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Pigeon pea *Fusarium* wilt: *In vitro* pathogenicity test of *Fusarium* isolates and its biological control

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Abstract
Fusarium udum L. (Butler) is the causal agent of a wilt disease on pigeon pea (*Cajanus cajan* L.) and cause huge economical loss by destroying half or complete plant. An experiment was conducted to isolate and check the pathogenicity of *Fusarium* strains isolated from the wilted Redgram plant. The results revealed that the isolate RGF1 has shown significant amount of disease incident in pre emergent and post emergence of plant. Further studies were carried out to control the wilt disease incident through the help of biocontrol agents isolated from rhizosphere soil of Redgram. The results shows that the treatment which includes consortium of microorganisms had controlled significant amount of disease incident.

Keywords: *Fusarium* wilt, pathogenicity test, biocontrol, greenhouse

Introduction
Fusarium wilt is the economically devasting fungal disease of Redgram and is common throughout India. It is prevalent in Andhra Pradesh, Maharashtra, Madhya Pradesh, Uttar Pradesh and Bihar. Redgram is susceptible to wilt pathogen throughout its development stages. However, the symptoms are more pronounced and the damage is greater at flowering and pod formation stage. Wilting of seedlings and grown up plants as if they have suffered from water shortage although there is plenty of moisture in the field, is the main symptom. Wilting is characterized by gradual, sometimes sudden yellowing, withering and drying of leaves followed by drying of the entire plant or some of its branches.
The disease was severe in Maharashtra, Bihar and Uttar Pradesh. In Karnataka, the incidence of wilt varied from 0 to 90%. It was severe in major crop growing areas of Gulbarga, Dharwad, Bidar and Bijapur (Kannaiyan *et al.*, 1981) [6]. A voluminous work has been done on *F. udum* Butler in India and abroad. The pathogen is a soil and seed borne. The genus *Fusarium* has wide host range and survives for long time in field in the absence of host plant. Therefore, chemical control is not satisfactory, adequate and non-economical as a long-term solution. Considering, the crop health and economic losses, the alternative to this is to explore the possibility of improving genetical disease resistance and integration of chemical and biological control, which can be successfully adopted in modern agriculture.
Plant-associated microorganisms bring about important functions for plant growth and health. Direct plant growth promotion by microbes is based on improved nutrient acquisition and hormonal stimulation. Diverse mechanisms are involved in the suppression of plant pathogens, which is often indirectly connected with plant growth. Plant growth promoting microorganisms (PGPM) and biological control agents (BCA) are shown to possess secondary beneficial effects that would increase their usefulness as bio-inoculants, regardless of the need for their primary function. Indeed, PGPM, such as *Rhizobium* spp., can promote plant growth and productivity (primary effect) but have now been shown to play a role in reducing disease incidence (secondary effect). Conversely, Biocontrol agents (BCA), such as *Trichoderma spp.* and *Pseudomonas* spp. can control disease (primary effect) but have recently demonstrated stimulation of plant growth (secondary effect) in the absence of a pathogen. Whereas the bacterial genera of *Bacillus*, *Pseudomonas* and *Rhizobium* are well-studied examples for plant growth promotion and *Trichoderma* are model organisms to demonstrate influence on plant health. In present study, attempts were made to isolate wilt pathogen and conducted pathogenicity test. Biocontrol of the most virulent *Fusarium* isolate among five isolates were also carried out with the help of biocontrol agents.

Material and Methods

Isolation of pathogen from wilt affected redgram plant

Wilt affected plants were collected from Zonal Agricultural Research Station, GKVK, Bengaluru. Plant samples were collected based on visual observation of wilt symptoms (yellowing of leaves, partial or complete drying of plant and dark coloration of xylem of plant when stem/root slit into half vertically).

Wilted red gram Samples were collected in a paper envelope and brought to the laboratory. The pathogens were isolated by direct culturing of infected parts according to Chopada *et al.* (2015) [2] with minor modifications. Briefly, the infected roots were washed with running tap water to remove all adhering soil particles, and then cut into small pieces prior to surface sterilization using 96% ethanol for 30s. All the sterilized pieces were placed onto Potato Dextrose Agar (PDA) plates. Plates were incubated under the standard incubation conditions (Chehri *et al.* 2010) [1] for 48h and the resulting single-spore of *Fusarium* colonies were transferred to fresh Potato Dextrose Agar (PDA) plates for further studies. The species were identified on the basis of macroscopic and microscopic characteristics such as, pigmentations of colony, types of conidiogenous cells, shape and size of conidia. Identification to the species level is based on the descriptions of Leslie and Summerell (2006) [8].

