**BIOCONTROL POTENTIALS OF PLANT GROWTH PROMOTING RHIZOBACTERIA AGAINST FUSARIUM WILT DISEASE OF CUCURBIT**

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**ABSTRACT**

*Fusarium* spp., are the major soil-borne as well as seed borne pathogens causing wilt and rot diseases in more than 80 plant species including cucurbit. *Fusarium* spp., causes up to 100 % yield loss in the worldwide. Eleven isolates including three standard isolates were tested both in-vitro and in-vivo. In-vitro assay was done by dual culture method. Maximum inhibition was in case of *Fusarium solani* by *Bacillus cereus* MIC5. *Sarratia* spp. MIC1 antagonized the *F. verticillodles* and *F. solani*2. *P. aeruginosa* MIC2 inhibits all tested isolates *F. oxysporum*1. *P. aeruginosa* MTCC2581 suppressed the radial growth rate of *F. oxysporum*2. The two systemic fungicides used were chlorothalonil + mefenoxam (1000 ppm) and carbendazim (75 ppm to 500 ppm) which checked the growth of *F. oxysporum*. Carbendazim was more effective compared to mefenoxam + chlorothalonil at all tested concentrations. The crude extract of *P. aeruginosa* MIC2 developed in chloroform: methanol (9:1) showed a metabolite at Rf 0.77 which it may be 2,4-diacetylphloroglucinol (DAPG), a broad-spectrum antimicrobial agent. Increased cucurbit seeds germination and seedling vigour was observed in *B. amyloliquefaciens* MIC6 (68% & 1576) and *P. aeruginosa* MTCC2581 (70% & 1929) in primed seeds. Further *P. aeruginosa* MTCC2581 can be tested in the field against the Fusarium wilt.

**Keywords**: *Fusarium* spp., PGPR, fungicides, biological control, DAPG.

**INTRODUCTION**

India is the second largest producer of vegetables in the world with 2.8 % of total cropped area and 13.38 % of total vegetable production (Kundu, 2012). Being the largest cash crop, about 4,929,400 million tonnes of cucurbit were produced in India (FAOSTAT, 2010). *Fusarium* spp., are the major soil-borne as well as seed borne pathogens causing wilt and rot diseases in more than 80 plant species including cucurbit (Mahfooz et al., 2011). *Fusarium* spp., causes up to 100 % yield loss in the worldwide (Santos et al., 2002). In cucurbitaceae *F. oxysporum* is the frequent pathogen in cucumber seeds (Farrag and Moharam, 2012). *Fusarium* spp., cause the crown rot, foot rot and fruit rot of squash as well as pumpkin (Zitter, 1996). Pathogens survive in soil and seed in the form of chlamydospores for many years. Mycelia enter the epidermal tissues invading through roots, extend to the vascular bundles and form spores in plants (Chehri et al., 2010). The pathogen causes seed abortion and rot, necrosis, reduction or elimination of germination capacity as well as plant damage at later stages of plant growth resulting in development of the disease as systemic or local infection (Khazanda et al., 2002). The diseases can be controlled by using resistant cultivars, chemical fungicides as well as fumigants (Favel et al., 2005) and biological control agents (Idris et al., 2007). Soil solarisation (Tamietti and Valentino, 2006), crop rotation and grafting (Zhao et al., 2011) are used to control the root diseases. Fungical seed treatment may kill or inhibit seed borne pathogens that may form a protective zone around seeds to reduce seed decay and seedling wilt resulting in vigorous seedlings and emergence of seeds (Khazanda et al., 2002). In-vitro inhibition of *F. solani* was observed by such fungicides as, Aliette, Benlate and Carbendazim. Carbendazim completely inhibited seed borne infection of *F. solani* in bitter gourd, cucumber and bottle gourd. Root infection was completely controlled by Benlate and Carbendazim in bitter gourd (Sultana and Gaffer, 2010). Different fungicides were tested against the seed borne fungi *F. moniliforme* in wheat. The fungicides retarded...
the growth of fungi and increased the seedling number (Khanzada et al., 2002).

