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Original Research Paper

Bacterial isolates for the Bio-control of *Fusarium* wilt of Pigeon Pea

Jyoti Rana[1], S. C Tiwari[2] , Vikas S. Jadon[1] and Sanjay Gupta[1*]

[1]Department of Biotechnology and Biochemistry, SBSPGI, Balawala, Dehradun, Uttarakhand, India
[2] Department of Botany and Microbiology, HNB Garhwal Central University, Srinagar, Garhwal, Uttarakhand, India.

*Corresponding author. E-mail: sanjay_gupta9999@rediffmail.com.

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ABSTRACT

Fusarium wilt is one of the major yield limiting factors of pigeon pea (*Cajanus cajan*). For an eco-friendly and sustainable management of such a disease, bacterial isolates were evaluated against fungal pathogen *Fusarium udum*, which is known to be infecting the susceptible variety of pigeon pea, commonly prevalent in India. To assess the antifungal efficacy of these bacterial isolates, the *in vitro* as well as *in vivo* studies were performed. Thirty isolates of *Pseudomonas* spp. and twenty isolates of *Bacillus spp*. have been isolated from soil of pigeon pea field. Among these, five isolates of *Pseudomonas* spp. (Pf_{05}, Pf_{14}, Pf_{19}, Pf_{23}, Pf_{25}) and four isolates of *Bacillus* spp. (Bc_{01}, Bc_{09}, Bc_{14}, Bc_{20}) were considered as potential biocontrol agents against the fusarium wilt, as they have been attributed to the production of antifungal metabolites including hydrogen cyanide, chitinase and siderophores. Under pot condition, Pf_{14} and Bc_{20} isolates treated pigeon pea seeds have shown 59% and 50% increase in seedling growth respectively and reduction in fusarium wilt incidence. When we further tested under field condition, Pf_{14} treated seeds resulted in higher grain yield than Bc_{20} treated seeds. This indicates *Pseudomonas* spp. Pf_{ss} had good potential as a biocontrol against *fusarium* wilt of pigeon pea.

Keywords: biocontrol, *fusarium* wilt, antifungal metabolites.

INTRODUCTION

Pigeon pea (*Cajanus cajan*) is an important pulse crop cultivated in the tropics and sub-tropics. Crop yield is significantly reduced due to wilt disease caused by *Fusarium udum* Butler, with an estimated yield loss of US$36 million in India and $5 million in eastern Africa (Karimi *et al.,*2010). Like any other soil-borne diseases, the wilt disease of Pigeon pea is difficult to control with chemical fertilizers. Some pesticides and chemicals have been recommended for the management of the disease, but none have been proven to give the desired success in controlling the disease (Voisard *et al.,*1989). Pesticides are reported to cause adverse effects on treated soil ecosystem because of their non-biodegradable nature and also because they induce resistance in pathogens (Garbeva *et al.,*2010). Biological pesticides have the potential to replace or augment conventional plant disease management. Several studies have demonstrated reduced incidence of diseases in different crops after supplementing the soils with fungal or bacterial antagonists (Van Elasas *et al.,*2000; Mohammed *et al.*, 2014; and Mishra *et al.,*2013). This study was undertaken to assess the efficacy of certain biocontrol agents against wilt disease of Pigeon pea.

Table 1. Morphological and biochemical characteristics of the test isolates (Bergey's Manual of Determinative Bacteriology).

Biochemical characters	Fluorescent *Pseudomonas* 30*	*Bacillus* species 20*
Pigmentation	diffusible fluorescent green pigment	Nil
Colony Morphology	Button shaped	Serrated, irregular Margins
Gram reaction	Negative	Positive
Cell shape	Rods	Rods
Spores/cyst	Negative	Positive
Growth on N_2 free medium	Negative	Positive
Catalase, Citrate test	100	100
Oxidase test	100	80
Starch	55.56	80
Lipid	77.78	80
Glucose	55.56	10
Lactose	11.11	70
Sucrose	33.33	60
Mannitol	11.11	70

MATERIAL AND METHODS

Collection of soil samples

Both rhizospheric and non-rhizospheric (bulk soil at ≈15 cm depth) soils were collected from different agricultural fields in the vicinity of Garhwal, Uttarakhand (India) during the season (May-August, 2009). Five replicates of each soil sample were collected from the same field at a little distance which denotes a composite sampling of the soil. These five samples were processed separately for microbiological studies. The description of the soil sampling sites is given in Table 1.