Morphological and Microscopic characterization of pathogen isolates

Fungal isolates were sub-cultured and cultural characters *viz*., colony color, nature of mycelia, pigmentation on the media were observed. Microscopic observations like conidial characters such as shape and color of conidia were done to confirm the *Fusarium* isolates. Confirmed *Fusarium* isolates were purified and further used for pathogenicity studies.

Maintenance of isolated *Fusarium* cultures

The cultures of pathogenic *Fusarium* spp. were grown for six days at 28 ± 2º C on potato dextrose agar (PDA) slants and preserved at 4º C in a refrigerator and maintained further by culturing once in a month. One set of all cultures were preserved in 50 per cent glycerol vials to serve as stock culture.

Pathogenicity test for *Fusarium* isolates

The isolated *Fusarium* strains were cultured in potato dextrose broth for three days under shaking condition. Redgram seeds (BRG-2) were soaked in the beaker containing *Fusarium* isolates for 3h followed by sowing in trays containing sterilized soil. Twelve seeds were treated for each isolate. The observations on wilt symptoms were recorded during pre-emergence and post emergence of crop up to where maximum of 50 per cent of seedlings got wilt in 10 days after inoculation.

Percent pre-emergence disease incidence = 100 (GA-GT)/ GA

GA-Germination percentage in absolute control

GT- Germination percentage in treatment

Percent post- emergence disease incidence = 100 (GP-ND)/GP

GP-Number of healthy plants left in control

ND- Number of healthy plants left in treatment

Isolation and screening of Biocontrol agents from rhizosphere soil of healthy Redgram plant

Antagonistic microorganisms were isolated by following serial dilution technique (Jhonson and Curl, 1977) [5]. The antagonistic potential of the bio-control agents against wilt pathogens were tested by dual culture method (Dennis and Webster, 1971a) [3] on PDA medium. *In vitro* screening of bacterial and fungal isolates against *Fusarium* in liquid culture (Opgenorth and Endo, 1983) [10]. Isolates RGB8, RGP7 and RGT4 were selected as effective biocontrol agents after screening against best pathogen used for biocontrol of *Fusarium* under greenhouse condition.

Preparation of bio inoculants in small scale for greenhouse

Nutrient broth for bacterial inoculants were prepared in conical flasks and the medium was sterilized at 121°C for 30 minutes. The conical flasks were inoculated with 1ml of the standard inoculum of selected bacterial isolates and for fungal isolate potato dextrose broth was prepared and fungal mycelial disc was inoculated. The conical flasks were incubated at 28°C (±2) on rotary shaker for 24h. Liquid inoculum was used for both field and greenhouse experiments.

Preparation of pots for greenhouse experiment

The experimental pots were filled with red soil. Composite soil sample (0-15cm depth) was collected from fields before initiation of the experiment. The soil was air-dried, powdered and allowed to pass through 2mm sieve and was analyzed for physical and chemical properties. Soil is mixed with sand and FYM with the proportion of 3:2:1. The experiment was laid out in Complete Block Design with nine treatments and three replications each.

Table 1: Treatment details for pot culture experiments

Treatment	Particulars
T_1	RGB8 + RGF1
T_2	RGP7 + RGF1
T_3	RGT4 + RGF1
T_4	RGB8 + RGP7 + RGF1
T_5	RGB8 + RGT4 + RGF1
T_6	RGP7 + RGT4 + RGF1
T_7	RGB8 + RGP7+ RGT4 + RGF1
T_8	RGF1
T_9	Control

Note: RGB- Redgram *Bacillus* isolate, RGP- Redgram *Pseudomonas* isolate, RGT- Redgram *Trichoderma* isolate, RGF- Redgram *Fusarium* isolate

Results and discussions

Totally five pathogenic isolates were isolated from diseased specimens and they were subjected to morphological and microscopic observations. The results obtained from these studies were presented in table 1. Colony diameter of these isolates varied from 6.7 to 8.0cm and all isolates appears to be white in color. Microscopic observations revealed that conidial color varied from light blue to blue and having sickle shape. Based on these morphological and microscopic observations five isolates were tentatively identified as *Fusarium* isolates.

Table 2: Morphological characterization of *Fusarium* isolates isolated from wilt affected Redgram plant

Isolate code	Diameter of colony growth at 4 days (cm)	Colour	Conidia character	
			Shape	Colour
RGF1	6.7	White	Sickle shaped	Light blue
RGF2	7.3	White	Sickle shaped	Blue
RGF3	6.9	White	Sickle shaped	Light blue
RGF4	7.6	White	Sickle shaped	Light blue
RGF5	8	White	Sickle shaped	Blue

Note: RGF- Redgram *Fusarium*.

