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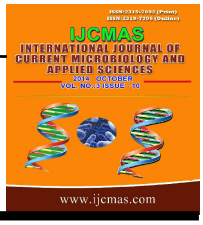

Original Research Article

A study on the Biocontrol of phytopathogens of *Vigna radiata* using *Pseudomonas fluorescens* in Sustainable Agriculture

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Keywords

Bacillus subtilis, Biocontrol, Biofilm, Mungbean, Phytopathogens, *Pseudomonas fluorescens*, *Rhizobium*

A B S T R A C T

Pulses are rich source of protein and integral part of balanced diet. They maintain soil fertility through biological nitrogen fixation. Thus play a vital role in sustainable agriculture. The phytopathogens cause serious damage in plants, resulting in critical losses of yield, quality and profit. In the present study, rhizobacteria isolated from soil and confirmed as *Pseudomonas fluorescens* . *Pseudomonas fluorescens* was checked for antibiosis against the phytopathogens *in vitro*. The biofilm formation with PGPRs like *Rhizobium* and *Bacillus subtilis* was analyzed to confirm that they can coexist. Thirty alginate beads entrapping *Pseudomonas fluorescens* and PGPRs were able to inhibit the growth of pathogens *in vitro*. Mungbean plant treated with *Pseudomonas fluorescens* and other PGPRs induced a significant increase in root and shoot length, nodules, weight and protein content. In test plants combined inoculation was superior to individual inoculation.

Introduction

Interactions between microorganisms and plants have undoubtedly had major effects on the development of civilization since humans began to rely extensively on cultivated crops for food. More recently, plant disease outbreaks have resulted in catastrophic crop failures that have triggered famines and caused major social change. Disease is not the only outcome of plant-microbe interactions. A number of mutually beneficial relationships between plants and microorganisms affect agricultural productivity and the health of plants in general. By altering the balance of microflora in the rhizosphere, symbiotic associations may also help to protect plants from disease causing microorganisms (Smith *et al*., 1998)

Legumes for human consumption constitute about 5% of the cultivated crops. Most leguminous vegetables are rich in phosphorus, calcium, iron, and a number of essential vitamins. Mungbean is one among the leguminous plants that is nutritious and economically important in India. India accounts for about 60% of the world's

mungbean area and harvests 47% of the world production (Aiyer, 1958). The bacterial and fungal pathogens cause serious damage in agriculture, resulting in critical losses of yield, quality and profit. Chemical insecticides are normally used against phytopathogens. Due to serious demerits of using chemical insecticides, bioinsecticides are used as alternatives.

Biological control is a natural and specific way to control pathogens and enhance crop yield by growth promoting attributes of environment friendly microorganisms. This method has been developed successfully during the last few years. It is based on the reduction of inoculum or of pathogenic activity due to the natural presence of one or more organisms, through the management of the environment, the host or antagonists (Zaki and Siddiqui, 2005)

The use of plant growth promoting rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the world. PGPR form biofilms on the root surface. The cells are densely packed and covered by an exopolysaccharide layer, and facilitate a suitable environment for gene – regulatory systems. The percentage of competent cells has been more in biofilms, compared to individual bacteria.

According to Scheepens and Van Zon (1982), a biopesticide formulation is aimed at "stability and conservation of the inoculums for a prolonged period; addition of constituents that may increase the efficiency of application; and addition of substances that increase the efficiency of the control agent". As an inoculant carrier, alginate is easy to use, is biodegradable and non-toxic, and does not contribute to pollution (Scheepens and Van Zon, 1982)

Materials and Methods

Biometric observations

Biometric observation was performed for root and shoot length, plant biomass, number of nodules, number of pods and total protein content of mungbean.

Root and shoot length

Root length was measured from the point of attachment of the stem base to the tip of the adventitious root. Shoot length was measured from the stem base to the tip of the longest leaf stretched at 15 days interval after treatment.

Total Protein Content (Lowry et al., 1951)

500µl of each sample was made up to 1ml by adding distilled water. To the diluted sample, 4 ml of alkaline solution was added. It was mixed well and allowed to stand for 10 minutes or longer at room temperature. 0.50 ml of 1X Folin – Ciocalteau reagent was added to each tube and mixed was immediately. The tubes were incubated in dark for 30 minutes. After 30 minutes the optical density (OD) reading was read spectrophotometrically at 650 nm. The concentration of the protein in sample was calculated using the standard protein (Bovine Serum Albumin) used.