Isolation and identification of bacterial isolates

The rhizospheric and non-rhizospheric soil samples were serially diluted and 10^6 dilution fraction was plated on yeast extract mannitol agar (YEMA) and King's B media, and were incubated at 27°C for 72 hours. The isolates were identified according Bergey's Manual of Systematic Bacteriology [(Arora *et al.*,2001).

Isolation and identification of fungal Pathogens

The fungal pathogens were isolated from wilted stem of *C.cajan*. Transverse sections of the wilted stem were placed on the PDA and RBA medium plates, incubated at 27°C for 4 days. On the basis of microscopic observations and morphological characteristics, the strains were identified as *F. udum*.

Screening of isolates for their biocontrol potential against phytopathogenic fungi

The bacterial isolates were screened against *F. udum* by modified dual culture method given by Liang *et al.* (1996). Observation was recorded for each plate independently for zone of inhibition formed. Percentage growth inhibition was calculated by the following equation:

$$PI = \frac{100\ (R_2-R_1)}{R_1}$$

Where R1 is the radius of colony in the direction of bacterial colony and R2 is the radius of the fungal colony in the direction with no bacterial colony.

Determination of HCN, siderophore production by biologically active agents

The test bacteria were screened for the production of hydrogen cyanide by the method of Lork 1948). Siderophore production was detected by universal assay of Shwyn and Nielands (1987).

Determination of chitinase activity

Chitinase activity was determined according to Robert and Selitrennikoff (1988).

Assay for the detection of antifungal activity

To test the antifungal activity of the bacterial isolates, the bacterial cultures were multiplied /cultured in nutrient broth medium. The test fungi, *F. udum* maintained on Potato dextrose agar (PDA) slants were sub-cultured. The spores were scrapped and suspended in 10 ml of sterile normal saline solution. A 0.1 ml of diluted spore suspension of the fungi was spread on Mueller Hinton agar (MH), nutrient agar (NA) and Sabouraud dextrose agar (SDA) plates. Wells of 08 mm in diameter were punched using a sterile cork borer. In to each well, 200 µl of bacterial culture was pipetted. Nutrient broth was taken as negative control and 100 µg ml^{-1} antifungal antibiotic (nystatin) was used as positive control. The plates were

incubated for 5 days at 28°C. Antifungal activity was evaluated by measuring the growth inhibition zone against test fungi.

Seed germination assay

In vitro seed germination test was performed on the seeds of *C. cajan*. The seeds were surface sterilized by 2-3 times washing them in sterile distilled water, then kept in 0.1% $HgCl_2$ solution for I min, followed by several washings with distilled water. They were kept in 70% ethanol for 1 minute and again washed several times with sterile distilled water. The bacterial cultures (10^7 cfu ml^{-1}) were centrifuged, supernatant was discarded and the pellet was suspended in NSS. The surface sterilized seeds were inoculated with bacterial cultures for 2 h and placed on petri plates containing soft agar. Germinated seeds were counted to determine percent germination. Seedling growth (seedling biomass, root and shoot length) was determined after ten days of incubation Weller (1988) and Cook *et al.* (1979) methods was adopted for seed bacterization.

Sterile soil assay

The surface sterilized seeds were sown in small pots filled with sterile soil. The experiment was conducted for following sets of combinations: 1. Soil inoculated with fungus + non-bacterial seeds (Negative control). 2. Soil inoculated with fungus + bacterized seeds. 3. Bacterized seeds. 4. Control (unbacterized seeds). Pots were watered routinely with sterile water. After 30 days, plants were uprooted and seedlings growth (shoot and root length, fresh shoot and root weight) were recorded. All experiments were carried out in triplicate.

RESULTS

150 soil and root samples were collected from different places of Garhwal region. The root and soil samples were examined for the presence of *F. udum* and soil samples having low number of *F.udum* were later selected for isolation of promising biological control bacterial agents. Viable plate count of bacterial as well as fungal isolates were determined both in rhizospheric and non-rhizospheric soil samples of different regions of Garhwal. The viable count of bacteria in the rhizospheric soils of different crops ranged from 1.23×10^6 to 1.28×10^8 CFU g^{-1} soil while in the non-rhizospheric soils it varied from 1.30×10^5 to 1.12×10^6 CFU g^{-1} soil. Bacteria were grouped into two types (i) Gram -ve short rods, (ii) Gram +ve bacilli.

Bacterial isolates

A total of 50 bacteria were isolated. Selected rhizobacteria of two major groups fluorescent *P. spp.*, *B. spp*. were considered in this study. These isolates were identified according to Bergey's Manual of Systematic Bacteriology. Out of the 50 isolates 20 isolates were of *B. sp*. and designated as Bc_{01} to Bc_{20} & 30 isolates were of *P. sp*. designated as Pf_{01} to Pf_{30}. The morphological and biochemical characteristics of groups of bacteria are presented in Table 1.