From the past three decades to minimize the use of the chemicals, synthetic fertilizers and pesticides sustainable agriculture was practiced as an ecofriendly concept. In sustainable agriculture soil borne pathogens can be controlled by biological agents like plant growth promoting rhizobacteria (PGPR) as they colonize host root and create competition for space as well as nutrition. PGPR is ecofriendly, stimulates the plant growth factors and reduces the incidence of crop diseases and supplies the nutrition for the growth of plant. *P. cepacia*, *P. fluorescense*, *B. polymyxa* and *B. subtilis* used as biocontrol agents against *Fusarium* wilt of melon. Among them *P. fluorescense* shows highest reduction in the *Fusarium* wilt incidence and good antagonistic activity compared to control (Hamed et al., 2009). *F. oxysporum* causes root and crown rots in sorghum in world. Seventy eight isolates of PGPR were tested against *F. oxysporum* in-vitro and in greenhouse. Four isolates viz. *Bacillus* spp. KBE2-5, *B. stearothermophilus* KBE5-7, *B. cereus* KBE5-1 and *B. cereus* NAE5-5 suppressed disease by root colonization. *F. oxysporum* is managed by PGPR as compared to control (Idris et al., 2007). Bafti et al. (2005) reported that, *Streptomyces olivaceus* strain 115 antagonised *F. oxysporum* f.sp. *melonis* caused root rot and *Fusarium* wilt of cucurbits in Kerman Province, southeast of Iran. In green house soil treated with *S. olivaceus* reduction in wilt incidence was observed, as compared to control. In modern agriculture PGPR is used as bio-fertilizer as well as biological control agent against certain seed and soil-borne plant pathogens. The present work relates to in-vitro and in-vivo evaluation of fungicides as well as effective use of PGPR against *Fusarium*.

**MATERIALS AND METHOD**

**Microorganisms used:** The eight PGPR isolates (Table 1) isolated from the rhizosphere samples of healthy *Cucurbitaceae* field soils were obtained from the culture collection of the Department of Studies in Microbiology, Manasagangothri, Mysore. Standard PGPR strains obtained from Microbial type culture collection (MTCC) Chandigarh, India, included *Pseudomonas aeruginosa* MTCC2581, *Bacillus coagulans* MTCC3543 and *Bacillus subtilis* MTCC2763, which were used as positive controls. The different *Fusarium* spp. were isolated from diseased *cucurbitaceae* field soil and materials on potato dextrose agar (PDA), incubated at 25°C for 5-6 days.

**In-vitro antagonistic activity**

**Biological control:** The in-vitro mycelial inhibition of *Fusarium* spp. was tested by dual culture technique as referred by Idris et al. (2009). Briefly each bacterial isolate was point inoculated at four sides, 3cm apart from the center on the PDA plate and incubated at 35±2°C for 24 h. After 24 h, six day old fungal culture was point inoculated at the center of the PDA plate, control plates were sealed with parafilm and incubated at 28±2°C for 4-5 days. Inhibition per cent was calculated as follows.

\%
\[
\text{of Inhibition} = \left( \frac{R - r}{R} \right) \times 100
\]

Where, *r* is the radius of the fungal colony opposite to the bacterial colony and *R* is the maximum radius of the fungal colony away from the bacterial colony.

**Fungicides:** The carbendazim and chlorothalonil + mefenoxam fungicides were evaluated by poison food method against *F. oxysporum*. Different concentrations (25, 50, 75, 100, 500, 1000 ppm) of fungicides were added to molten PDA after sterilization and poured to 9 cm diameter petri plates. Six days old culture 5 mm mycelial disks were point inoculated at the center of the plate to check the toxicity. The plates without fungicides were maintained as control and incubated at 28±2°C for 4-5 days (Faravel et al., 2005).

**Antibiotic Production**

The antibiotic activity of selected PGPR isolates were assessed by extracting and testing toxicity of metabolites produced by them by the method of Ayyadurai et al. (2005). *P. aeruginosa* MIC2 was grown for 48 h in nutrient broth (NB) and centrifuged at 10,000 rpm for 10 min. The filtrate was extracted three times by shaking with an equal volume of ethyl acetate (1:1 ratio). *P. aeruginosa* MIC2 culture extract was collected and dried in a rotary evaporator (Buchi, Switzerland). The extract residue was re-dissolved in ethyl acetate and stored in refrigerator at 4°C. The residue was spotted in ethyl acetate on to thin layer chromatography plate (Silica gel 60 F254, 20×20 cm, 0.2 mm thickness, Merck). The plate was chromatogrammed using chloroform: methanol (9:1) as solvent system. Later the plates were observed under UV light at 254 nm.

