After 72 hrs. A total 400 ml of all bacterial broth suspension containing  $1\times 10^8$  CFU/ml, 1 kg of the carrier material (lignite), 100 g calcium carbonate (to adjust the pH to neutral) and 5 g Carboxy Methyl Cellulose (CMC) (adhesive) were mixed under sterile conditions by following the method described by Vidhyasekaran and Muthamilan (1995). For bacterial strain mixture the bacterial isolates were grown separately in respective broths. The isolates were added equally (v/v) to lignite and mixed according to the procedure as described above. Lignite formulations of the isolates were mixed equally where makes a consortium of microbes at the time of application and applied to the saplings and soil.

**Treatment**

The experimental design used was a Randomized Block Design with Three treatments, namely:

T1= 5 % (PGPR consortium + *M. phaseolina* + *F. oxysporum* + *F. solani*)

T2= 15 % (PGPR consortium + *M. phaseolina* + *F. oxysporum* + *F. solani*)

T3= 30 % (PGPR consortium + *M. phaseolina* + *F. oxysporum* + *F. solani*)

Control, without fertilization only pathogen,

Each treatment consists of 5 (FIVE) replications so the total was 18 plants. The length of used was 120 cm.

The following Observations were recorded: (i) Number of branch/plant (ii) Number of Leaf / plants (iii) Shoot length: from each fresh sample plant (iv) Number of yellowish leaves/ plant.

**Effect of PGPR consortium against root rot of mulberry under pot culture conditions:****Sapling treatment:**

A pot culture experiments was conducted with 4 treatments with five replications in completely randomized block design with above treatment saplings were dipped and soil application of PGPR consortium suspension for 30 min and transplanted in the pots. The root rot incidence was observed at fortnightly intervals up to 120 days after planting (DAP).

**RESULT**

Compatibility of the four PGPR isolates (*P. fluorescens*, *Bacillus velezensis*, *Azotobacter* sp. and *Azospirillum* sp.) were tested for their compatibility under *in vitro* conditions. The results indicated that inhibition zone and there was overgrowth of bacterial isolates between (*P. fluorescens*, *Bacillus velezensis*, *Azotobacter* sp. and *Azospirillum* sp.) Suggesting that the biocontrol agents were compatible with each other.

**In vitro screening of antagonistic activity of PGPR isolates:**

The results revealed that in four PGPR isolates were effective in reducing mycelial growth of the pathogen. The four PGPR isolates were tested against three fungal pathogens for antagonistic activity such as *M. phaseolina*, *F. oxysporum*, *F. solani*. The radial growth of pathogen (mm), zone of inhibition was recorded (Table 1).

Among the four PGPR isolates against three fungal pathogens, the maximum RG (mm) PI% and IZ (mm) were recorded in the isolate *Pseudomonas fluorescens* (22mm,74.71%, 26mm), (24mm, 72.41%, 28mm), (23mm,73.56%, 26mm) followed by *Bacillus velezensis* (25mm, 71.26 %, 24mm),(26mm,70.11%, 26mm),(27mm, 68.96%, 25mm), *Azotobacter* sp. (34mm, 60.91%, 21mm),(32mm,63.21%, 24mm),(31mm, 64.36%, 22mm) showed broad antifungal activity against in all the three fungal pathogens. However *Azospirillum* sp. (36mm, 58.62%, 20mm), (34mm,60.91%, 23mm), (32mm,63.21%, 21mm) were recorded the minimum antagonistic activity against all the three pathogens (Fig- 2).

**Effect of PGPR consortium against root rot infected mulberry under pot culture conditions:**

The effect of PGPR isolates against root rot disease of mulberry in pot culture work (Figure 3) depicts a comparison of plant growth. The PGPR consortium T3 treatment enhanced the shoot length to (106.2) and increased the number of leaves to (37.3) and increased the number of branches to (6.2) in terms of growth promotion over the inoculated control and less yellowing symptoms.

Among the inoculations of PGPR consortium of various concentrations T3 - (1.8, 3.2, 2.4) in 30 % PGPR consortium recorded the minimum of 100%, 50%, 25% yellowing of leaves / plant in Followed by 5%, 10 % of PGPR consortium inoculation, and recorded maximum 100%, 50%, 25% yellowing of leaves / plant in T1- (3.4), (5.4), (4.2) and T2- (2.6), (3.4), (3.2). The outcome shows that applying biocontrol agents in consortium resulted in more growth promotion and less diseases induced was observed (Table -2), (Fig-3&4).