Screening the antagonistic activity of isolates against *Fusarium udum*

Five isolates of fluorescent *Pseudomonas* and four isolates of *Bacillus* demonstrated antifungal activity against *F. udum*. In further experiments *Bacillus sp.* Bc_{20} showed a very prominent 17% inhibition of *F. udum*, while *Bacillus sp.* Bc_{14} showed 14% inhibition. *Pseudomonas sp*. Pf_{19} showed 15% inhibition of *F. udum*. As experimental conditions affect the inhibition zone, same experimental conditions were maintained while repeating the experiments. The results are given in Figure1.

Screening of isolates for biocontrol activity

The fluorescent *Pseudomonas* isolates were grouped into four biocontrol activity groups. BCA type I constitute 40% isolates and showed one BCA traits (Chitinase production) followed by BCA type II which constitute, 30% isolates and were positive for production of chitinase and HCN. BCA type III, constitute only 10.33% isolates and were positive for antifungal activity and chitinase production. BCA type IV contained only 10.66% representing five isolates (Pf_{14}, Pf_{19}, Pf_{05} Pf_{23} Pf_{25}) with three BCA traits (Table 2). Chitinase production with 100% value is common among *Pseudomonas* followed by hydrogen cyanide production, 40.66% and antifungal activity, 30.33 %. Only five isolate produced siderophore and observed positive for antifungal activity (Table 2).

On the basis of multiple BCA traits, certain isolates from *Bacillus* and fluorescent *Pseudomonas* were analyzed for quantitative estimation of BCA traits. The output of the assessment is as follows:

HCN production

HCN production was seen in Bc_{20}, Pf_{19} and Pf_{23}, as there is a remarkable change in color from yellow to reddish–brown, therefore, had strong production of HCN. Some other strains were also positive for HCN production but were not active against the phytopathogens (Tables 2 and 3).

Siderophore production

The production of Siderophore by test isolates were assayed in term of zone diameter of orange-yellow halo produced on CAS agar plates. Fluorescent *Pseudomonas* (Ps_{19}) and *Bacillus* isolate (Bc_{20}) showed almost equal zone size ≈13.50mm.

