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Research Article

EFFECT OF *PSEUDOMONAS FLUORESCENCE*, *P. AERUGINOSA* AND *BACILLUS SUBTILIS AS* BIOCONTROL AGENT FOR CROP PROTECTION

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ABSTRACT
Pseudomonas fluorescence, *P. aeruginosa* and *Bacillus subtilis* are the beneficial rhizobacteria possessing biocontrol activity against plant pathogenic fungus. In present research the biocontrol effect of *P. fluorescence*, *P. aeruginosa* and *B. subtilis* were evaluated on the seedling growth of Cotton (*Gossypium arboretum* L.), Castor (*Ricinus communis* L.), Peanut (*Arachis hypogaea* L.) and Mung bean (*Vigna radiate* (L.) R. Wilczek) challenged by plant pathogenic fungus *viz. Fusarium oxysporum*, *Aspergillus niger* and *Alteneria alternate*. The antagonist activity of all three bacteria against pathogenic fungus was evaluated in *in vitro* condition on solidified medium. The most active fungus inhibitor was *P. aeruginosa*, than *P. fluorescence* and least was *B. subtilis*. In plant inoculation study, the seeds were treated with pathogenic fungus as well as *P. fluorescence*, *P. aeruginosa* and *B. subtilis* respectively. The shoot and root length were measured as growth parameters after tenth day of the germination. *P. fluorescence* has shown the highest growth promoting effect, followed by *P. aeruginosa* and least was of *B. subtilis*. The most positive response was observed with *P. fluorescence* in Caster seedlings and the least effect was observed with Peanut seedlings. All three bacterial species can be use as potential biopesticides for economical plants of Gujarat.

Key Words: *Pseudomonas fluorescence, Pseudomonas aeruginosa, Bacillus subtilis, Biocontrol, Biopesticides, Antagonism, Plant Pathogen*

INTRODUCTION
Pseudomonas are gram negative rod shaped bacteria (Palleroni, 1984), and are aerobic, produce exopolysaccharides those generate biofilms (Hassett *et al.,* 2002). Certain *Pseudomonas fluorescens* strains *viz.*CHA0 and Pf-5, are having biocontrol properties and thus protecting the roots of some plant species against pathogenic fungi such as *Fusarium* or *Pythium*, as well as some phytophagous nematodes (Haas and Keel, 2003). Even certain strains such as Pf-5 and JL3985 have natural resistance against ampicillin and streptomycin (Sarniguet *et al.,* 1995). *P. fluorescens* shows plant growth-promoting properties and even induce resistance in plants against pathogen. There are several mechanisms involving in the bacteria resulting in biocontrol activity. Even the competitive outcome with soil microbes reduce the growth of pathogens like, it secretes siderophores which are ever known soluble compound which chelate iron (Fe^{3+}) and thus other microbes are scavenging for iron. The siderophore with iron complex is taken by active transport mechanism of bacteria itself. Even the production of secondary metabolite 2,4-diacetylphloroglucinol (2,4-DAPG) which have antagonistic effect on other soil microbes (Bangera and Thomashow 1999). There are antibiotics reported from *P .fluorescens* like, phenazine 1- carboxylic acid

(Zhengyu *et al.*, 2004), 2,4-diacetyl phloroglucinol (Weller *et al.*, 2007; Kumar *et al.*, 2002) and oomycin A (Ursula, 1995). All these theories have experimental evidence and beautifully summarized in a review written by Haas and Defago (2005). The biocontrol activity of *P. fluorescence* was reported on 16 yrs old sweet orange *Citrus sinensis* L. during the field trial against the infection of *Fusarium spp.* and citrus nematode *Tylenchulus semipenetrans* Cobb (Abd-Elgwad *et al.*, 2010). The *in vitro* effect of *P. fluorescence* was evaluated against various fungi including *Fusarium spp.* (Srivastava and Shalini, 2008; Thangavelu and Mari, 2006). *P. fluorescence* has reported antagonistic effect for *Verticillium dahliae* causes Verticillium wilt disease on Cotton (Erdogan *et al.*, 2011).