**In-vivo evaluation of PGPR**

All PGPR strains were grown in NB for 48 h and centrifuged at 8000 rpm for 5 min to get the pellet and washed with sterile distilled water. The concentration of PGPR inoculum were adjusted with sterile distilled water to 1×10⁸ cfu/ml at A₆₁₀ nm using UV-visible
Table 1. In-vitro mycelial inhibition of Fusarium spp. by PGPR isolates from different cucurbitaceae crops.

<table>
<thead>
<tr>
<th>PGPR</th>
<th>F. graminarum</th>
<th>F. solani</th>
<th>F. oxysporum1</th>
<th>F. verticilliodes</th>
<th>F. solani 2</th>
<th>F. semitectem</th>
<th>F. oxysporum 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>74.0 ± 0.57^a</td>
<td>84.66 ± 0.88^a</td>
<td>74.0 ± 0.57^a</td>
<td>64.0 ± 0.57^a</td>
<td>46.0 ± 0.57^ab</td>
<td>67.33 ± 0.88^a</td>
<td>86.33 ± 0.88^a</td>
</tr>
<tr>
<td>Sarratia spp. MIC1</td>
<td>0</td>
<td>0</td>
<td>36.33 ± 0.88^cd</td>
<td>52.36 ± 0.85^b</td>
<td>50.33 ± 1.49^a</td>
<td>0</td>
<td>15.63 ± 1.68^e</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa MIC2</td>
<td>46.06 ± 1.94^b</td>
<td>44.86 ± 0.92^b</td>
<td>50.23 ± 0.98^b</td>
<td>42.3 ± 1.35^d</td>
<td>35.3 ± 2.4^ab</td>
<td>0</td>
<td>36.86 ± 1.21^d</td>
</tr>
<tr>
<td>Bacillus cereus MIC3</td>
<td>43.73 ± 1.32^bc</td>
<td>46.43 ± 2.14^b</td>
<td>29.3 ± 1.61^e</td>
<td>0</td>
<td>44.1 ± 2.68^ab</td>
<td>0</td>
<td>23.3 ± 1.57^f</td>
</tr>
<tr>
<td>Bacillus subtilis MIC4</td>
<td>0</td>
<td>0</td>
<td>30.13 ± 0.78^e</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacillus cereus MIC5</td>
<td>27.0 ± 1.15^d</td>
<td>32.1 ± 1.87^de</td>
<td>49.86 ± 10.92^a</td>
<td>30.53 ± 1.48^c</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacillus amyloliquefaciens MIC6</td>
<td>15.26 ± 1.53^e</td>
<td>0</td>
<td>34.46 ± 1.75^e</td>
<td>0</td>
<td>20.63 ± 1.28^d</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacillus cereus MIC7</td>
<td>16.26 ± 0.81^e</td>
<td>0</td>
<td>28.9 ± 0.77^c</td>
<td>0</td>
<td>0</td>
<td>43.76 ± 1.22^b</td>
<td>0</td>
</tr>
<tr>
<td>Bacillus licheniformis MIC9</td>
<td>39.6 ± 0.87^c</td>
<td>32.76 ± 1.45^c</td>
<td>33.1 ± 1.51^de</td>
<td>38.6 ± 0.75^d</td>
<td>31.0 ± 0.57^b</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa MTCC 2581</td>
<td>0</td>
<td>0</td>
<td>40.93 ± 0.98^c</td>
<td>47.16 ± 0.53^c</td>
<td>36.33 ± 0.57^ab</td>
<td>0</td>
<td>55.53 ± 0.6^b</td>
</tr>
<tr>
<td>Bacillus subtilis MTCC2763</td>
<td>0</td>
<td>11.83 ± 0.43^e</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>41.60 ± 1.09^e</td>
<td>0</td>
</tr>
<tr>
<td>Bacillus coagulans MTCC3543</td>
<td>0</td>
<td>21.8 ± 1.03^d</td>
<td>48.43 ± 0.69^bc</td>
<td>0</td>
<td>0</td>
<td>29.26 ± 0.54^e</td>
<td>0</td>
</tr>
</tbody>
</table>

spectrophotometer and test fungicides along with an adhesive carboxymethyl cellulose (CMC) were used to treat surface sterilized cucurbit seeds for 6 h on rotary shaker at 150 rpm. Seeds soaked in distilled water amended with CMC (0.1g/10 ml) served as control (Ramamoorthy et al., 2002). The overnight drained seeds were subjected to germination test by paper towel method (ISTA, 2005) and seedling vigor was calculated as per mentioned by Abdul-Baki and Anderson (1973). Each rhizobacterial isolate was maintained in triplicate and all experiments were carried out in triplicate.