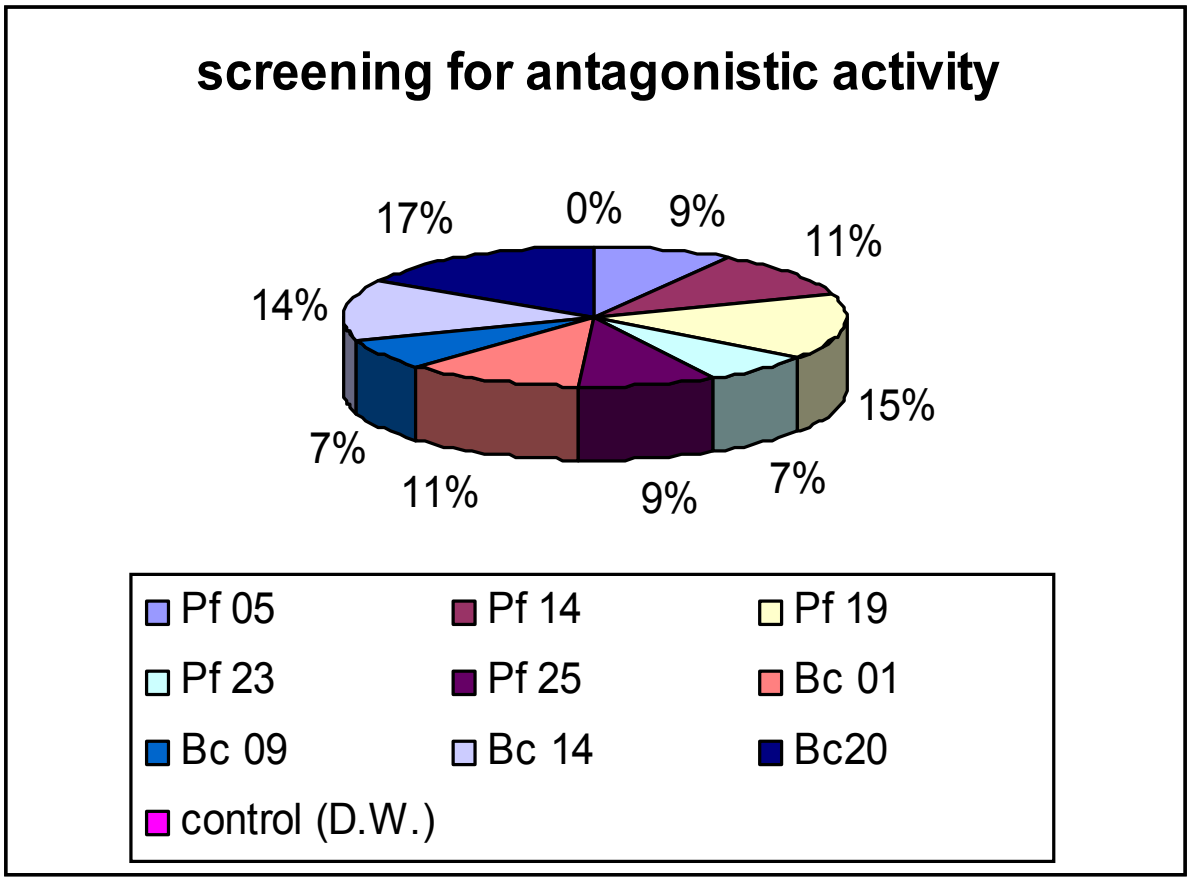

Figure 1. Screening for antagonistic activity.

Table 2. Biocontrol activity based typing of *Pseudomonas* isolates.

Isolate designation	No. of isolates	Chitinase production	Siderophore production	Antifungal activity	HCN production	Activity profile
Pf_{02}, Pf_{04}, Pf_{06}, Pf_{08}, Pf_{11}, Pf_{13}, Pf_{15}, Pf_{17}, Pf_{20}, Pf_{22}, Pf_{24}, Pf_{26}	12 (40.00)	+	–	–	-	C, H
Pf_{01}, Pf_{07}, Pf_{09}, Pf_{10}, Pf_{16}, Pf_{18}, Pf_{28}, Pf_{29}, Pf_{27}	9 (30.00)	+	–	–	+	C, H
Pf_{03}, Pf_{12}, Pf_{21},Pf_{30}	4 (10.33)	+	–	+	–	C
Pf_{14}, Pf 19, Pf_{05},Pf_{23},Pf_{25}	5 (10.66)	+	+	+	+	C, S, A,H
Total number of isolates	30 (100)	30(100)	5 (16.67)	9(30)	14 (40.66)	

C-Chitinase production, S- Siderophore production, A- Antifungal activity, H-HCN production,
Figure in parenthesis indicates the percentage.

Screening of Chitinase activity

Both the Bacillus sp. and Pseudomonas sp. were screened for the production of chitinase enzyme. They showed growth when inoculated on the colloidal chitin agar plate, which contained colloidal chitin as the sole

Table 3. Biocontrol activity based typing of Bacillus isolates.

Isolate designation	No. of isolates	Chitinase production	Siderophore production	Antifungal activity	HCN production	Activity profile
Bc_{02} ,Bc_{04} , Bc_{06}, Bc_{08}, Bc_{10}, Bc_{12}, Bc_{16},Bc_{18},	8 (40.00)	+	–	–	–	C
Bc_{05}, Bc_{07}, Bc_{09} ,Bc_{15}, Bc_{17} ,Bc_{19},	6 (30.00)	+	–	–	+	C, H
Bc_{03}, Bc_{13}	2 (10.00)	+	–	–	+	C, H
Bc_{14}, Bc_{20}, Bc_{01},Bc_{09}	4 (20.00)	+	+	+	+	C, S, A, H
Total number of isolates	20	20 (100)	4(20)	4(20)	12 (60)	

C-Chitinase production, S- Siderophore production, A- Antifungal activity, H-HCN production,
Figure in parenthesis indicates the percentage.