P. aeruginosa inhibits the production of *Aspergillus niger* enzymes polygalacturonase and cellulase which degrading the plant cell wall and thus inhibit the infection of fungus. Even it induce systemic acquired resistance in plants indicated by the rapid accumulation of defence related enzymes like chitinase, 1,3-glucanase, peroxidase and phenylalanine ammonia lyase in the groundnut seedlings (Kishore *et al.*, 2006). *P. aeruginosa* reported as biocontrol agent for *Colletotrichum gloeosporioides* in Papaya inhibited 68.45% spore germination during *in vitro* screening on potato dextrose agar (PDA) medium (Rahman *et al.*, 2007).

P. fluorescence and *B. subtilis* both are reported as biocontrol agent for fungus *Hemileia vastatrix* causal organism for leaf rust of coffee (Daivasikamani and Rajanaika, 2009). *B. subtilis* is well known for its biocontrol property and produce the antibiotics iturin A and surfactin (Asaka and Shoda, 1996). Even the other species like *Pseudomonas chlororaphis* and *Bacillus amyloliquefaciens* also have biocontrol effect evaluated *in vitro* against *Sclerotinia sclerotiorum*, create stem rot of canola plant (Fernando *et al.*, 2007). Biocontrol activity of *B. subtilis* was evaluated against another bacterial pathogen *Pseudomonas syringae* infecting Arabidopsis roots (Pal *et al.*, 2004), *Fusarium verticillioides* (Cavaglieri *et al.*, 2005), *Pythium aphanidermatum* and *Phytophthora niotianae* to improve tomato and cucumber yield against yield looses caused by these pathogens (Grosch *et al.*, 1999).

In the present work the antagonistic activity of *P. fluorescence*, *P. aeruginosa* and *B. subtilis* was confirmed against three plant pathogen fungi *Fusarium oxysporum*, *Aspergillus niger* and *Alteneria alternate* through *in vitro* assay. The biocontrol effect of three bacteria was evaluated on four economical important crop plants of Gujarat *viz*. Castor (*Ricinus communis* L.), Cotton (*Gossypium arboretum* L.), Peanut (*Arachis hypogaea* L.) and Mung bean (*Vigna radiate* (L.) R. Wilczek). The seeds were treated with the combine culture of fungus and biocontrol bacteria and the effect was observed on the growth of seedlings.

MATERIALS AND METHODS

Microorganism evaluated as biocontrol agent on selected crop plants

The bacterial species were procured from Microbial Type Culture Collection Centre (MTCC), Chandigarh designated with MTCC numbers *Pseudomonas flurescence* MTCC 4828 and *Pseudomonas aeruginosa* MTCC 424. *Bacillus subtilis* was our labs isolate.

Culture of plant pathogenic fungi

Fusarium oxysporum (MTCC 1755) procured from MTCC whereas *Aspergillus niger* was isolated from the fruit surface of infected Pomegranate (*Punica granatum* L.) and *Alteneria alternata* obtained from lab collection. The disease infected fruit of Pomegranate (*Punica granatum* L., Lythraceae) collected from the field located 9km away from Palanpur (Umiyanagar, Gujarat, India). The fruit has black colure spots on the surface and on flashy seeds. The infected pomegranate was washed under running tap water and surface sterilised by 70% ethanol for 1 min, followed by sodium hypochlorite for 3 min. Subsequently the fruits were washed thrice with sterilized distilled water. The black sport of fruit rind was inoculated on potato dextrose agar (PDA) in sterile condition. Plates were incubated at 37 °C for 4 to 6 days, resulted in black coloured sporulated fungus identified as *Aspergillus niger*.

