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In vitro biocontrol activity of rhizosphere isolates against wilt causing pathogen of Redgram (*Cajanus cajan*)

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Abstract
An experiment was conducted in the Department of Agricultural Microbiology, GKVK, UAS, Bengaluru, to screen the bacterial isolates from rhizosphere soil of Redgram against the wilt causing pathogen of Redgram under *in vitro* condition. Forty bacterial isolates were tested *in vitro* condition for their biocontrol activity against fungal plant pathogen using Dual plate technique and mycelial dry weight reduction assay. The study revealed that bacterial isolates from Redgram rhizosphere found to inhibit the growth of wilt causing *Fusarium* spp. in the laboratory conditions. The isolates RGB8 (63.33% inhibition in dual plate and 70% inhibition of dry mycelial weight) and RGP7 (60.00% inhibition in dual plate and 65.78% inhibition of dry mycelial weight) found to be effective against the *Fusarium* wilt pathogen which can be used as bio inoculant against the wilt pathogen.

Keywords: Dual plate, *Fusarium* spp., Redgram wilt, biocontrol

Introduction
PIGEONPEA [*Cajanus cajan*] is widely grown by small farmers in the semi-arid tropics as a backyard subsistence crop. It is produced commercially in India, Myanmar, Kenya, Malawi, Uganda and a few countries of Central America. Pigeonpea commonly known as arhar or tur, is the second most important pulse crop after chickpea in India. It is one of the extensively used pulses in India as an important source of protein in human diet. The diseases of economic importance at present are *viz*., *Fusarium* wilt, Sterility Mosaic Disease (SMD), Phytophthora Blight (PB), Macrophomina root rot, Stem canker, Alternaria blight and pearly cyst nematode in the Indian sub-continent.

The wilt is caused by *Fusarium udum* Butler is one of most serious and oldest known disease (Butler, 1906) [1] and it is known to cause heavy losses every year in India (Kannaiyan *et al.*, 1981). The pathogen is a soil and seed borne. The genus *Fusarium* have wide host range and survives for long time in the field in the absence of host plant. Therefore, chemical control is not satisfactory, adequate and 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 fulfil 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.

Materials and Methods
Isolation of pathogen: Samples of plants (red gram) with disease symptoms were collected in a paper envelope and brought to the laboratory. The pathogen is isolated by direct culturing of infected parts according to Chopada *et al.* (2015) [3] 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 (Nash and Snyder 1962) [9]. Plates were incubated under the standard incubation conditions (Chehri *et al.* 2010) [2] for 48 h 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 growth rates, pigmentations of colony, types of conidiogenous cells, shape and size of conidia, and presence or absence of sporodochia and chlamydospore.

Isolation of antagonistic bacteria: Antagonistic bacteria were isolated by following serial dilution technique. Composite soil sample was collected from rhizosphere of healthy redgram plants. The soil was dried under shade and then used for serial dilution. To get 10^{-1} dilution, 10 g of soil was dissolved in 100ml of sterile distilled water and one ml of soil suspension was taken and added to 9ml of sterile distilled water to get 10^{-2} dilution. This step was repeated until a dilution of 10^{-4} for the isolation of fungi and 10^{-6} for bacteria.

Screening of bacterial isolates for their antagonistic activity against *Fusarium* spp.

Potato dextrose agar was prepared and 20ml poured into Petri plates allowed for the solidification. Isolated bacterial strains were inoculated on one side of the plate and pathogen on other side. The assay plates were incubated at 28 ± 1 °C for four days and observations will be made on inhibition of mycelial growth of the test pathogen. Three replicates with suitable controls were maintained for each test and the per cent inhibition over control was calculated by adopting the following formula,

$$\text{Per cent Inhibition of pathogen} = \frac{\text{Growth of pathogen in control plate} - \text{Growth of pathogen in presence of bio agent}}{\text{Growth of pathogen in control plate}} \times 100$$

Evaluation of bacterial isolates on growth inhibition of fungal plant pathogen in liquid culture.

