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

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ABSTRACT
Pseudomonas fluorescense, P. aeruginosa and Bacillus subtilis are the beneficial rhizobacteria possessing biocontrol activity against plant pathogenic fungus. In present research the biocontrol effect of P. fluorescense, 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. fluorescense and least was B. subtilis. In plant inoculation study, the seeds were treated with pathogenic fungus as well as P. fluorescense, P. aeruginosa and B. subtilis respectively. The shoot and root length were measured as growth parameters after tenth day of the germination. P. fluorescense 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. fluorescense 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 fluorescense, 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 (Fe³⁺) 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-diacyethyl 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. fluorescense 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. fluorescense was evaluated against various fungi including Fusarium spp. (Srivastava and Shalini, 2008; Thangavelu and Mari, 2006). P. fluorescense 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. fluorescense and B. subtilis both are reported as biocontrol agent for fungus Helminthosporium oryzae 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. fluorescense, 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 fluorescense 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% HgCl₂ 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. Flasks were incubated at 30°C on orbital incubator shaker for overnight. Spores of fungus (Aspergillus niger, Fusarium oxysporum, Altoeneria 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 inoculants by soaking 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

<table>
<thead>
<tr>
<th>Bacterial Species as Biological control</th>
<th>Activity of test bacteria on fungus (zone of inhibition in mm)</th>
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<tr>
<td></td>
<td>Fusarium oxysporum</td>
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<td>Pseudomonas fluorescence</td>
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<td>Pseudomonas aeruginosa</td>
<td>20</td>
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<tr>
<td>Bacillus subtilis</td>
<td>18</td>
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Antagonistic activity of \textit{P. fluorescens} culture suspension was observed against \textit{Pythium ultimim} (18mm), \textit{Macrophomina phaseolina} (14mm) and \textit{Pyricularia oryzae} (10mm) (Goud and Muralikrishnan, 2012). Our strain of \textit{P. fluorescens} directly showed similar result (Table 1) with different pathogenic fungus.

Antifungal activity (zone of inhibition) of \textit{P. aeruginosa} against fungus varies like; 10mm for \textit{Aspergillus flavus}, 14mm for \textit{Aspergillus niger}, 15.5mm for \textit{Rhizoctonia bataticola}, 15.5mm for \textit{Rhizoctonia solani} and 14.5mm for \textit{Sclerotium rolfsii} (Kishore et al., 2005). The another report indicated zone of inhibition by \textit{P. aeruginosa} against fungi were 2mm for \textit{Macrophomina phaseolina}, 6mm for \textit{Fusarium solani} and 10mm for \textit{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 \textit{P. fluorescens}, \textit{P. aeruginosa} and \textit{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. \textit{P. fluorescens} 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 \textit{P. fluorescens} followed by \textit{B. subtilis} and \textit{P. aeruginosa}.

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

![Shoot Height](chart1.png)  ![Root Length](chart2.png)

The growth promoting application of \textit{P. aeruginosa} was reported with the combine treatment of medicinal plant \textit{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 \textit{P. aeruginosa}, and the highest response was of \textit{P. fluorescens} increased shoot length from 40mm (control) to 160mm (treated).

Even the same combine treatment of \textit{P. aeruginosa} and \textit{L. nudicaulis} (medicinal plant powder) reduced the infection of \textit{Macrophomina phaseolina}, \textit{Rhizoctonia solani} and \textit{Fusarium solani} in Mng 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. fluorescense 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 alcaligines, 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. fluorescense* and *B. subtilis*. For *in vitro* inhibition of *A. niger*, *P. aeruginosa* and *P. fluorescense* 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. fluorescense*. After infection *P. aeruginosa* has reported batter action for shoots growth where *P. fluorescense* 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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Research Article


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Research Article