## DISCUSSION

This study describes PGPR isolates against root rot disease caused *M. phaseolina*, *F. oxysporum*, *F. solani* under *in vitro* conditions. The inhibition of mycelial growth and PI % was ranged from (20 to 26 mm and 58 to 74 %) for *M. phaseolina*, (23 to 28 mm and 60 to 72 %) for *oxysporum*, (21 to 26 mm and 63 to 73 %) for *F. solani*. The experiments were conducted under *in vitro* revealed the formulation of zone of inhibition due to siderophores production, HCN, antibiotic production which suppresses the plant pathogen [14]. Under these condition PGPR strains may grow well and produce siderophore that affect the growth of fungal pathogen by reduces Ferric iron availability [15], Hydrogen cyanide is a volatile secondary metabolites released by many rhizospheric bacteria that exhibits fungicide activity against root rot fungi and protects the plant against infection [15],[16].

In the next study, PGPR consortium significantly positive effects on mulberry plant growth and disease reduction under pot culture *in vivo* condition. The 30% PGPR consortium treated with saplings and soil application produces more number of leaves, number of branches and highest shoot length compared to pathogen inoculated control. The PGPR strains able to produce the plant growth hormones IAA, which improves plant growth [15], [16].

In the present study PGPR strains exhibits the potential of bioinoculant increases the availability of nutrients from soil and enhance the phytological processes leading to increases the mulberry growth and reduced the disease incidence similar to in sunflower plant [16].

The various PGPR isolates consortium (5%, 15%, and 30%) were used as sapling treatments and soil application is to control the root rot disease of mulberry under pot culture condition. The present study revealed that among treatments, T3 consortium of PGPR isolates (*P. fluorescens*, *Bacillus velezensis*, *Azotobacter* sp. and *Azospirillum* sp.) as sapling treatment and soil application recorded significantly lesser of 100%, 50%, 25% of yellowing of leaves per plant (1.8), (3.2), (2.4) followed by T2 (2.6), (3.4), (3.2) in 5% of PGPR consortium and T1 (3.4), (5.4), 4.2) in 15 % of PGPR consortium.

Similar reports of use of PGPR in combination for better disease control were [17], [18]. The consortium of 30% PGPR strains showed better diseases suppression than control.

PGPR increased plant growth directly by mediating the production of secondary metabolites and phytohormones such as Auxine, Cytokinins or Gibberellic acid [19] or indirectly suppression of pathogen reported by [20]. In conclusion consortium of PGPR strains recorded better disease control due to the different mechanism of actions produced by them.

**Table 1: *In vitro* Effect of PGPR isolates extract of antagonistic activity against root rot pathogen:**

S.No	PGPR	<i>M. phaseolina</i>		<i>Fusarium oxysporum</i>			<i>Fusarium solani</i>			
		RG (mm)	PI %	IZ (mm)	RG (mm)	PI %	IZ (mm)	RG (mm)	PI %	IZ (mm)
1	<i>Pseudomonas fluorescens</i>	22 <sup>e</sup>	74.71	26	24 <sup>d</sup>	72.41	28	23 <sup>d</sup>	73.56	26
2	<i>Azospirillum</i> sp.	36 <sup>b</sup>	58.62	20	34 <sup>b</sup>	60.91	23	32 <sup>b</sup>	63.21	21
3	<i>Azotobacter</i> sp.	34 <sup>c</sup>	60.91	21	32 <sup>c</sup>	63.21	24	31 <sup>b</sup>	64.36	22
4	<i>Bacillus velezensis</i>	25 <sup>d</sup>	71.26	24	26 <sup>d</sup>	70.11	26	27 <sup>c</sup>	68.96	25
5	Control	87 <sup>a</sup>	0	0	87 <sup>a</sup>	0	0	87 <sup>a</sup>	0	0

Values were the mean of replications. Same letter on suffix showed data were statistically same (non-significant) while different letter indicates they were significantly different at 5% level by DMRT RG, radial growth of pathogen (mm); IZ inhibition zone (mm), PI, per cent inhibition of mycelial growth.