Statistical analysis: Statistical significance was measured using GraphPad Prism 5 software and figure 2 by Origin 6. Data on the In-vitro mycelial inhibition of Fusarium spp. by PGPR and suppression of F. oxysporum pv. cucumerianum by fungicides were subjected to two way analysis variance (ANOVA). Mean values among treatments were compared by Tukey’s HSD test (p<0.0001).

RESULTS

In-vitro antagonistic activity

Biological control: In the tested 11 isolates, three standard and eight isolated PGPR were used for the dual culture assay against Fusarium spp. Maximum inhibition was observed in case of F. verticilliodes (52.36) and F. solani 2 (50.33) by Sarratia sp. MIC1. P. aeruginosa MIC2 inhibited all types of Fusarium spp. to the maximum extent. P. aeruginosa MTCC2581 inhibited mycelial growth of F. oxysporum 2 (55.53) maximum (Table 1). Control plates not treated with bacterial isolates were completely covered by phytopathogens. All the seven Fusarium spp. were not inhibited by single isolate, different isolates are effective on the different species. The mean mycelial growth inhibition of the Fusarium sp. by bacteria showed significant (p <0.0001).

Fungicides: The toxicity of the two tested fungicides carbendazim and mefenoxam + chlorothalonil used was evaluated against F. oxysporum. The fungal radial growth rate was reduced as a result of increasing the fungicide concentration. The carbendazim inhibited F. oxysporum at 500 ppm and mefenoxam + chlorothalonil inhibited at 1000 ppm as maximum concentration. As summarised in Table 2. carbendazim was more effective against F. oxysporum as compared to mefenoxam + chlorothalonil in the different tested concentrations.
Table 2. In-vitro suppression of *Fusarium oxysporum* pv. *cucumeriamun* by fungicides

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>% Inhibition Mefenoxam+ chlorothalonil</th>
<th>Carbendazim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>86.78 ± 0.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>84.52 ± 0.66&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>25</td>
<td>13.71 ± 0.44&lt;sup&gt;c&lt;/sup&gt;</td>
<td>55.73 ± 0.89&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>45.83 ± 0.58&lt;sup&gt;c&lt;/sup&gt;</td>
<td>61.50 ± 0.68&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>75</td>
<td>65.86 ± 1.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>71.64 ± 0.90&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>100</td>
<td>75.52 ± 0.62&lt;sup&gt;c&lt;/sup&gt;</td>
<td>86.33 ± 0.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>500</td>
<td>83.97 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>92.63 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1000</td>
<td>93.84 ± 0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

**Antibiotic Production**

Crude extract obtained from *P. aeruginosa* MIC2 was brownish colored having broad-spectrum antimicrobial activity. The TLC plates were analysed in chloroform: methanol (9:1) system (Fig. 1) and revealed the presence of metabolite at R<sub>f</sub> - 0.77 confirmed by comparing with standard 2,4-diacetylphloroglucinol (DAPG) under UV at 254nm.

**In-vivo evaluation**

The 13 primed seeds showed no increase in germination and higher vigour index compared to control (Fig. 2). Among these *B. amylophilafaciens* MIC 6 and *P. aeruginosa* MTCC 2581 showed higher germination and vigour index. Both the chemicals showed maximum germination and vigour.

![Figure 1. TLC summary of crude extracts of *P. aeruginosa* MIC2.](image)

![Figure 2. Effect of PGPR and fungicides priming on cucurbit seed germination and seedling vigour cucurbit after 8 days of germination.](image)