The results obtained were similar to the experiment conducted by Kiprop *et al*. 2002 [7] where they characterized seventy-nine single-spore isolates of *Fusarium udum*, the causal agent of wilt disease of pigeonpea, from Kenya, India and Malawi were according to their cultural characteristics, pathogenicity and vegetative compatibility group (VCG). The isolates exhibited high variation in pathogenicity on a wilt-susceptible pigeonpea variety, and in mycelial growth and sporulation on potato dextrose agar medium.

The observations were in agreement with Santram *et al*. (2017) [13] who isolated eight *Fusarium oxysporum* f. sp. *udum* and studied cultural and morphological variability among the isolates.

Pathogenicity test of *Fusarium* isolates

Isolated pathogens were subjected to pathogenicity test under greenhouse conditions. Sterilized soil was filled into plastic trays and used for pathogenicity test. Five isolates were inoculated to wilt sensitive Redgram variety BRG-2 by soaking seeds in liquid broth containing isolated pathogen for 3h. Population count was checked before treatment inoculation by plating technique and confirmed the presence of 10^{-4} CFU/ml. The results obtained from pathogenicity test were presented in table 3 and figure 1.

Table 3: Pathogenicity of *Fusarium* isolates isolated from wilt affected Redgram plant.

Isolate	% Germination	% Pre-emergence	% Post emergence
RGF1	25.00	75.00	91.67
RGF2	41.67	58.33	75.00
RGF3	41.67	58.33	75.00
RGF4	58.33	41.67	58.33
RGF5	50.00	50.00	67.67
Control	100.00	---	---

Pathogenicity test revealed that all five isolates showed typical wilt symptoms while there is no sign of visual symptoms in case of control which is not inoculated with any of pathogen. Out of five isolates inoculated, isolate coded RGF1 showed less germination percentage (25%) compared to other treatments followed by isolates RGF2 and RGF3. In case of post emergence disease incident isolate RGF1 showed 91.67% disease incidence and it is highest compared to all other treatments. There is no symptoms of wilt in plants under controlled conditions. With the results obtained by pathogenicity tests isolate RGF1 is again isolated, purified and maintained for further studies.

The results observed were similar to the work done by Ghante *et al*., (2019) [4] where they isolated twenty two isolates of *Fusarium oxysporum* and subjected them to pathogenicity tests by sick pot method. Ten isolates *viz*., FOU 2, FOU 3, FOU 6, FOU 12, FOU 13, FOU 16, FOU 17, FOU 22, FOU 19 and FOU 30 were highly pathogenic and these were carried further for studies.

Naik *et al*. (2017) [9] conducted an experiment where they isolated 50 isolates of *Fusarium udum*. Out of 50 isolates 15 isolates were found to show typical wilting symptoms and these isolates were able to prove Koch's postulate and proved to be truly pathogenic to pigeonpea cultivar '*Bahar*'.

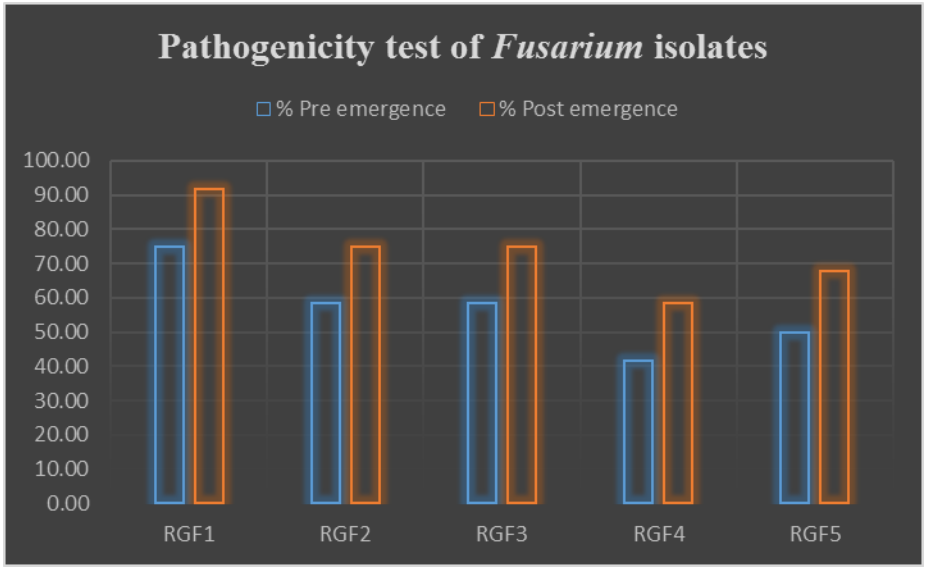

Fig 1: Pathogenicity test of *Fusarium* isolates under greenhouse conditions.

