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

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A B S T R A C T

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 cucurbits. *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. verticillodes* and *F. solani2*. *P. aeruginosa* MIC2 inhibits all tested isolates *F.* oxysporum1. *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 R_f - 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 cucurbits 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 cucurbits (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 (Khanzada *et al.,* 2002). The diseases can be controlled by using resistant cultivars, chemical fungicides as well as fumigants (Fravel *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.

Fungicidal 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 (Khanzada *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

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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. ceparcia, P. fluorescence, B. polymyxa and B. subtilis* used as biocontrol agents against *Fusarium* wilt of melon. Among them *P. fluorescence* 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 25^0 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.

% of Inhibition = (R- r)/R × 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 (Fravel *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 were 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 1×10^8 cfu/ml at A_{610} nm using UV-visible

Table 1. In-vitro mycelia inhibition of Fusarium spp. by PGPR isolates from different cucurbitaceae crops.

PGPR	% Inhibition						
	F. graminarum	*F. solani* 1	*F. oxysporum*1	*F. verticillodes*	*F. solani* 2	*F. semitectem*	*F.oxysporum* 2
Control	74.0 ± 0.57[a]	84.66 ± 0.88[a]	74 ± 0.57[a]	64 ± 0.57[a]	46 ± 0.57[ab]	67.33 ± 0.88[a]	86.33 ± 0.88[a]
Sarratia spp. MIC1	0	0	36.33 ± 0.88[cd]	52.36 ± 0.85[b]	50.33 ± 1.49[a]	0	15.63 ± 1.68[g]
Pseudomonas aeruginosa MIC2	46.06 ± 1.94[b]	44.86 ± 0.92[b]	50.23 ± 0.98[b]	42.3 ± 1.35[d]	35.3 ± 2.4[ab]	0	36.86 ± 1.21[d]
Bacillus cereus MIC3	43.73 ± 1.32[bc]	46.43 ± 2.14[b]	29.33 ± 1.61[e]	0	44.1 ± 2.68[ab]	0	23.3 ± 1.57[f]
Bacillus subtilis MIC4	0	0	30.13 ± 0.78[e]	0	0	0	0
Bacillus cereus MIC5	27.0 ± 1.15 [d]	0	32.10 ± 1.87[de]	0	49.86 ± 10.92[a]	30.53 ± 1.48[c]	0
Bacillus amyloliquefaciens MIC6	15.26 ± 1.53[e]	0	0	34.46 ± 1.75[e]	0	20.63 ± 1.28[d]	0
Bacillus cereus MIC7	16.26 ± 0.81[e]	0	28.9 ± 0.77[e]	0	0	43.76 ± 1.22[b]	0
Bacillus licheniformis MIC9	39.6 ± 0.87[c]	32.76 ± 1.45[c]	33.1 ± 1.51[de]	38.6 ± 0.75[d]	31.0 ± 0.57[b]	0	0
Pseudomonas aeruginosa MTCC 2581	0	0	40.93 ± 0.98[c]	47.16 ± 0.53[c]	36.33 ± 0.57[ab]	0	55.53 ± 0.6[b]
Bacillus subtilis MTCC2763	0	11.83 ± 0.43[e]	0	0	0	0	41.60 ± 1.09[c]
Bacillus coagulans MTCC3543	0	21.8 ± 1.03[d]	0	48.43 ± 0.69[bc]	0	0	29.26 ± 0.54[e]

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 by 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. verticillioides* (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. bybacteria showed significant ($p <0.0001$).

Fungicides: The toxicity of the two tested fungicides carbendezim 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. carbendezim 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

Dose	% Inhibition	
ppm	Mefenoxam+ chlorothalonil	Carbendezim
Control	86.78 ± 0.69[b]	84.52 ± 0.66[b]
25	13.71 ± 0.44[f]	55.73 ± 0.89[e]
50	45.83 ± 0.58[e]	61.50 ± 0.68[d]
75	65.86 ± 1.09[d]	71.64 ± 0.90[c]
100	75.52 ± 0.62[c]	86.33 ± 0.62[b]
500	83.97 ± 0.25[b]	92.63 ± 0.19[a]
1000	93.84 ± 0.49[a]	

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_f - 0.77 confirmed by comparing with standard 2,4- diacetylphloroglucinol (DAPG) under UV at 254nm.

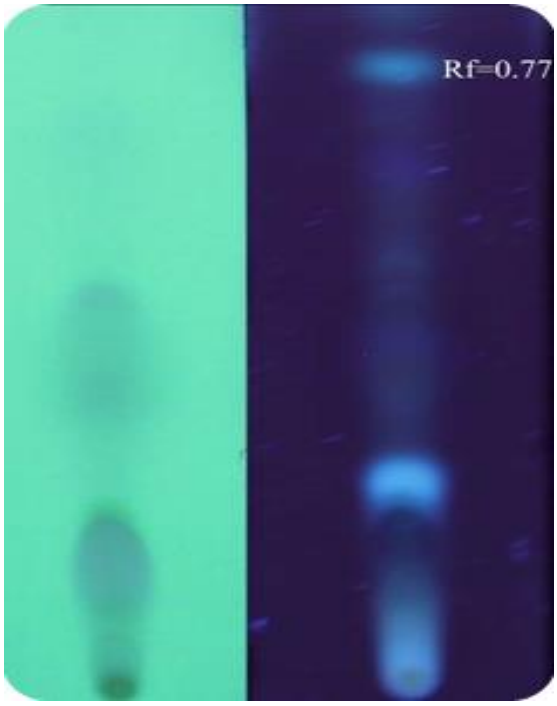

Figure 1. TLC summary of crude extracts of *P. aeruginosa* MIC2.

***In-vivo* evaluation**

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

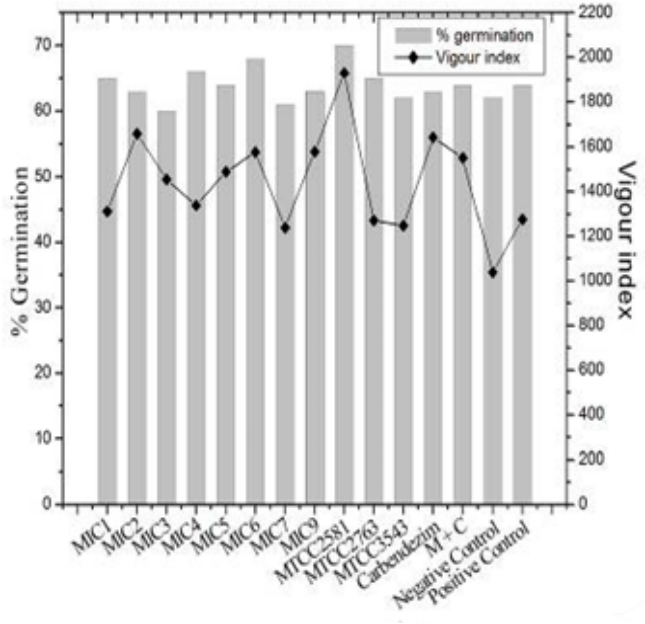

Figure 2. Effect of PGPR and fungicides priming on cucurbit seed germination and seedling vigour cucurbit after 8 days of germination.

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.*

verticillioides (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. verticilloids* 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* UPMSP3, *Erwinia* UPMSP10 *Pseudomonas* UPMSP20 and UPMSP6 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. areroginosa* 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 R_f - 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 carbendizm 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.

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