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## Biocontrol and PGPR potential of marine sponge *Callyspongia diffusa* associated *P.fluorescens* BCPBMS-1

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### ABSTRACT

Plant growth promoting rhizobacteria (PGPR) exhibit direct and indirect mechanisms as plant growth promoters and biological control agents. Sponge associated microbes rich in bioactive metabolites. *P.fluorescens* BCPBMS-1 was isolated from the sponge *Callyspongia diffusa*. *P.fluorescens* BCPBMS-1 having antagonistic activity against *Fusarium oxysporum*. It was observed by using cross streak method. In blotting paper test more number of seeds were found to germinate when *P. fluorescens* was inoculated along with the *Fusarium oxysporum*, the pathogen. Out of 50 seeds treated *P. fluorescens* and *Fusarium oxysporum*, 48 seeds germinated, 50 seeds treated with *F. oxysporum*, only 5 seeds were germinated. The antagonistic effect of *P.fluorescens* was confirmed also by in vitro. *P.fluorescens* also having phosphate solubilizing activity. PGPR activity was remarkable in pot culture experiment. Seed lings with *P.fluorescens* grew fastly compared to control. In the present observation pH of the uninoculated *P. fluorescens* soil sample was 7.5. while pH of the inoculated *P. fluorescens* was 7.1. Available nitrogen was 6.4 mg/g for uninoculated *P. fluorescens* soil sample whereas 7.2 mg/g was observed with *P. fluorescens* inoculated soil. Available phosphorus was maximum in *P. fluorescens* inoculated soil 12.0mg/g, whereas in *P. fluorescens* uninoculated soil sample available phosphorus content was only 8.0mg/g. These results endorsed *P.fluorescens* BCPBMS-1 can be used as biofertilizer.

**Key words:** PGPR, biocontrol, antagonistic, *P.fluorescens* BCPBMS-1.

### INTRODUCTION

Despite the use of available means of plant protection, about one third of the crops produced are destroyed by pests and diseases. The discovery of synthetic chemicals has contributed, greatly to the increase of food production by controlling pests and other disease causing agents. However, the use of these synthetic chemicals during the last few decades has raised a number of ecological problems. In recent years, scientists have diverted their attention towards exploring the potential of beneficial microbes, for plant protection measures. Bio-control agents are easy to deliver, improve plant growth, and activate resistant mechanisms in the host, and increase biomass production and yield. These antagonists act through various mechanisms like antibiosis, secretion of volatile toxic metabolites, mycolytic enzymes, parasitism and through competition for space and nutrients. In addition, the present day bio-products can be further improved to obtain disease control to a greater extent.

Application of PGPR either as single strain or as a consortium based on formulations checked pest and disease spread besides increasing growth and yield. Though bio-control with PGPR is an acceptable green approach, development of PGPR formulations with increased shelf life and broad spectrum of action with consistent performance under field

conditions remain as a challenge even today. When export oriented agricultural and horticultural crops faced the problem of residues, a great potential and demand for the incorporation of biopesticides in crop protection emerged. It facilitated the industries to involve in commercial production of PGPR.

PGPR with wide scope for commercialization include *P. fluorescens*, *P. putida*, *P. aeruginosa*, *B. subtilis* and other *Bacillus* spp. The potential PGPR isolates are produced through solid or liquid fermentation technologies and formulated using different organic and inorganic carriers. They are used for various purposes like seed treatment, bio-priming, seedling dip, soil application, foliar spray, fruit spray, hive insert, sucker treatment etc.,. Preparation of PGPR formulations with many strains mixture performs better than individual strains, for the management of pest and diseases of crop plants, in addition to plant growth promotion [1].

Plant growth promoting rhizobacteria has diverse applications for the management of plant diseases in agriculture, horticulture and forestry. In addition, it also plays a vital role in environmental remediation [2]

Among several PGPR strains, *Bacillus*, an endospore former based products gained momentum for commercialization, due to its tolerance to extremes of abiotic environments such as temperature, pH, pesticides, fertilizers etc.,. Hence many different commercial products of *Bacillus* origin were developed in China to mitigate soilborne fungal diseases [3].