Control of Fusarium wilt of redgram using biocontrol agents

As the main aim of the research disease incidence by artificial inoculation of pathogen and its control by applied biocontrol agents was observed. The results obtained were presented in Table 4. Out of nine different treatments applied treatment T_7 (*Bacillus* sp. + *Pseudomonas* sp. + *Trichoderma* sp. + *Fusarium* sp.) showed significantly highest (70.00%) biocontrol efficiency than other treatments followed by treatment T_5 which controlled 35.00% of disease incidence. Treatment T_8 having *Fusarium* alone showed no control of disease after 30 days of sowing. These results may be due to the production of antimicrobial compounds, cell wall degrading enzymes and competition for nutrients and space which is more in combination of all three biocontrol agents.

Results were in accordance with Siddiqui and Shakeel (2007) [14] who evaluated five *Bacillus* isolates for biocontrol potential under pot conditions. Isolates B615 and B603 were found to be the most promising and used for field experiments.

Similar results were observed by Rini and Sulochana (2007) [12], they tested *Trichoderma* isolates and *Pseudomonas fluorescens* isolates against *Fusarium oxysporum* diseases in tomato and revealed that the combined application of both *Trichoderma* and *Pseudomonas* isolates has given highest disease suppression.

Rajasekhar *et al.* (2016) [11] widely studied and found most promising antagonists *Trichoderma harzianum* (TH), *Pseudomonas flourescens* (PF), *Rhizobium* (Rh) and *Bacillus subtilis* (BS) have been evaluated in their study for plant health management of pigeonpea in different combinations to make consortia. Results in this study indicating that the consortia having, treatments T7 (TH+PF+BS+Rh) (86%), T2 (TH+BS) (82%) and T5 (PF+Rh) (77%) have shown remarkable wilt disease reduction. These results are showing role of biological consortia to play in integrated disease management and plant health management of pigeonpea.

Table 4: Biocontrol activity of rhizosphere isolates against wilt causing pathogen of Redgram (*Cajanus cajan* L.) under greenhouse conditions.

Treatments	% Pre-emergence	% Biocontrol efficiency
T_1: RGB8 + RGF1	26.67^{d}	26.67 (4.47)^{b}
T_2: RGP7 + RGF1	41.67^{b}	21.67 (4.70)^{ab}
T_3: RGT4 + RGF1	35.00^{c}	26.67 (4.47)^{b}
T_4: RGB8 + RGP7 + RGF1	33.33^{c}	26.67 (4.33)^{b}
T_5: RGB8 + RGT4 + RGF1	33.33^{c}	35.00 (5.92)^{ab}
T_6: RGP7 + RGT4 + RGF1	28.33^{d}	28.33 (5.31)^{ab}
T_7: RGB8 + RGP7+ RGT4 + RGF1	6.67^{e}	70.00 (8.30)^{a}
T_8: RGF1	50.00^{a}	0.00 (0.70)^{c}
T_9: Control	---	----

Note: Means with same superscript, in a column do not differ significantly at P=<0.05 as per Duncan Multiple Range Test (DMRT), RGB- Redgram *Bacillus* isolate, RGP- Redgram *Pseudomonas* isolate, RGT- Redgram *Trichoderma* isolate, RGF- Redgram *Fusarium* isolate