The bacterial isolates were tested against the fungal plant pathogen in liquid media (Potato dextrose broth). Potato dextrose broth was prepared and 100 ml broth was distributed in each flask and autoclaved. Mycelial disc of (5mm size) respective pathogens were inoculated to liquid broth along with one ml of 24 hour old bacterial endophytes each as per treatment wise. Control flasks without any bacterial endophytes were maintained for each pathogen. All the flasks were kept in incubator at 30 °C under static conditions for 10 days. After incubation, the contents in the flasks were filtered through a pre weighed Whatman filter No.1 paper and fresh weight of contents were recorded. The filter papers along with contents were dried in hot air oven at 105 °C for 48 h and reweighed along with the mycelium to get the constant dry weight values. The weight of the fungal mycelial mat was calculated by subtracting the weight of the pre weighed filter paper from the weight of the filter paper + mycelial mat. The reduction in weight of mycelium in co-inoculated flasks were determined by comparing with the control flasks.

Results and discussion

Totally forty isolates of bacteria were isolated from rhizosphere soil of Redgram. Morphological and biochemical characterization studies revealed that twenty isolates belongs to *Bacillus* spp. and other twenty belongs to *Pseudomonas* spp. These isolates were used for further studies to test their biocontrol activity against the *Fusarium* wilt of Redgram.

Screening of isolates through antagonistic activity against *Fusarium* spp. in dual plate assay

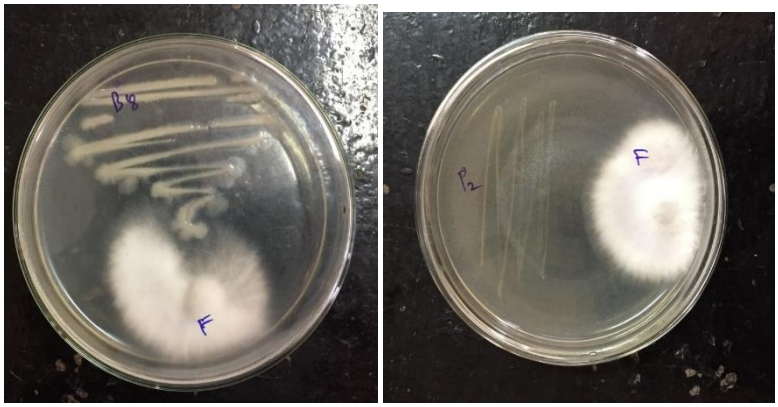
Bacterial isolates were streaked on PDA plates at one side and pathogen on other side. The pathogen alone on PDA grown covering almost a petri plate, on the other hand in dual plate culture where bacteria and pathogen both inoculated, the diameter of pathogen reduced significantly showing the biocontrol activity of bacterial isolates by the production of volatile and nonvolatile compounds (Plate 1).

Twenty *Bacillus* isolates were streaked against the pathogen, results revealed that pathogen grown in a plate where isolate RGB8 was streaked showed a significant reduction in colony diameter of 3.3cm indicating the 63.33% of growth inhibition by the isolate followed by the isolates RGB6 and RGB7 both showed the growth inhibition of pathogen by 61.11%.

In another experimental setup where *Pseudomonas* isolates were streaked, results revealed that isolate RGP7 significantly reduced the diameter of pathogen (3.4cm) as compared to control plate (where it has grown 8.5cm). The isolate RGP7 inhibited the growth of pathogen by 60% followed by the isolate RGP8 which inhibited 55.25% of pathogen growth (Table 1). The inhibition in radial growth of two interacting organisms in dual culture is attributed to inhibitory substance released by one or both organisms through competition, mechanical obstruction and hyper parasitism (Dennis and Webster 1971) [4].