Antagonistic effect of bacteria on pathogenic fungi by dual culture method

Fungal culture was spread on PDA and allows growing for 5 to 6 days. Spore from plates were obtain by re-suspending spores in one ml 30% glycerol solution and it was store at 4°C until use. Spore suspension was plated on PDA and 100 µl of bacterial culture suspension was inoculated in 10mm well in the centre of plates. Incubate all the plates at 37 °C for 4-7 days and observed the plates for the zone of inhibition. Clear zone around well was indicator of inhibition of fungus growth measured in mm.

Preparation of seedling for plant inoculation study

Castor (*Ricinus communis* L.), Cotton (*Gossypium arboretum* L.), Peanut (*Arachis hypogaea* L.) and Mung bean (*Vigna radiate* (L.) R. Wilczek) seeds were surface sterilized by 0.1% $HgCl_2$ for 3min and rinsed with sterile distil water for several times then blotted on a sterile filter paper, dried and kept for application.

Plant inoculation study

Pseudomonas spp. and *Bacillus subtilis* were inoculated in 50ml King's B and nutrient broth respectively in 250ml Erlenmeyer flasks. Flaks were incubated at 30°C on orbital incubator shaker for overnight. Spores of fungus (*Aspergillus niger, Fusarium oxysporum, Alteneria alternata*) was inoculated in 50ml potato dextrose broth (PDB) in 250ml Erlenmeyer flasks individually. All were incubated at 30°C on orbital incubator shaker for 5 days at 130 rpm. Mixer of bacterial and fungal inoculants in the ration of 1:1 was prepared in 10% Jaggery slurry. Pot experiment was conducted with the treatment of bacterial and fungal inoculums by socking the seeds for 2 hrs. Set of control prepared by seeds coated with (1:1) PDB and jiggery (10%) without any inoculums. Seeds were kept for drying on a clean plastic sheet in a sterile condition for maximum 2hrs.

Air- dried seeds were immediately sown at 2cm depth in plastic pots (6 seeds/pot) containing double autoclaved 250g soil. The pots were sprinkled with water and covered with perforated polyethylene (for aeration) about 2 days to prevent the moisture lost. Plants were watered periodically as per need. The seed germination percentage was calculated after 5 and 10 days of sowing. Shoot length was measured after 5 days and both root and shoot length was recorded after 10 days of sowing.

RESULTS AND DISCUSSION

Antagonistic effect of bacteria against fungal pathogens

The results of antagonistic effect of three bacteria species against plant pathogenic fungi are shown in Table 1. The optimum antagonistic activity was observed by *P. aeruginosa* and even inhibits the growth of *A. alternata*. None of the bacterium have zone of inhibition against *A. alternata* except *P. aeruginosa*. *P. fluorescence* has moderate zone of inhibition against *F. oxysporum* and *A. niger* , and none against *A. alternata*. The least activity was observed by *B. subtilis* especially against *A. niger* and *A. alternata.*

Table 1 *In vitro* effect of bacterial as biological control against pathogenic fungi