In addition certain PGPR strains also activate octadecanoid, shikimate and terpenoid pathways, which in turn alter the production of volatiles in the host plant leading to the attraction of natural enemies (6). Identification of entomopathogenic PGPR strains that have the capability to colonize phylloplane in a stable manner will be a breakthrough in the management of foliar pests [4]. Since PGPR has its own potentiality in plant disease and pest management, several products have been registered for the practical use of farming community.

## MATERIALS AND METHODS

### Isolation of *P. fluorescens* and identification

The sponge *Callyspongia diffusa* was collected from Mandappam Coast, TamilNadu, India. The sample soon after collection was transferred to a sterile polyethylene bag and transported at 4°C to the laboratory for the isolation of associated microbes. On reaching the laboratory, the invertebrate was brought to room temperature and cut aseptically into small pieces (2 × 2 cm) using a sterile scissors. The pieces were freed from adhering particles by vortexing twice for 20 sec. with 2 ml of sterile seawater. The seawater was decanted, which was once again replaced with sterile seawater with continued vortexing between washings. Finally, sample in sterile seawater was homogenized using sterilized mortar and pestle in a Laminar flow chamber. The homogenate was serially diluted up to 10<sup>-6</sup> dilutions and then spread plated on Kings B agar Peptone-20.0 g, Glycerol-10.0 mL, Dipotassium phosphate-1.5g, MgSO<sub>4</sub>·7H<sub>2</sub>O-1.5g, Agar-15g, 50% Sea water-1000ml) agar plates. The plates were incubated at room temperature for 24-48 hrs. Then strain was identified by Biochemical Method and 16 s Partial sequencing.

### In vitro test

The efficacy of antagonistic activity of *Pseudomonas fluorescens* was tested by cross streak method [5] *P. fluorescens* strain was streaked at one side of petridish (1cm away from the edge) containing PDA medium. *Fusarium oxysporum*, a plant pathogen was streaked on the opposite side in the petridish perpendicular to the *P. fluorescens* streak and plates were incubated at room temperature for 3 to 7 days.

### Blotting paper test

The antagonistic effect of *P. fluorescens* was tested by paper towel (blotter) method, to assess its effect on germination of the seeds. 50 seeds were first soaked in the 15 ml of *F. oxysporum* (1.3×10<sup>6</sup> cfu/ml) in a Potato dextrose broth then soaked with suspension of the 5 ml of *P. fluorescens* (1.0×10<sup>6</sup>cfu/ml), rolled in moist blotter and incubated at room temperature for 2 to 3 days. The seeds soaked only in *F. oxysporum* suspension served as control [6].

### Sterilization of soil

10 kg of soil samples were collected from agriculture lands of Keezhamoongiladi village, Cuddalore district, TamilNadu, India. Soil sample was sterilized in an autoclave at 121°C for 1hr.

**Test tube experiment**

The antagonistic effect of *P. fluorescens* was tested in test tube, to check their effect on germination of green gram seeds. 5 g of sterile soil was taken in 3 tubes, 10 seeds were first soaked in the 3 ml suspension of *F. oxysporum* ( $1.3 \times 10^6$  cfu/ml) in a Potato dextrose broth, then soaked with 1 ml suspension of the *Pseudomonas fluorescens* ( $1.0 \times 10^6$  cfu/ml), seeds were inoculated to tubes and incubated for 7 days. The seeds soaked only in *F. oxysporum* and untreated seeds were inoculated to separate test tubes [6].

**Phosphate solubilizing activity of *P. fluorescens***

Phosphate solubilizing activity was observed by using Pikovskaya's Agar (Glucose -1.000 g, Calcium phosphate-5.000g, Ammonium sulphate-0.500g, Potassiumchloride-0.200g, Manganese sulphate-0.100g, Magnesium sulphate-0.100g, Ferrous sulphate-0.0001g, Agar-15.00g, 50% sea water- 1000ml, pH<sup>-</sup> 7.0).

Pikovskaya's agar was sterilized and poured into petriplate, strain was streaked and the plate was incubated at room temperature for 48-96 hrs. Phosphate solubilization was indicated by the formation of a clear zone around the strain [5].