Similar results were observed by Karimi *et al*. (2012) [7] who studied the antagonistic effects of six isolates of *Pseudomonas* and six isolates of *Bacillus* genera which are isolated from the rhizosphere of chickpea were evaluated against *Fusarium oxysporum* f. sp. *ciceris* as potential biocontrol agents *in vitro* and *in vivo*. Fungal inhibition tests were performed using plate assay. Twelve isolates were selected based on their high antagonistic efficiency under *in vitro* condition by using dual plate assay.

These results were in accordance with the work of Lahlali *et al*. (2007) [8] where they evaluated 220 bacterial strains isolated from different organs of healthy potato plants and rhizosphere soils, out of which 25 isolates were selected using screening methods based on *in vitro* dual culture assays. The mycelial growth inhibition rate of the pathogen ranged from 59.40 to 95.00%.



A. B.

Plate 1: Dual plate assay indicating the antagonistic activity of bacterial isolates. A: *Bacillus* isolate B: *Pseudomonas* isolate.

Table 1: Biocontrol activity of bacterial isolates using dual plate assay.

Isolates	Radial growth of *Fusarium* (diameter in cm)	% inhibition	Isolates	Radial growth of *Fusarium* (diameter in cm)	% inhibition
RGB1	5.2	42.22^{gh}	RGP1	6.50	23.50^{j}
RGB2	4	55.55^{d}	RGP2	5.80	31.76^{h}
RGB3	5	44.44^{g}	RGP3	6.20	27.00^{i}
RGB4	4.5	50.00^{ef}	RGP4	4.80	43.50^{de}
RGB5	3.4	62.22^{a}	RGP5	4.30	49.40^{c}
RGB6	3.5	61.11^{ab}	RGP6	4.90	42.30^{ef}
RGB7	3.5	61.11^{ab}	RGP7	3.40	60.00^{a}
RGB8	3.3	63.33^{a}	RGP8	3.80	55.29^{b}
RGB9	4.5	50.00^{ef}	RGP9	4.20	50.50^{c}
RGB10	5.3	41.00^{h}	RGP10	5.10	40.00^{f}
RGB11	5.7	36.60^{i}	RGP11	6.20	27.00^{i}
RGB12	3.7	58.80^{bc}	RGP12	6.10	28.20^{i}
RGB13	4.6	48.80^{f}	RGP13	4.60	45.80^{d}
RGB14	4.3	52.20^{e}	RGP14	7.10	16.40^{k}
RGB15	5.8	35.50^{i}	RGP15	3.90	54.11^{b}
RGB16	4.3	52.22^{e}	RGP16	5.10	40.00^{f}
RGB17	5.9	34.40^{i}	RGP17	4.90	42.30^{ef}
RGB18	4.3	52.20^{e}	RGP18	4.30	49.40^{c}
RGB19	3.9	56.60^{cd}	RGP19	5.70	32.90^{h}
RGB20	4.6	48.80^{f}	RGP20	5.40	36.40^{g}
Control	9	--	control	8.50	--

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*, **RGP-** Redgram *Pseudomonas*.