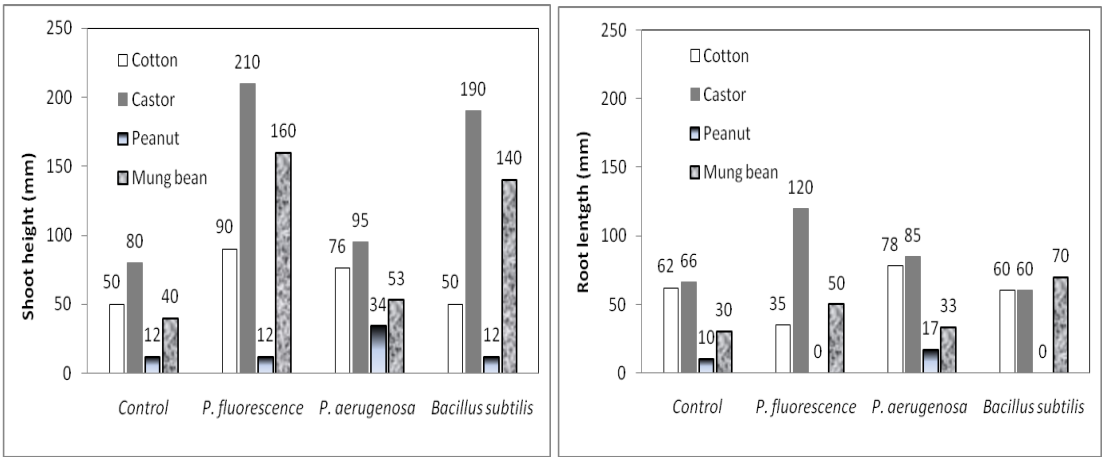
Bacterial Species as Biological control	**Activity of test bacteria on fungus (zone of inhibition in mm)**		
	Fusarium oxysporum	***Aspergillus niger***	***Alteneria alternata***
Pseudomonas fluorescence	12	17	00
Pseudomonas aeruginosa	20	18	12
Bacillus subtilis	18	05	00

Antagonistic activity of *P. fluorescens* culture suspension was observed against *Pythium ultimim* (18mm), *Macrophomina phaseolina* (14mm) and *Pyricularia oryzae* (10mm) (Goud and Muralikrishnan, 2012). Our strain of *P .fluorescens* directly showed similar result (Table 1) with different pathogenic fungus.
Antifungal activity (zone of inhibition) of *P. aeruginosa* against fungus varies like; 10mm for *Aspergillus flavus*, 14mm for *Aspergillus niger*, 15.5mm for *Rhizoctonia bataticola*, 15.5mm for *Rhizoctonia solani* and 14.5mm for *Sclerotium rolfsii* (Kishore *et al.*, 2005). The another report indicated zone of inhibition by *P. aeruginosa* against fungi were 2mm for *Macrophomina phaseolina*, 6mm for *Fusarium solani* and 10mm for *F. oxysporum* (Mansoor *et al.*, 2007). The present work shows equally or even high potential results to inhibit the evaluated fungi species (Table 1).

Biocontrol effect of bacteria against pathogenic fungus on the growth of seedlings

Crops seeds (Castor, Cotton, Peanut and Mung bean) (Figure 1) treated with bacteria *P. fluoresces*, *P. aeruginosa* and *B. subtilis* without any infection of fungus showed plant growth promoting effect of bacteria. Initial results were recorded after fifth and tenth days of sowing but it was found that no significant difference was observed between control and test results within 5 days. The results observe after 10 days were significantly differ from the each groups of the treatment.
Treatment of all three bacterial species was given to the seeds of crop plants and the height of shoots and length of roots were shown in Figure 4 after the tenth day of growth. *P. fluorescence* showed maximum growth promoting effect on Castor seedlings followed by Mung bean and Castor seedlings (Figure 1). The least response was observed in Peanut seedlings. The all over growth promoting effect was shown by *P. fluorescence* followed by *B. subtilis* and *P. aeruginosa*.

Figure 1: Growth promoting effect of bacterial species on the shoot height and root length of seedlings without any fungal treatment



The growth promoting application of *P. aeruginosa* was reported with the combine treatment of medicinal plant *Launaea nudicaulis* dried powder as soil amendment with 0.1% w/w concentration, increased the shoot length of Mung bean form 93mm (control) to 121mm (treated) without any fungus infection (Mansoor *et al.*, 2007). The results of present work increase such length in Mung bean from 40mm (control) to 53mm (treated) by *P. aeruginosa*, and the highest response was of *P. fluorescence* increased shoot length from 40mm (control) to 160mm (treated).
Even the same combine treatment of *P. aeruginosa* and *L. nudicaulis* (medicinal plant powder) reduced the infection of *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium solani* in Mnng bean roots,

measured by root plating on PDA after 6 weeks (Mansoor *et al.*, 2007). In the reported work the shoot length was not measured after the combine treatment of *P. aeruginosa* and fungal infection, unlike in present work the shoot and root lengths were recorded with the infection of pathogenic fungus and *P. aeruginosa* treatment.