***P. fluorescens* in plant growth promotion****Pot culture experiment**

3 kg of sterile soil was mixed with 10 ml of 24 hrs culture of *P. fluorescens* containing  $5.6 \times 10^2$  cfu/ml mixed with 90 ml of water. 50 seeds were inoculated in pot containing with *P. fluorescens* and uninoculated *P. fluorescens* soil incubated for 7 days [7].

**Soil analysis**

pH of the samples was observed by pH meter (Eutech instrument). Soil samples were analysed for nutrients like nitrogen and phosphorus following the procedures given below.

**Estimation of soil available nitrogen (Alkaline-permanganate method)**

Available nitrogen was estimated for plant growing pot soil containing both inoculated with *P. fluorescens* and uninoculated soil and sterile soil (without *P. fluorescens* and seeds).

A quantity of 4 g soil sample was placed in a 500 ml flat bottom flask, and 20ml of 0.32%  $\text{KMnO}_4$  solution and 20ml of 2.5% NaOH solution were added. The flask was then connected to the distillation unit and 50ml of the distillate was collected in 5 ml of boric-acid indicator mixture. The absorbed ammonia was titrated with 0.005N  $\text{H}_2\text{SO}_4$  to determine the amount of available nitrogen [8].

**Estimation of available phosphorus**

Available phosphorus was estimated in plant growing pot soil samples containing both inoculated with *P. fluorescens*, uninoculated and sterile soil (without *P. fluorescens* and seeds). Phosphorus content of the soil was estimated [9].

Reagent A was prepared as follows:

12.0 g of ammonium molybdate was added to 250 ml of deionized water, whereas 0.291 g of antimony potassium tartrate was added to 100 ml of deionized water in separate flasks and both solutions were added in turn to 1,000 ml of 5.76 N  $\text{H}_2\text{SO}_4$ . It was made up to 2,000 ml and labeled as reagent A. Reagent B was prepared by dissolving 1.056g of ascorbic acid in 200 ml of reagent A and was prepared as fresh every time. 3.0 g of soil was mixed with 9.0 ml of deionized water, to which 3.0 ml of Reagent B was added. OD was taken at 882 nm after 10 mins. Phosphorus concentration for blank and unknown samples was calculated from standard curve [9].

**RESULTS**

Isolated potent strain was identified as *Pseudomonas fluorescens* by both biochemical and 16sr- DNA sequencing. In the present study Phylogenetic tree revealed that *P. fluorescens* BCPBMS-1 (bioactive compound producing bacteria) which was isolated from marine sponge *Callyspongia diffusa* was submitted to NCBI.

In the present study the growth of the fungus was inhibited when it grew towards the bacterial colony on PDA. The clear zone was observed after 6 days of incubation which indicated the antagonistic activity of *Pseudomonas fluorescens* (Figure.1).

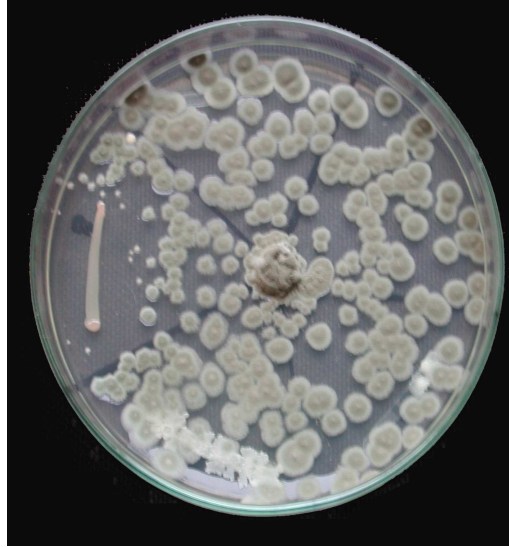


Figure:1 Antagonistic activity of *P.fluorescens* against *Fusarium oxysporum*

Regarding blotting paper test more number of seeds were found to germinate when *Pseudomonas fluorescens* was inoculated along with the *Fusarium oxysporum*, the pathogen. Out of 50 seeds treated *Pseudomonas fluorescens* and *Fusarium oxysporum*, 48 seeds germinated (i.e) 96% germination was observed. On the contrary, of 50 seeds treated with *F. oxysporum*, only 5 seeds (i.e) 10% were germinated (Figure.2).