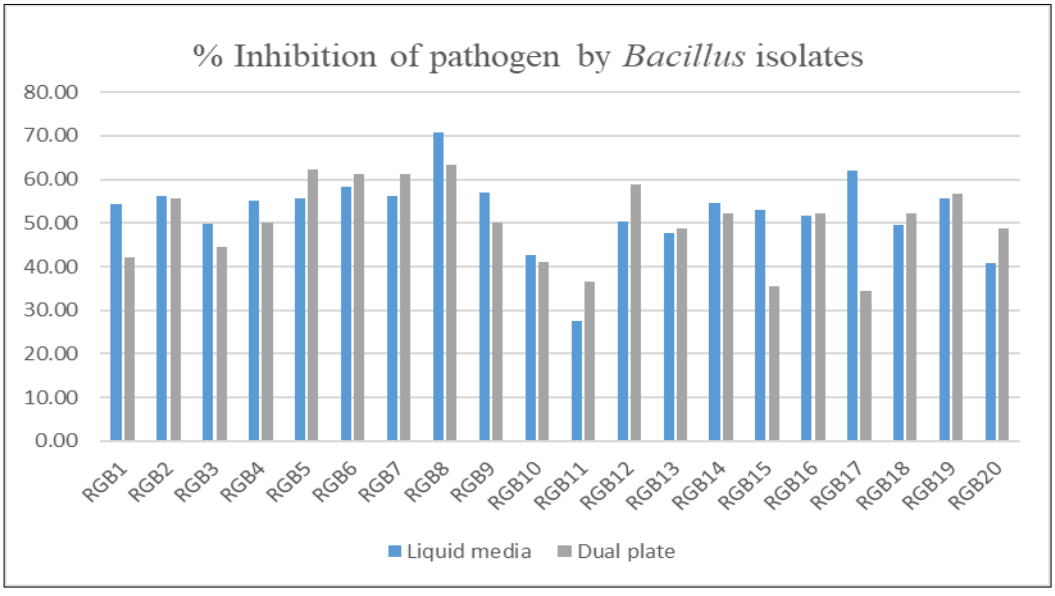


Fig 1: Biocontrol activity of *Bacillus* isolates Inhibition of fungal plant pathogen by bacterial isolates in liquid culture

Fungal growth inhibition in liquid broth was conducted to test the biocontrol activity of bacterial isolates in liquid media. The results (Table 2) revealed that the isolate RGB8 was reducing the mycelial dry weight significantly *i.e.*, 1.21 g dry

weight of mycelial growth as compared to the control which has mycelial dry weight of 4.13 g indicating 70.70% of growth inhibition which is highest of all *Bacillus* isolates. This is followed by isolate RGB17 which inhibited 61.99% of growth. In other hand the flask treated with *Fusarium* and *Pseudomonas* isolate RGP7 shows highest growth inhibition of 65.78% with dry mycelial weight of 1.28 g as compared to control which shown 3.74 g of dry mycelial growth. This is followed by isolate RGP20 which inhibited 54.81% of mycelial growth.

These results are in evidence with Kai *et al*. (2007) [5], where they demonstrated that small volatile organic Compounds (VOCs) emitted from bacterial antagonists negatively influenced the mycelial growth of the soil borne phytopathogenic fungus *Rhizoctonia solani* Kühn and reported that the strong inhibitions (80 - 99%) were observed with *Stenotrophomonas maltophila* R3089, *Serratia plymuthica* HRO – C48, *Stenotrophomonas maltophila* P 69, *Serratia odorifera* 4Rx13, *Pseudomonas trivialis* 3Re2-7, *S. plymuthica* 3Re4-18 and *Bacillus subtilis* B2g, *Pseudomonas fluorescens* L13-6-12 and *Burkholderia cepacia* 1S18 achieved 30% growth reduction.

Plate 2: Mycelial growth inhibition in liquid broth.

Table 2: Inhibition of fungal plant pathogen by bacterial isolates in liquid culture