Preparation of sample

5N NaOH was added to each tube of 1 ml grinded mungbean extract so that the final concentration was 0.5N. The samples were autoclaved with aluminium foil. After autoclaving, 1 ml of sample was transferred to corresponding eppendorf tubes. The tubes were centrifuged at 10,000 g for 5 minutes. 500µl of clear supernatant were transferred to fresh tubes.

Results and Discussion

Isolation of *Pseudomonas fluorescens*

Rhizobacteria that establish positive interaction with plant roots are called plant growth promoting rhizobacteria (PGPR). PGPR play important role in phytostimulation, phytoremediation and biofertilization. The important traits of PGPR that are involved in plant growth stimulation include production of plant hormones, siderophores, bacteriocins, exopolysaccharides, phosphate solubilization, nitrogen fixation, etc. Among the PGPRs, *Pseudomonas fluorescens* stand out because of their high level of genetic variability and competitiveness in the rhizosphere, their ability to release plant growth promoting substances, alter the root physiology, increase the nutrient availability to plants and to control plant pathogens (Kloepper, 1993). The rhizosphere soil sample was serially diluted and plated on King's B agar plate. The plate was observed under UV transilluminator after 24 hours of incubation. The colonies producing fluorescens under UV light were *Pseudomonas fluorescens.*

In vitro screening of *Pseudomonas fluorescens* for their biocontrol activity against phytopathogens

The inhibitory activity of *Pseudomonas fluorescens* against plant pathogenic organisms is due to production of secondary metabolites such as phenazines, acetyl phloroglucinols and cyanides (Davison, 1986). *Pseudomonas fluorescens* produce pyoverdine type siderophores, which are high affinity iron chelaters (Keel *et al*., 1989). Besides, the aggressive root colonization character of *Pseudomonas fluorescens* is also reported to play an important role in rhizosphere competence and associated biocontrol activity (Neilands and Leong, 1986, Defago and Haas, 1990) The isolated *Pseudomonas fluorescens* was tested for its *in vitro* biocontrol potential against three fungal pathogens (*Rhizoctonia solani, Colletotrichum truncatum* and *Macrophomina phaseolina*), two bacterial pathogens (*Xanthomonas axonopodis* and *Pseudomonas syringae*). The results clearly indicated the potential of *Pseudomonas fluorescens* to inhibit all the pathogens tested. The *Pseudomonas fluorescens* isolate was tested for its biocontrol potential against two bacterial pathogens. Zone of inhibition was observed against all bacterial pathogens. *Xanthomonas* with zone of inhibition diameter of 3cm and *Pseudomonas syringae* with 2cm was observed after 2 days incubation (Fig. 1).

Pseudomonas fluorescens isolate showed biocontrol potential against all fungal pathogens. Maximum zone of inhibition was observed for *Colletotrichum truncatum* with a zone diameter of 1.4 cm. *Macrophomina phaseolina* and *Rhizoctonia solani* was observed with a zone of inhibition diameter of 1.3 cm and 1.2 cm respectively (Fig. 2). Relatively higher percentage inhibition was observed with *Rhizoctonia solani* (39.2%) compared with the control plate. The percentage inhibition of *Colletotrichum truncatum* and *Macrophomina phaseolina* were 30% and 28% respectively.

Production of biofilm at the air – liquid interface of broth (pellicle)

Traces of biofilm on walls, slight veil, sediment of thin pellicle, was observed at the interface between air and liquid (pellicle). Biofilm formation on abiotic surfaces initiates in response to environmental cues, and the same is true for attachment to the plant surfaces (Kamali *et al*., 2011).