Figure: 2 Blotting paper test A-Infected with *F. oxysporum*  
B- Seeds treated with *P. fluorescens* and *F. oxysporum*

In the present study, out of 10 infected seeds only one seed germinated and after 7 days 3.5 cm of plant growth was observed in it. On the other hand out of the 10 treated seeds, four germinated. The plant growth was observed to be 13 cm. In control all the 10 seeds germinated and the plant growth was 19 cm (Figure: 3).

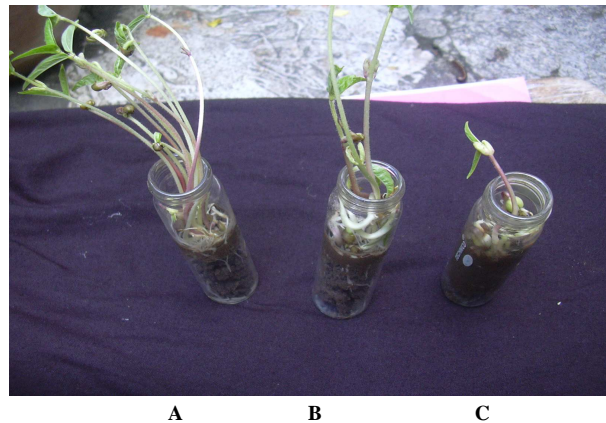


Figure:3 Test tube study A – Control, B - *P. fluorescens* and *F. oxysporum* treated green gram ,C - *F. oxysporum* infected green gram

In the present study phosphate solubilizing activity was observed in the presence of zone around the colony of about 3mm (Figure: 4) .



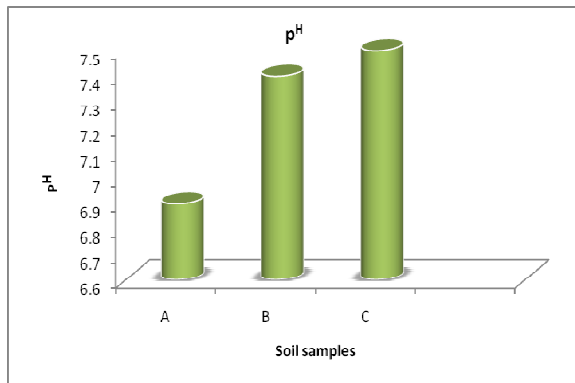
Figure:4 Phosphate solubilizing activity of *P. fluorescens*

In the present study, results showed that green gram seed sowed in the soil inoculated with *P. fluorescens* has shown more growth in 10 days compared to the seed sowed in the soil without *P. fluorescens* in the same period (Figure:5).



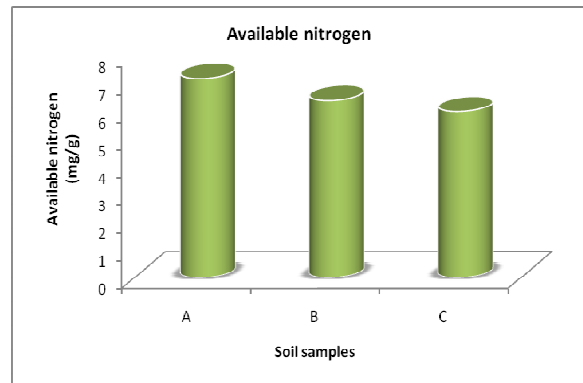
Figure:5 Pot culture experiment A- Green gram seedlings in *P. fluorescens* inoculated soil, B- Green gram seedlings without *P. fluorescens* inoculated soil

In the present study pH of the uninoculated *P. fluorescens* soil sample was 7.5. while pH of the inoculated *P. fluorescens* was 7.1. In sterile soil (without *P. fluorescens* and seeds) pH was 7.5(Figure.6).In the present study available nitrogen was 6.4 mg/g for uninoculated *P. fluorescens* soil sample whereas 7.2 mg/g was observed with *P. fluorescens* inoculated soil.In sterile soil (without *P. fluorescens* and seeds) available nitrogen was 6.4 mg/g (Figure.7). Available phosphorus was maximum in *P. fluorescens* inoculated soil (i.e.,) 12.0mg/g, whereas in *P. fluorescens* uninoculated soil sample available phosphorus content was only 8.0mg/g. In control it was 6.0 mg/g (Figure:8).