Isolates	Dry weight of mycelia (g)	% inhibition	Isolates	Dry weight of mycelia (g)	% inhibition
RGB1	1.89	54.24^{def}	RGP1	2.47	33.96^{j}
RGB2	1.81	56.17^{cde}	RGP2	2.20	41.18^{gh}
RGB3	2.07	49.88^{ghi}	RGP3	1.89	49.47^{de}
RGB4	1.85	55.21^{cde}	RGP4	1.81	51.60^{cd}
RGB5	1.83	55.69^{cde}	RGP5	2.49	33.42^{j}
RGB6	1.72	58.35^{c}	RGP6	2.31	38.24^{i}
RGB7	1.81	56.17^{cde}	RGP7	1.28	65.78^{a}
RGB8	1.21	70.70^{a}	RGP8	2.39	36.10^{ij}
RGB9	1.78	56.90^{cd}	RGP9	1.70	54.55^{b}
RGB10	2.37	42.62^{g}	RGP10	1.93	48.40^{c}
RGB11	2.99	27.60^{k}	RGP11	1.83	51.07^{cde}
RGB12	2.05	50.36^{ghi}	RGP12	2.47	33.96^{j}
RGB13	2.16	47.70^{i}	RGP13	2.07	44.65^{f}
RGB14	1.88	54.48d^{ef}	RGP14	1.69	54.81^{b}
RGB15	1.94	53.03e^{gf}	RGP15	1.82	51.34^{cd}
RGB16	2.00	51.57^{fgh}	RGP16	1.76	52.94^{bc}
RGB17	1.57	61.99^{b}	RGP17	2.30	38.50^{hi}
RGB18	2.08	49.64^{hi}	RGP18	2.10	43.85^{fg}
RGB19	1.83	55.69^{cde}	RGP19	1.90	49.20^{de}
RGB20	2.44	40.92^{j}	RGP20	1.69	54.81^{b}
Control	4.13	--	Control	3.74	--

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*, **RGP-** Redgram *Pseudomonas.*

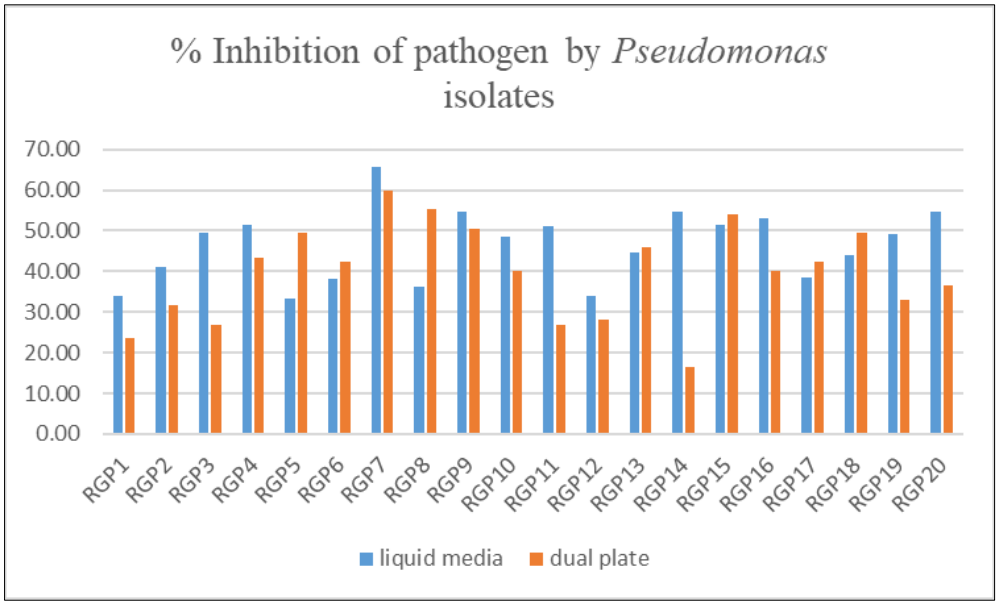


Fig 1: Biocontrol activity of *Pseudomonas* isolates

Summary and Conclusion

In this experiment, forty bacterial cultures were isolated and screened against the wilt causing pathogen *i.e.*, *Fusarium* spp., through their biocontrol mechanisms. Out of forty isolates twenty found to be *Bacillus* and other twenty were *Pseudomonas* spp. based on the morphological and biochemical characterization. The isolates RGB8 and RGP7 recorded maximum inhibition against *Fusarium* spp. in both dual plate assay and mycelial weight reduction assay. So these isolates can be further characterized at molecular level and can be used as biocontrol agents against redgram wilt disease as an ecofriendly control measure of disease.

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