The seeds were treated with three individual biocontrol bacterial species against the infection with *F. oxysporum*, *A. niger* and *A. alternate*. The biocontrol effect of bacteria on the growth of shoot and roots are shown in Figure 2-4. The pathogenic effect of fungus was successfully overcome by the presence of bacterial. The best growth of shoot was observed with *P. aeruginosa* where root was observed with *P. fluorescence* and *B. subtilis* in all the plant species. The shoot and root growth of Castor seedlings was best among all. The subsequent healthy growth was observed in Mung bean, Cotton and least in Peanut. In the case of infection with *A. niger* the growth of Peanut was adversely affected and even inhibited (Figure 3). The infection of *A. alternate* was successfully over came with the biocontrol efficiency of all three bacterial species (Figure 4).

The similar kind of study with plants seedlings were reported with different species of test organisms and plants by Akhtar et al (2010). They reported the biocontrol effect of *Bacillus pumilus*, *Pseudomonas alcaligenes*, and *Rhizobium sp.* on wilt disease caused *Fusarium oxysporum* with positive results on lentil (*Lens culinaris*) edible pulse plant indicated by length of shoots and number of root nodules per plant developed with *Rhizobium sp*.

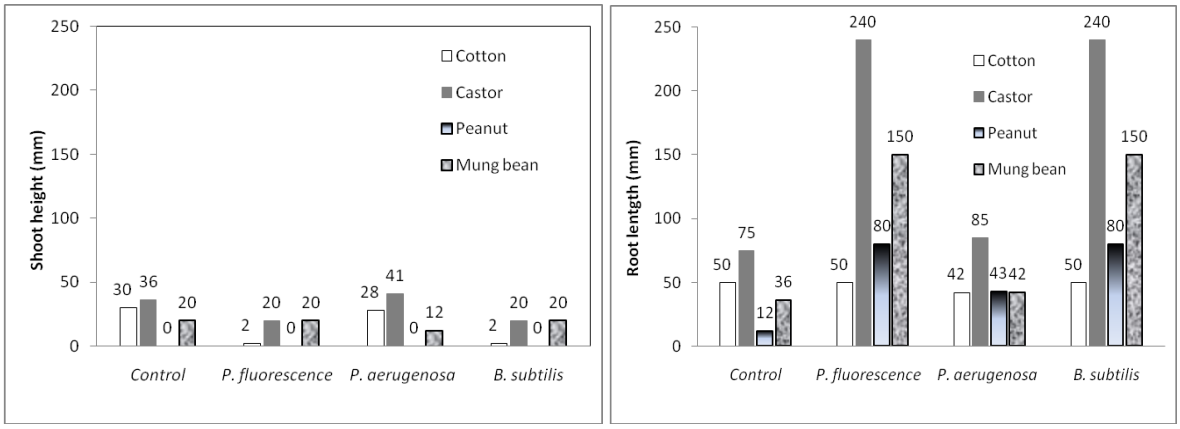


Figure 2: Biocontrol effect of bacterial species against the infection of *F. oxysporum* on the seedlings