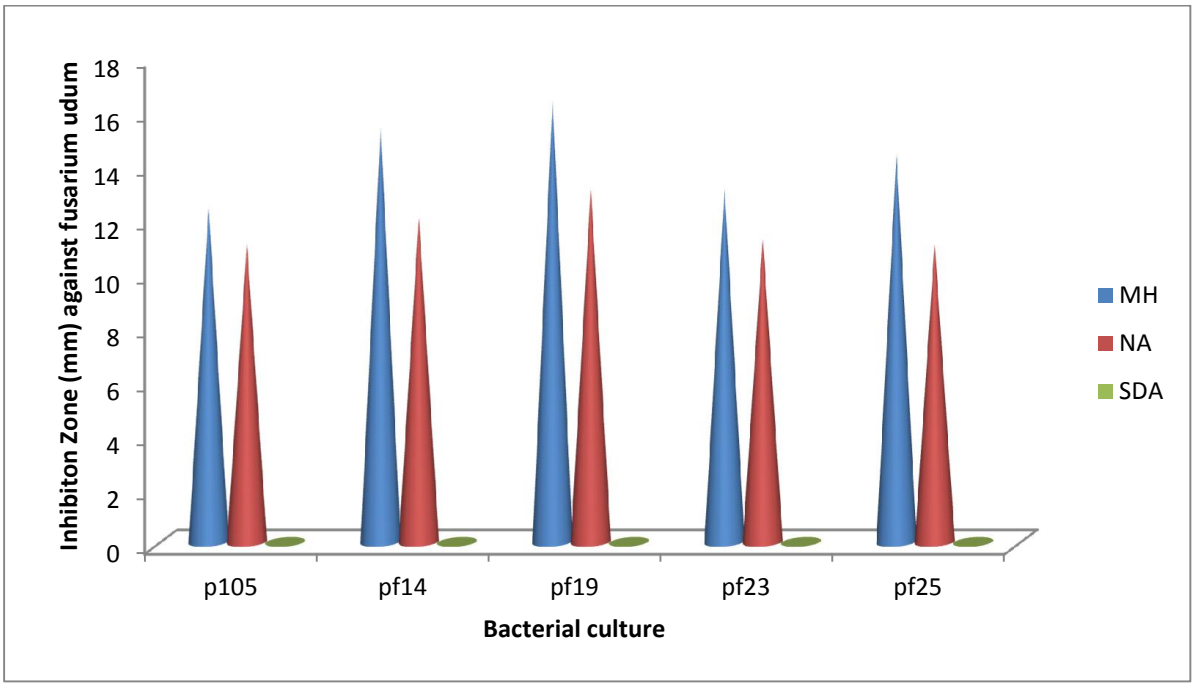

Figure 2. Antifungal activity of Pseudomonas isolates on different media.

source of carbon and nitrogen for the antagonists. Hence, both the strains were positive for chitinase (Tables 2 and 3).

Antifungal activity of test bacterial isolates:

Antifungal activity of thirty isolates of fluorescent *Pseudomonas* isolate (Pf_{01} to Pf_{30}) and twenty *Bacillus* isolate (Bc_{01} to Bc_{20}) were checked against *F. udum* using three different media, Muller-Hinton (MH), Nutrient agar (NA) and Sabouraud Dextrose Agar (SDA). The isolates Pf_{19} and Bc_{20} also exhibit broad-spectrum activities against test fungi. Among thirty *Pseudomonas* isolates five isolate (Pf_{05}, Pf_{14}, Pf_{19}, Pf_{23}, and Pf_{25}) showed activity against fungal growth and out of the five isolates only, Pf_{19} proved to be the best bacterial isolate exhibiting strong antifungal activity against four fungi. Similarly, among twenty isolates of *Bacillus* only four strains (Bc_{01}, Bc_{09}, Bc_{14}, Bc_{20}) showed positive antifungal activity and above all, Bc_{20} proved to be the best bacterial isolate exhibiting antifungal activity against *Fusarium* sp. on all the three test media (Figure 2 and 3). No antifungal activity was