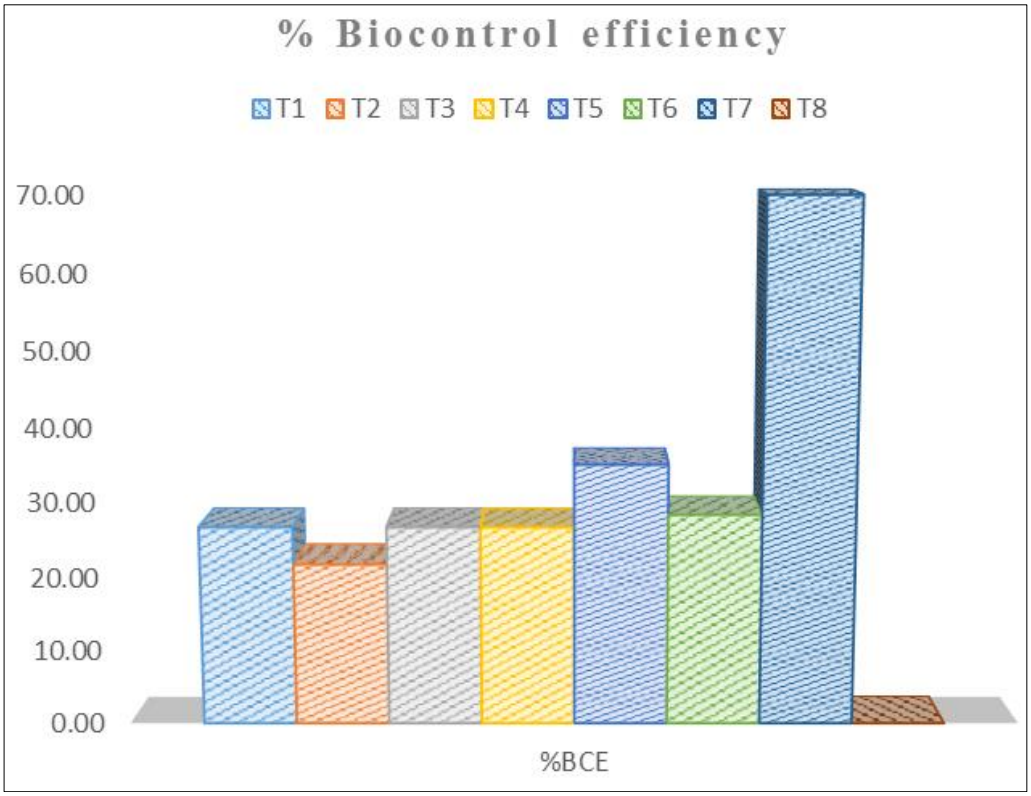

Fig 2: Biocontrol activity of rhizosphere isolates against wilt causing pathogen of Redgram (*Cajanus cajan* L.) under greenhouse conditions

Summary and conclusion

Among five *Fusarium* isolates which have been isolated from wilted samples, isolate RGF1 showed most disease incidence and when this isolate was used as artificial or challenged inoculation to test biocontrol efficiency of biocontrol agents isolated from redgram rhizosphere, the results revealed that, use of microbial consortia (T_7: RGB8 + RGP7+ RGT4 + RGF1) against the pathogen reduced the disease incidence than single inoculation. So use of microbial consortia against wilt of redgram can be recommended as an eco-friendly and cost effective measure of disease control.

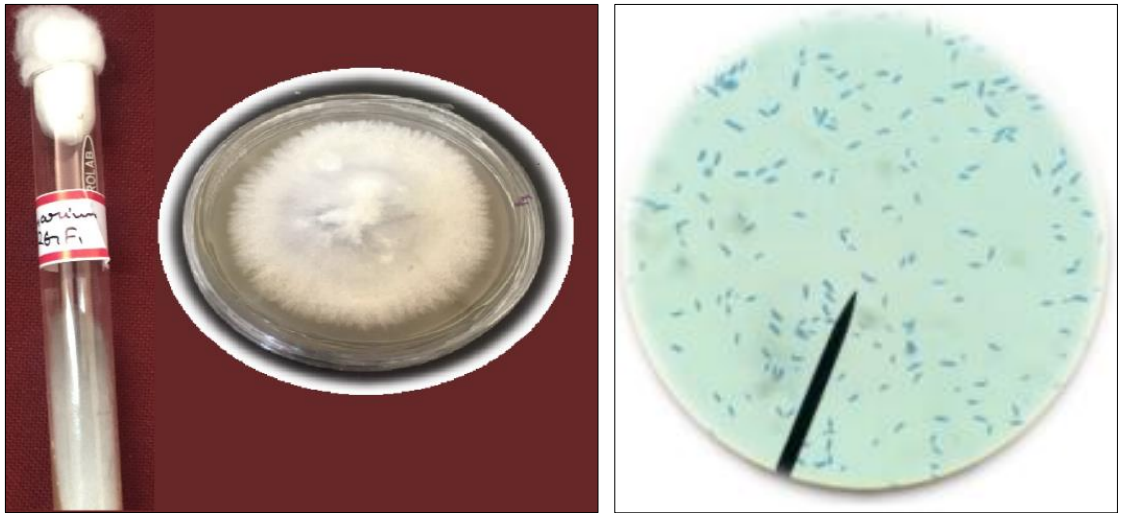

Fig 1: Pure culture of *Fusarium* isolate RGF1 and its conidial structure.

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