Figure.1 Antibiosis activity of *Pseudomonas fluorescens* against *Xanthomonas* sp and *Pseudomonas syringae*

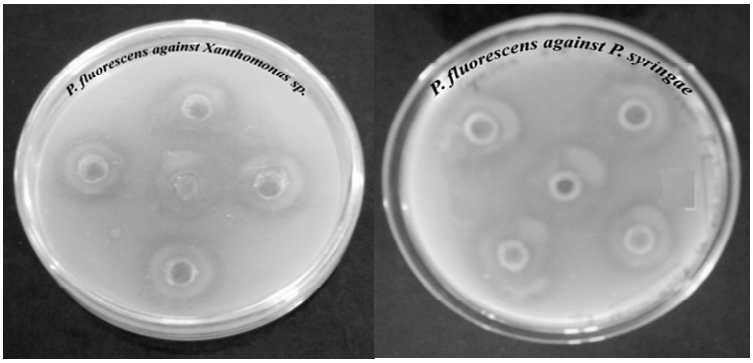

Figure.2 Antibiosis activity of *Pseudomonas fluorescens* against *Colletotrichum*, *Macrophomina* and *Rhizoctonia solani*

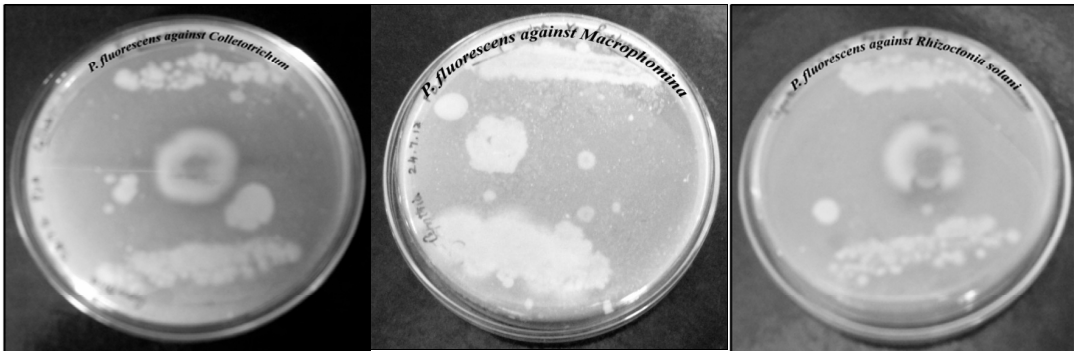

Table.1 Treatment schedule for pot culture experiment

Pot	Treatments	Formulation
Control	No Organism	---------
A	*Pseudomonas fluorescens* + PGPRs Biofertilizer	Carrier
B	*Pseudomonas fluorescens*	Carrier
C	PGPRs Biofertilizer	Carrier

Table.2 Studies on the growth promoting efficiency of inoculants on green gram in pot culture experiment

TREATMENT	Shoot length (cm)	Root length (cm)	Total biomass (gram)	No. of Nodules per plant	No. of pods per plant	Protein content per 10 g mungbean (gram)
No Organism	19.2	3.8	4.04	4	4	0.57
Only *Pseudomonas fluorescens*	22.5	4	4.38	8	5	0.59
PGPRs Biofertilizer	26.7	4.8	5.72	36	6	0.69
Pseudomonas fluorescens + PGPRs Biofertilizer	30.2	5.5	6.89	42	9	0.86

Beneficial PGPR play a key role in agricultural approaches through quorum sensing in their biofilm mode. The *in vitro* production of biofilmed PGPR can be used to give increased crop yields through a range of plant growth mechanisms. They can be used as biofertilizers through improved N_2 fixation and micro- and macronutrient uptake (Gamini *et al*., 2011).

Influence of temperature on biofilm density Crystal Violet method

The attachment of biofilm to the surface of eppendorf was seen more in tubes incubated at room temperature compared to tubes incubated at 30□C. *Pseudomonas fluorescens, Bacillus subtilis* and *Rhizobium* coexist and thick veil of pellicle was observed on the sides of the tube and on the surface. In the persent study, biofilm formation of *Pseudomonas fluorescens, Rhizobium* and *Bacillus subtilis* was determined by crystal violet method. The effect of temperature on biofilm density was also analyzed.

Preparation of Alginate beads entrapping PGPRs

Pseudomonas fluorescens, Bacillus subtilis and *Rhizobium* were entrapped in 5% alginate as beads. The beads were hard and eventual dislodging of the entrapped beads was absent. The formulation of inocula is reliable and consistent effect under field conditions. The preparation of beads containing bacteria is fairly easy and involves a multistep procedure. The main advantages of alginate preparations are their nontoxic nature, biodegradability, and their slow release of microorganisms into a soil (Fages, 1992). Finely saturated cultures of *Pseudomonas fluorescens, Rhizobium* and *Bacillus subtilis* were used for entrapment in alginate beads. The minimum number of beads inhibiting the growth of phytopathogens was determined as 30 beads for 5ml medium inoculated with a loop full culture.