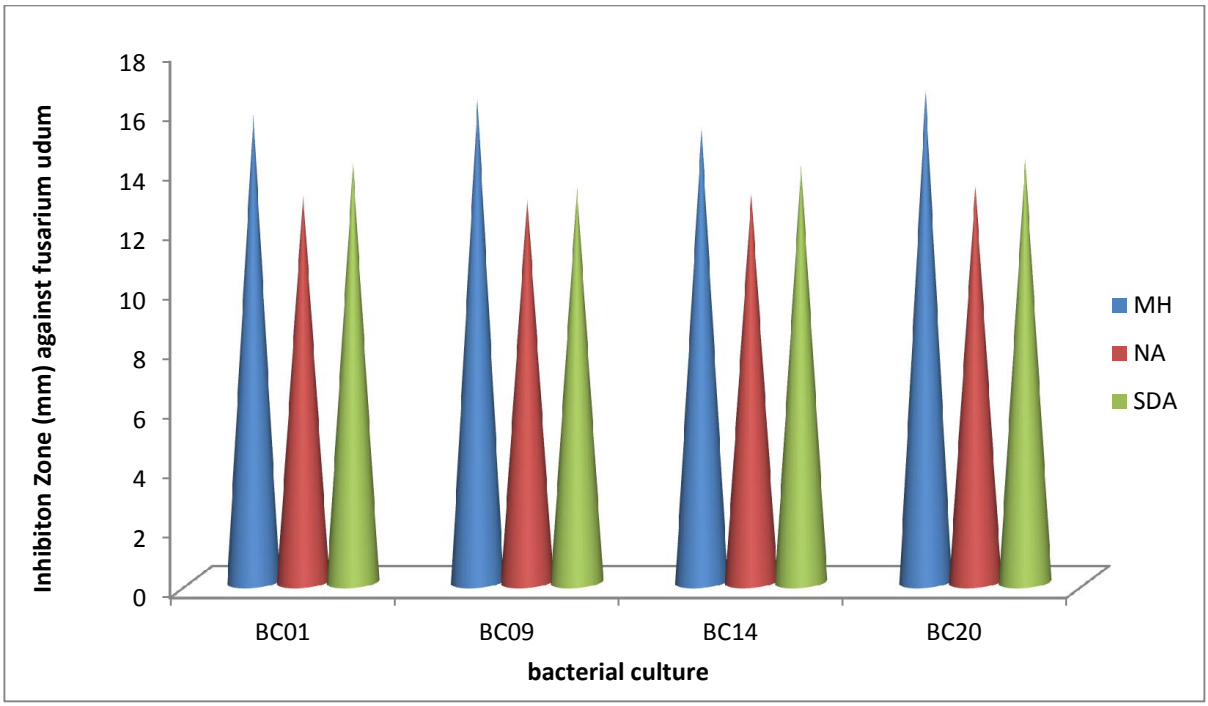

Figure 3. Antifungal activity of *Bacillus* isolates on different media.

observed on SDA medium as there is interfering component in SDA medium which limits the growth of *Psuedomonas spp.* Similar results were reported by Ahmad et al., (2008) where, *Psuedomonas* sp. exhibit broad spectrum antifungal activity on Muller-Hinton medium against *Aspergillus, Fusarium* and *Rhizoctonia bataticola*.

Seed germination Assay

On the basis of seedling biomass, root length and shoot length growth, these bacterial isolates were tentatively grouped into four types, influencing the increase in biomass by 1.6-15.8% (I group), 16.7- 31% (II group), 32.0-46.8% (III group.) and 47.6-50.8% (IV group). However, seedling biomass in untreated seeds (control) ranged from 1.26-1.30 g. The positive growth influence on seedling was more visible in isolates belonging to group IV. None of the test isolate adversely affected the seedling growth up to 10 day observation. In the same run increase in the root and shoot length were recorded as 9.7-19.4% to 41.9-48.4% and 12.5-20.8%, respectively. As a result, group IV had been observed as the best group of isolates which had given best index for growth of biomass, root length as well as shoot length.

Sterile soil Assay (*In vivo* interaction of phyto-pathogen and antagonist)

Compared to fungal infested soil, treatment of test isolates resulted in increase in total plant weight. Among five efficient isolates of fluorescent *Pseudomonas* Pf_{19} was resulted best as in its respective treatment length and weight of plant recorded the best of it. When treatment was given with four selected *Bacillus* isolates only BC_{20} reported working efficiently against the infectious fungus as resulted in terms of plant length and weight. Decrease in plant length by 35.3% and 16% decrease in plant biomass was recorded in presence of phytopathogens. When Pf_{05}, Pf_{14}, Pf_{19}, Pf_{23}, Pf_{25}) were applied along with *Fusarium* it showed 3.12%, 3.22%, 4.54%, 3.90% and 4.19% increase in plant length and 12%, 11%, 14%, 12% and 13% increase in plant biomass with respect to positive control and 23% increase in plant biomass with respect to negative control. When bacteria Bc_{01}, Bc_{09}, Bc_{14}, and Bc_{20}) were applied along with *Fusarium,* it showed 5.94% increase in plant length and 12% increase in variation in root and shoot weight reveals the effect of stress caused by pathogenic fungi present in same rhizospheric environment, the data confirmed that two isolates used were not only able to promote the growth of *C. cajan* because of direct effect, but also able to restrict the fungal pathogens (Figures 4 and 5).