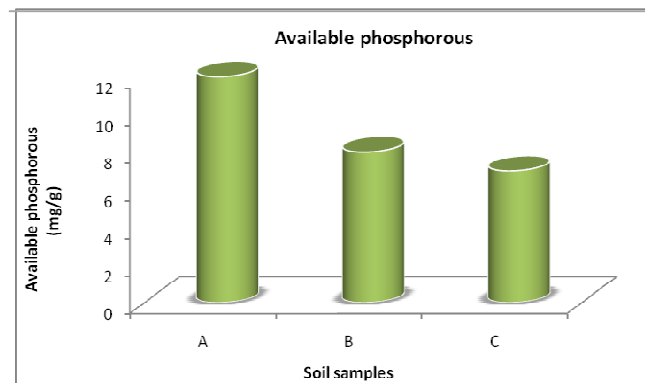
A *P. fluorescens* treated soil  
 B without *P. fluorescens* treated soil  
 C without *P. fluorescens* and seeds

Figure:6 pH of the soil samples



A-*P. fluorescens* treated soil  
 B-without *P. fluorescens* treated soil  
 C-without *P. fluorescens* and seeds

Figure: 7 Available nitrogen of the soil samples



A *P. fluorescens* treated soil  
 B without *P. fluorescens* treated soil  
 C without *P. fluorescens* and seeds

Figure: 8 Available phosphorus of the soil samples

### DISCUSSION

The clear zone was observed after 6 days of incubation indicating the antagonistic activity of *P. fluorescens* against *F. oxysporum*. Kumar *et al.* [10] confirmed that *P. fluorescens* had a strong antifungal activity against *F. oxysporum* mainly by the production of the antifungal metabolites. Karkachi *et al.* [11] also observed that *P. fluorescens* had activity against *Fusarium oxysporum* f.sp. *lycopersici*. In the present study blotting paper experiment was carried out to confirm the direct effect of antagonistic organism on pathogenic microbe. In this, out of 50 seeds treated with *P. fluorescens* and *F. oxysporum* 48 seeds germinated (i.e) 96% germination was observed. Out of 50 seeds treated with *Fusarium oxysporum* only 5 seeds (i.e) 10% were germinated and in test tube experiment shoot length of seedlings grown from seeds treated with *P. fluorescens* and *F. oxysporum* was 13 cm. The shoot length of seedlings grown from seeds infected with *F.oxysporum* was only 3.5 cm. In control plant the shoot length was 20cm.

This study proved the growth promoting as well as disease controlling ability of *Pseudomonas fluorescens*. Fluorescent pigments producing Pseudomonads are known to have a significant role in the suppression of fungal pathogens, apparently via the production of antifungal metabolites such as phenazine-1-carboxylate [12], 2, 4-diacetyl phloroglucinol [13]. Fluorescent *Pseudomonas* GRC2 isolates from potato rhizosphere [14] and *Pseudomonas chlororaphis* SRB 127 from sorghum rhizosphere [15] showed strong antagonistic activity against *M. phaseolina*, a charcoal rot pathogen of peanut and sorghum. Anis *et al.* [6] reported the use of *Trichoderma viridi* and *Paecilomyces variotii* in enhanced germination of sunflower seeds. The widely recognized mechanism of biocontrol mediated by PGPR are competition for an ecological niche or a substrate, production of inhibitory allelochemicals, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens [16, 17, 18]. Plant growth promoting bacteria including *Pseudomonas* spp. have been reported to stimulate the development of healthy root system [19] and rapid root colonization by beneficial bacteria [20].

In the present study, green gram seed sowed in the soil inoculated with *P. fluorescens* had shown more growth in 10 days compared to the seed sowed in the soil without *P. fluorescens* in the same period. This study proved the plant growth promoting effect of *P. fluorescens*. Ardakani *et al.* [21] observed that bioformulations using two isolates of *P. fluorescens* Q<sub>18</sub> (B<sub>1</sub>) and CKK-3 (B<sub>2</sub>) which were isolated from rhizosphere soil and cotton roots in Varamin's cotton fields increased seedling height compared to the control.