Estimating the minimum number of PGPRs entrapped beads inhibiting the bacterial pathogens

Turbid growth of organisms was observed in control tubes and tubes inoculated with 20 *Pseudomonas fluorescens, Bacillus subtilis* and *Rhizobium* entrapped beads. No growth was observed in tubes

containing 30 beads.

Estimating the minimum number of PGPRs entrapped beads inhibiting the fungal pathogens

Fungal growth was observed in control tubes and tubes inoculated with 20 *Pseudomonas fluorescens, Bacillus subtilis* and *Rhizobium* entrapped beads. Fungal growth was inhibited in tubes containing 30 beads.

Biometric Observations

The plant shoot length was estimated for 3 months, once in 15 days interval. After a period of 3 months the plants grown with *Pseudomonas fluorescens* & PGPR biofertilizers was observed to have a longer shoot of 30.2 cm, followed by plants inoculated with Only PGPR biofertilizers 26.7 cm and the control plant with 19.2 cm.

After a period of 3 months the plants grown with *Pseudomonas fluorescens* & PGPR biofertilizers was observed to have a longer root of 5.5 cm, followed by plants inoculated with Only PGPR biofertilizers 4.8 cm and the control plant with 3.8 cm. After a period of 3 months the plants grown with *Pseudomonas fluorescens* & PGPR biofertilizers was observed to have a high biomass of 6.8 gram, followed by plants inoculated with Only PGPR biofertilizers 5.7 gram and the control plant with 4 gram. In addition to their biocontrol potential, these antagonistic *Pseudomonas fluorescens* are known to produce plant growth promoting substances such as indole acetic acid and gibberellic acid and solubilize insoluble phosphates which might have resulted in increased plant growth (Suneesh, 2004) After 3 months, the plants grown with *Pseudomonas fluorescens* & PGPR biofertilizers had 42 and plants grown with only PGPR biofertilizers was observed to have 36 nodules and the control plant with 4 nodules.9 pods were observed in plants grown with *Pseudomonas fluorescens* & PGPR biofertilizers, 6 pods in plants grown with only PGPR and the control plant with 4 pods per plant.

The protein content of mungbean was estimated by Lowry's method. The mungbean of plants grown with *Pseudomonas fluorescens* & PGPR biofertilizers was estimated to have 0.86 g protein per 10g mungbean, followed by plants inoculated with Only PGPR biofertilizers 0. 69 g protein per 10g mungbean and the control plant with 0.57g protein per 10g mungbean. *Pseudomnas fluorescens* produces plant growth promoting substances and they are also capable of producing phosphate solubilising substances which accelerates plant growth (Megha, 2006).

The pot experiments conducted in the present investigation on growth promotion in *Vigna radiata* revealed significant increase in plant growth parameters, *viz.* plant shoot, root length, fresh biomass, nodules and pod numbers plants treated with *Pseudomonas fluorescens, Rhizobium* and phosphate solubilizing bacterial biofertilizers.

The findings of the present investigation have clearly brought out the potential of *Pseudomonas fluorescens* to reduce the incidence of different diseases of crop plants as well as to enhance the nutrient uptake and yield of mungbean plants under green house conditions. This strain could be of potential to develop as biofertilizer with biocontrol potential after testing its performance under field conditions either alone or as components of integrated disease/nutrient management

systems. As agricultural practices become more sustainable, there is an increasing need for ecologically sound methods of disease control. Biological control, which exploits the natural antagonistic activity of certain root-colonizing bacteria against bacterial and fungal pathogens, is one such approach. Biological control agents often perform inadequately under field conditions and this has impeded acceptance of the technology as an alternative to chemical pesticides. Soil *Pseudomonas fluorescens* possess a variety of promising properties which make them better biocontrol agents. Although the present study is not an initiative, it helps in better understanding and utilization of *Pseudomonas fluorescens* as biocontrol agent. Further investigations on the type of antimicrobial components and *in vivo* experiments will make *Pseudomonas fluorescens* as one of the most suitable biocontrol agent in suppressing the phytopathogens and replace the chemical pesticides.

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