DISCUSSION

Plant rhizosphere is known to be preferred ecological niche for various types of soil microorganisms due to rich nutrient availability. Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP

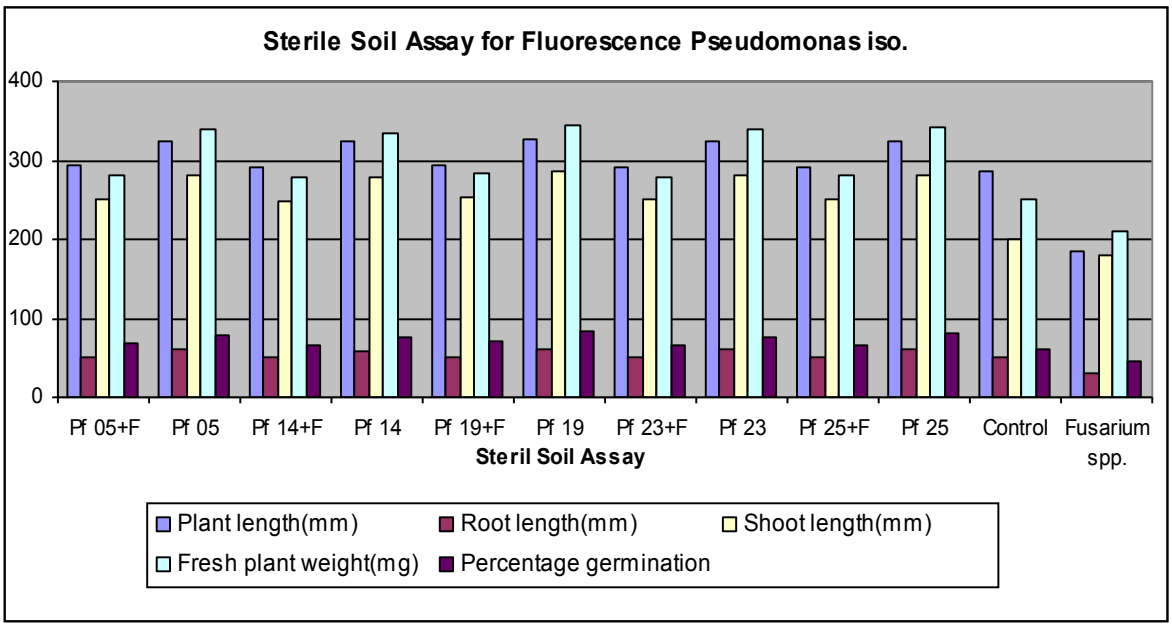

Figure 4. Sterile soil assay for *Pseudomonas* isolates.

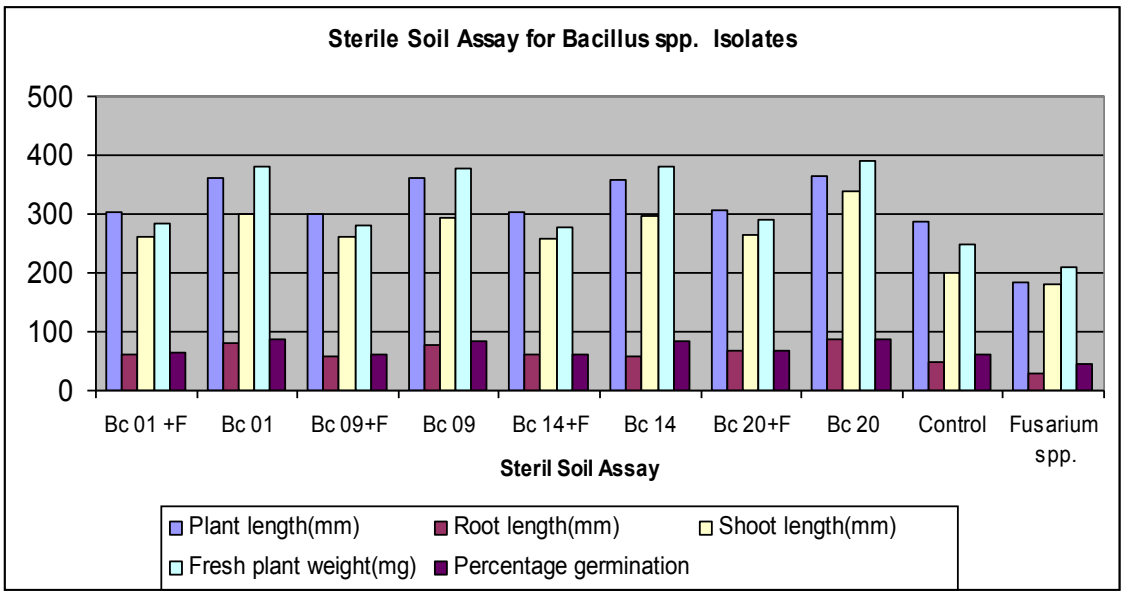

Figure 5. Sterile soil assay for *Bacillus* isolates.