**Table -2: *In Vivo* effects of PGPR consortium against root rot infected mulberry plants**

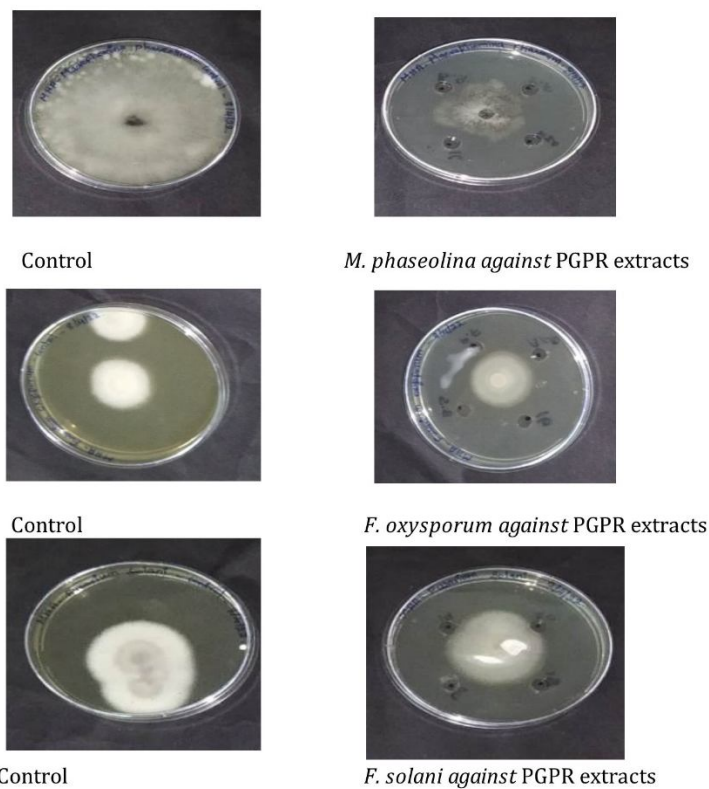
Treatment	No. of leaf / plant	100% yellowing leaves / plant	50% yellowing leaves / plant	25% yellowing leaves / plant	No. of Braches / plant	shoot length(cm) / plant
	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD	MEAN ± SD
T1	29.6± 9.476 <sup>c</sup>	3.4±0.577 <sup>b</sup>	5.4±1.140 <sup>b</sup>	4.2 ± 1.643 <sup>b</sup>	4 ± 1.581 <sup>b</sup>	82.3±11.015 <sup>c</sup>
T2	31± 7.483 <sup>b</sup>	2.6 ± 1.521 <sup>b</sup>	3.4 ± 1 <sup>c</sup>	3.2±2.081 <sup>c</sup>	4.6±1.816 <sup>b</sup>	96± 23.51595 <sup>b</sup>
T3	37.3 ±4.722 <sup>a</sup>	1.8 ±0.836 <sup>b</sup>	3.2± 2.081 <sup>c</sup>	2.4±1.140 <sup>c</sup>	6.2±0.836 <sup>a</sup>	106.2± 15.143 <sup>a</sup>
Inoculated Control	20.6 ±2.081 <sup>d</sup>	7.0 ±1 <sup>a</sup>	9.3± 2.081 <sup>a</sup>	10.33±0.577 <sup>a</sup>	1.7±0.577 <sup>d</sup>	72.3± 6.429 <sup>d</sup>
F-test	*	*	*	*	*	*

Observations were made from the first day after planting. To see the effect of the treatment, an analysis of variance was performed on the collected data. If the results of the ANOVA showed a significant effect on the variables being tested, then the data analysis continued to test the average difference of each treatment with DMRT, significance at F test.



*Macrophomina phaseolina*      *Fusarium oxysporum*      *Fusarium solani*

Fig -1: Isolation of root rots pathogen from mulberry infected root and rhizosphere soil.



Control

*M. phaseolina* against PGPR extracts

Control

*F. oxysporum* against PGPR extracts

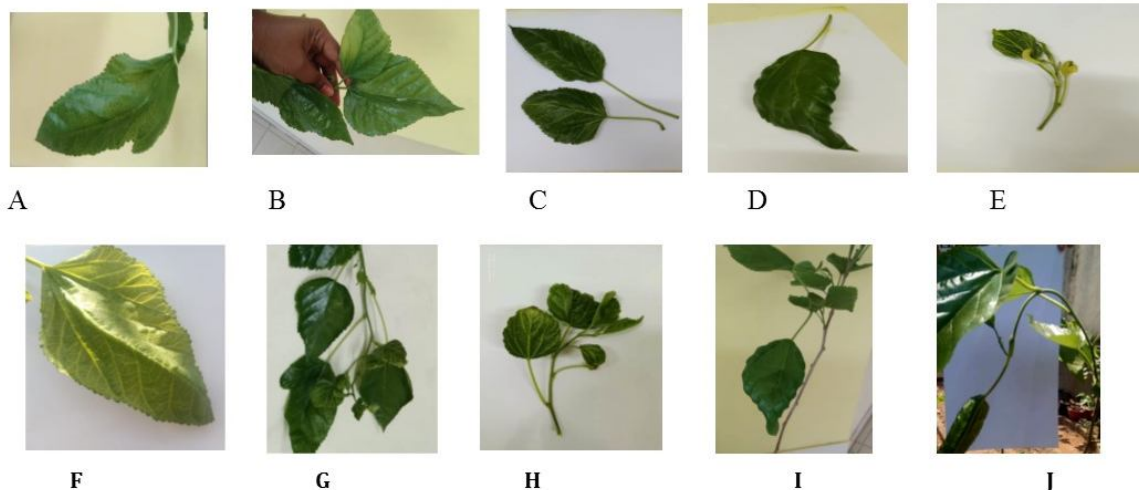
Control

*F. solani* against PGPR extracts

Fig -2: *In Vitro* Antagonistic activity of PGPR isolate extracts against root rot pathogens of mulberry.



Fig-3: Effects PGPR consortium of the growth of mulberry plants that were applied by the rhizobacteria consortium compared to the control at the observation 1 week after planting.



**Fig- 4: MULBERRY – ROOT ROT (FOLIAGE) SYMPTOMS**

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