Plant growth-promoting bacteria, isolated from rhizosphere soils, stimulate growth directly by nitrogen fixation [17]. Plant growth promoting rhizobacteria (PGPR) has a potential role in developing sustainable systems for crop production. Sharma *et al.* [22] observed that *P. fluorescens* was more effective in increasing seed germination as well as seedling growth than *B. megaterium*. *Pseudomonas* spp. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability and uptake in soybean crop [23].

In the present study phosphate solubilizing activity was observed in the presence of zone around the *P. fluorescens* strain. Promod and Dhevedaran [24] studied the ability of *Bacillus* spp. and *Vibrio* spp, *Pseudomonas* spp to solubilize the phosphate which was isolated from seawater and marine sediments. Terrestrial PSB like *B. amyloliquefaciens* have been used together with ectomycorrhizal fungi for inoculation of Douglas fir seedlings to promote their growth [25]. Ramachandran [26] reported the phosphate solubilizing activity of *Pseudomonas* spp and *Azospirillum* spp. isolated from rhizosphere soil sample of black pepper. Keneni *et al* [27] isolated and identified based on phenotypic characters as *Pseudomonas* spp Anb-105, Meh-008, Meh-101, Meh-303 and Meh-305.

In the present study pH of the uninoculated *P. fluorescens* soil sample was 7.5. while pH of the inoculated *P. fluorescens* was 7.1. In the present study available nitrogen was 6.4 mg/g in uninoculated *P. fluorescens* soil whereas 7.2 mg/g was observed with *P. fluorescens* inoculated soil. Available phosphorus was maximum in *P. fluorescens* inoculated soil (12.0mg/g) and in uninoculated *P. fluorescens* soil it was only 8.0mg/g. Rodelas *et al.* [28] observed that mixed inoculation of *Vicia faba* L. with four different PGPR strains changed the total accumulation, concentration and distribution of the macro-and micronutrients. Shamsuddin *et al.* [29] found increased amounts of P and K uptake in banana plants inoculated with PGPR and also combined inoculation of *A. brasilense* and the phosphate-solubilizing bacteria *Pseudomonas* grown sorghum field significantly increased grain weight. Hatayama *et al.* [30] isolated the nitrogen-fixing bacterium, designated strain 6H33bT, was isolated from a compost pile in Japan it was identified as *P. azotifigens*. Artursson *et al.* [31] reported that free-living P-solubilizing bacteria release phosphate ions from sparing soluble in inorganic and organic P compounds in soil and thereby contribute to an increased soil phosphate pool available for the plant.

Microorganisms with phosphate solubilizing potential increase the availability of soluble phosphate and enhance the plant growth by improving biological nitrogen fixation [32,33]. *Azospirillum* and rhizobacterial inoculation increased the photosynthetic rate of oil palm seedlings [34]. Phosphate-solubilizing microorganisms convert insoluble phosphates into soluble forms through the process of acidification, chelation, exchange reactions and production of gluconic acid [35]. Liu *et al.* [36] proved that two beneficial agents *Bacillus subtilis* SY1 and *P. fluorescens* W1 can be used to improved soil physical and chemical properties and fertility, promote soil nutrient content and accelerate the plant growth. The inoculation of groundnut with PGPR like *Bacillus* spp. and *Pseudomonas* spp. enhanced the growth, nodulation and yield [37]. The results of the present study also proved the same.

## CONCLUSION

The isolation of PGPR from the marine environment open new doors to design strategies for improving the efficacy of biocontrol agents. Many years of continuous farming practices in agriculture soil damage the soil health. The soil-borne pathogens and the deadly chemical residues are two serious problems of soil pollution which affect the yield and quality of agricultural products. Ecological remediation of soil is an effective way to resolve these problems and maintain the sustainable development of agriculture. From the above observations *P. fluorescens* seems to be an ideal candidate to be used to improve the ecosystem function and reduce the disease occurrence.

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