activities (Whipps,2001; and Yadav and Dadarwal,1997). A model was also developed for lowering the plant ethylene concentration by plant growth promoting bacteria (Glick and Penrose,1998). The primary interest of present study was selection of wild type rhizosphere competent bacteria having biological control activity against phytopathogen of *C. cajan* mainly *F. udum*, which cause wilting and drooping of host plant. As expected, a significant increase in the microbial density of rhizospheric soil was observed compared to non-rhizospheric soils which could possibly be due to the nutrient rich environment and availability of nutrients from root exudates, which includes an array of low and high molecular weight compounds (Weller,1988). Our observation demonstrated increase in the rhizospheric microbial density (Watanabe *et al.,*1993;O'Sullivan,1992**).**

The biocontrol activity of bacterial antagonists against plant disease has been attributed to the production of antifungal metabolites, including HCN hydrolytic enzymes like chitinase, and siderophores (Weller and Thomashow, 1994; Vassilev *et al*.,2006;Dal *et al.,*2002)). Isolates showed the production of both HCN and siderophore, as means of biological control (Weller and Thomashow, 1994), and suppress the disease caused by them **(**Glick, 1995). These rhizospheric isolates were found to produce chitinase Lytic enzymes produced by *B. circulans* WL-12 were noted to affect the integrity of fungal cell walls (Johnson *et al.,*1979). Improved efficiency of biocontrol and plant growth promoting was also observed in *Bacillus subtilis* AF1 isolates by chitin supplemented formulations as reported by Manjula and Podile, (2001). Chitinolysis plays an important role in biological control of plant disease control by chitin-supplemented application of chitinolytic biocontrol agents (Thomashow and Weller, 1995). *Bacillus* isolate Bc_{20} and *Pseudomonas* isolate Pf_{14} had a broad spectrum antifungal activity and thus effective biocontrol agent of *Fusarium* wilt of pigeon pea (Thomashow and Weller, 1995)**.** Many factors like production of antifungal compounds including antibiotics, HCN as well as siderophore may contribute to such activity as reported by others (Gupta *et al.,*2002;Jassen *et al*.,2002 and Robert and Selitrennikoff,1988). Voisard *et al.,(*1989) reported the cyanide production by *Psuedomonas fluorescence* which help to suppress black root rot of tobacco under genobiotic conditions. Further, the production of siderophores influences the plant growth. They bind to the available form of iron Fe3+ in the rhizosphere, thus making it unavailable to the phytopathogens and protecting the plant health (Lork, 1948; Liang *et al.* 1996).

Results given in preceded section clearly indicates that isolates of *P. spp* and *B. spp.* have potential as an effective biocontrol against fusarium wilt of pigeon pea crop. Isolate Pf_{19} and Bc_{20} provided better biocontrol than other isolates screened for biological control against *F. udum* (Karimi *et al.,*2010). The above-tested isolates could exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically. *Pseudomonas* isolates used in this study produced, both benzoate and salicylate type of siderophores. Similarly, Bacillus isolates also produced benzoate type of siderophores. There is a little contribution of bacterial siderophores to the overall requirement of plants. However, the role of microbial siderophores in PGPP is focused on biocontrol activities due their competitive effects with the plant pathogens (Bai *et al*.,2009; Zaidi *et al.,*2010**)**. Further studies on the performance of these isolates and their mutants on the growth of plant will uncover the mechanism and potential of these biocontrol agents exhibiting multiple traits.

Conclusion

Food production for world population required an integrated approach combining the concepts of IPNS, IPDM and integrated water and land management for the development of crop productivity and maintaining soil and environmental health. In this context plant growth promoting rhizobacteria (PGPR) may influence the plant growth through biofertilization, phytostimulation and indirect mechanisms including biocontrol activity. In the present investigation four major group of free living rhizobacteria that is, fluorescent *Psuedomonas, Bacillus*, *Azotobacter* and putative nitrogen fixing bacteria were isolated and screened for one or more plant growth promoting activities and were further analyzed for quantitative estimation of IAA and other PGP traits. Appliance of combination of compatible bio-control agents possessing differential mechanism of pathogen suppression is suggested as a reliable and potential means of disease suppression. The present investigation clearly indicates no antagonism of one PGPR strain towards other PGPR strains in parallel strek or overlay methods and high potentiality of bacterial (fluorescent *Pseudomonads* and *Bacillus*) biocontrol agents against economically important plant disease that is, *Fusarium* wilt of pigeon pea. The results further showed that development of bacterial consortium based on their interaction studies can reduce the possibilities of failure of potential microbial inoculants in rhizosphere.

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