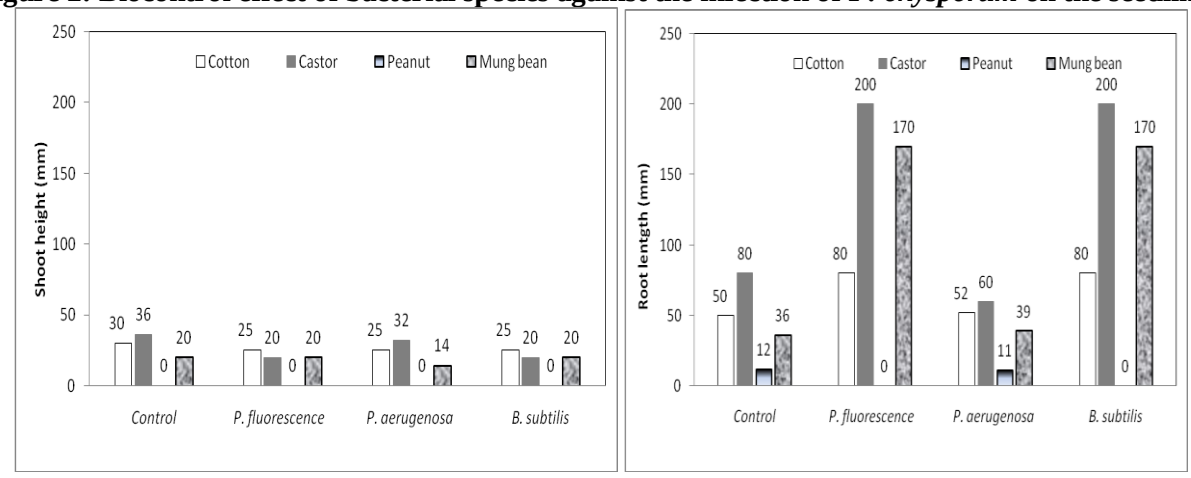


Figure 3: Biocontrol effect of bacterial species against the infection of *A. niger* on the seedlings

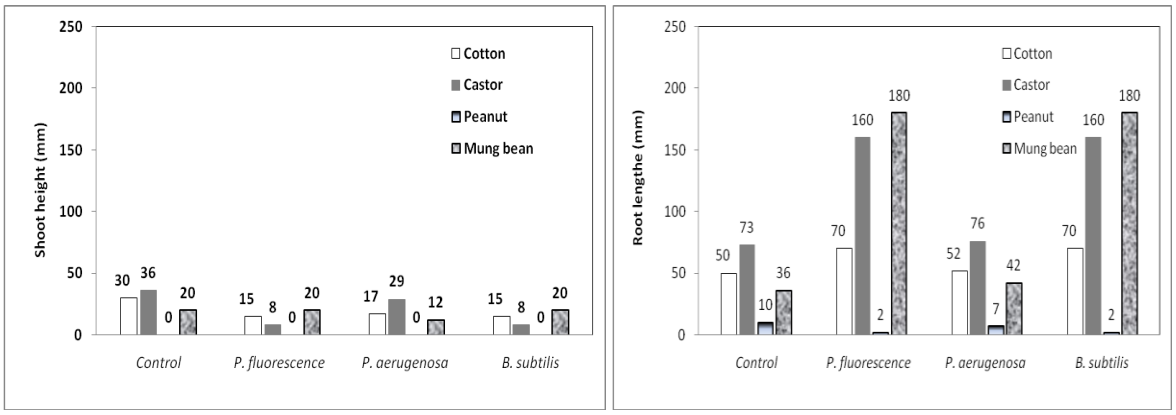


Figure 4: Biocontrol effect of bacterial species against the infection of *A. alternata* on the seedlings

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CONCLUSION

In vitro antagonistic effect against *F. oxysporum* was maximum with *P. aeruginosa*, followed by *P. fluorescence* and *B. subtilis*. For *in vitro* inhibition of *A. niger*, *P. aeruginosa* and *P. fluorescence* both were highest but *B. subtilis* was least active against it. *A. alternate* only control by *P. aeruginosa*. Concluding the maximum *in vitro* efficiency was observed in *P. aeruginosa* on all three plant pathogenic fungi. Growth promoting activity was highest in *P. fluorescence*. After infection *P. aeruginosa* has reported batter action for shoots growth where *P. fluorescence* and *B. subtilis* were batter for root growth. Highest biocontrol activity of bacterial species was observed on Castor plant than Mung bean, Cotton and least in Peanut.

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