**DISCUSSION**

Both the fungicides in all tested concentrations showed considerable effect on the growth of *F. oxysporum* compared to the control. Maximum inhibition of the colony growth by carbendazim at 500 ppm (92.63%) and mefenoxam + chlorothalonil at 1000 ppm (93.8%) was evident when compared to the control in the present study. Soil-borne diseases caused by *F. oxysporum* can be managed by using chemical fungicides. (Sultana and Ghaffar, (2010) reported that Carbendazim (100ppm) and Ridomil (500ppm) inhibited *Fusarium solani* a seed rot, seedling and root infection pathogen on gourds and cucumber. Carbendazim and Carbendazim + Mancozeb gave 100% inhibition of mycelial growth of *F. solani* at 0.2 and 0.3% concentrations (Chavan et al., 2009). Among seven different fungicides, Bravo was most toxic to *F. oxysporum* CS-20 at 50 ppm were as Azoxystorin and Chlorothalonil inhibits at 10ppm or greater concentration compared to control (Fravel et al., 2005). *F. graminearum*, *F. avenaceum* and *F. verticillioides* isolates were tested at different concentrations of carbendazim, tebuconazole, flutriafol, metconazole and prochloraz by Ivic et al., 2011. Carbendazim was toxic at 10mg/l with an IC50 of *F. graminearum* (0.39-1.41 mg/l), *F. avenaceum* (0.91-1.35 mg/l) and *F.
verticalioides (0.47-0.6 mg/l). Shakoor et al. (2011) reported that fungicides Bavistin (30mg/10ml) and Ridomil gold MZ (30 & 40 mg/10ml) concentrations controlled successfully Fusarium, Aspergillus as well as Myrothecium.

Eleven tested PGPR isolates, P. aeruginosa MTCC2581 controlled mycelial growth of F. oxysporum 2 and Sarratia spp. MIC1 inhibited F. verticilloides as well as F. solani 2 by more than 50%. P. aeruginosa MIC2 suppressed the growth of all Fusarium spp. to the maximum (50.23%). The efficacy of PGPR strains Serratia UPMS3, Erwinia UPMS10 Pseudomonas UPMS20 and UPMS6 isolated by Yasmin et al., (2009) was reported to show antagonistic activity against species of Rhizoctonia and Pythium . Hariprasad et al., (2009) reported that the antagonistic activity of the B. amyloliquefaciens IRB36 (40%), P. fluorescens IRB26 (43%), P. fluorescens PSRB19 (31%) and P. putida PSIRB15 against F. oxysporum in tomato. Among 14 PGPR isolates three inhibit the growth of F. oxysporum (20%). Remaining isolates inhibited Sclerotium rolfsii and R. solani (11-20%), being used as inoculants of soybean plant (Wahyudi et al., 2011). Singh et al., (1999) reported that Paenibacillus sp. 300 and Streptomyces sp. 385, suppressed Fusarium wilt of cucumber by chitinase and β-1,3-glucanase production. Antifungal metabolites isolated from a small portion of the soil micro-flora were able to produce certain types of antibiotics, hydrogen cyanide, proteases and chitinolytic enzymes (Kumar et al., 2002; Hariprasad et al., 2013). Ayyadurai et al., (2005) reported that P. aeruginosa produced an antimicrobial agent 2,4-diacetylphloroglucinol (DAPG) in the chloroform: methanol (9:1) solvent system. In our study P. aeruginosa MIC2 with the same solvent system produced a metabolite at Rf - 0.77 on TLC plate with a standard DAPG confirming the metabolite as DAPG (Fig.1).

In cucurbit seeds primed with PGPR, P. aeruginosa MTCC2581 showed maximum vigour followed by P. aeruginosa MIC2, B. amyloliquefaciens MIC6 and B. licheniformis MIC9. Compared to the control treated with sterile distilled water and pathogen. The roots from the control treatment rendered growth of F. oxysporum compared to an incidence upto 55% for plants treated with B. subtilis, B. licheniformis, B. cereus (Idris et al., 2007; Awatif and Al-Jedabi, 2009). Manikandan et al. (2010) reported that liquid formulation of P. fluorescens strain Pf1 applied against Fusarium wilt increased the tomato fruit yield compared to untreated control under glasshouse and field conditions. Chen et al. (2010) confirmed that among the 158 PGPR isolates, B. subtilis B579 suppressed the cucumber rot causing pathogen F. oxysporum f. sp. cucumerinum by production of hydrolytic enzymes. B. subtilis B579 increased the seedlings vigour and growth of plants.

In conclusion, among the tested PGPR strains P. aeruginosa MIC2 gave 35-50% inhibition of all Fusarium spp. and among the fungicides carbendim inhibited F. oxysporum at lower concentration. The metabolite may be DAPG present in the crude extract of P. aeruginosa MIC2. Even though percent germination was not affected, vigour of plants primed with B. amyloliquefaciens MIC6 and P. aeruginosa MTCC2581 was considerably higher than the control. The two isolates were further tested for their performances in the field.

REFERENCES


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