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Induced Systemic Resistance and Evaluation of Bio-control Agents for Management of Pigeonpea Wilt Caused by *Fusarium udum*

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The performance of the four fungal and two bacterial bioagents were evaluated for the bicontrol efficacy and ability to induce systemic resistance against *Fusarium udum* causing wilt of pigeonpea which is posing a serious threat to pigeonpea growing regions of India. Among the six isolates, maximum mycelial inhibition was noticed in *Trichoderma harzianum* (Th- R) as compared to other biocontrol agents. Among contact fungicides, maximum inhibition (> 75 %) of mycelium was recorded in Mancozeb and capton at 0.20 and 0.3 % concentrations. More than 90% inhibition was recorded among the systemic fungicides at all the all the concentrations except thiophanate methyl which recorded 53.67 % inhibition at 0.05 % concentration. Among different treatment combinations of biocontrol agents, the highest vigour index was recorded in *P. fluorescense* (RP- 46) + *P. putida* (RP- 56) treated seeds in both the cultivars (Moderately resistant and susceptible). The level of expression of defense related enzymes (PO, PPO & PAL) was more in moderately resistant cultivar(BSMR- 736) rather than susceptible one(ICP- 2376). In glass house experiment seeds treated with *P. flourescens* (RP- 46) + *P. putida* (RP-56) recorded least wilt incidence as compared to other treatments. In both *Kharif* seasons of 2013/14 and 2014/15 recorded significantly lowest wilt incidence and highest yield in soil drenching with 0.2 % Carbendazim fungicide. Among the biocontrol agents, seed treatment @4 g / kg seeds + soil application of PGPR (*P. flourescens* & *P. putida*) consortium @ 25kg/ ha in FYM @ 50 kg/ ha, recorded least wilt incidence and highest yield.

Keywords: Pigeonpea; Fungicide; Biocontrol; Induced systemic resistance; *Fusarium udum*; *Pseudomonas; Trichoderma*; PGPR.

Pigeonpea is an important pulse cum grain legume crop and the area under the crop is increasing due to the productivity, favorable conditions and economics of the crop. India is a principal pigeonpea growing country contributing nearly 90% of total world production. Currently, it occupies an area of 5.2 million ha with an annual production of 4.2 million tonnes[1]. Pigeonpea is attacked by more than 100 pathogens including fungi, bacteria, viruses, nematodes, and mycoplasma-like organisms, but only a few of them cause economic losses[2]. The diseases of considerable economic importance at present are fusarium wilt (*Fusarium udum*), sterility mosaic (Sterility mosaic virus), and phytophthora blight (*Phytophthora dreschsleri* f. sp. *cajani*). Among

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them, wilt caused by *F. udum* is considered the most important soil borne pathogen of pigeon pea[3].

The disease is typically soil borne and the pathogen perpetuates in soil for several years by means of chlamydospores[4]. Chemical control of a soil borne plant pathogen is frequently ineffective because of the physical and chemical heterogeneity of the soil, which may prevent effective concentration of the chemical from reaching the pathogen. Hence, the best alternative measure is to look for bio control agents, which colonize the rhizosphere, the site requiring protection and leave no toxic residues, as opposed to chemicals. A multitude of microbes has been implicated to be biocontrol agents of plant pathogens sometimes with excellent documentations[5-8]. Hence, experiments were carried out to find out the efficacy of some fungal and bacterial bio control agents against *F. udum* on pigeonpea and were also tested for induction of systemic resistance. Fluorescent *Pseudomonas* and *Trichoderma* species are important groups of plant growth-promoting microorganism reported to protect plants against pathogens by evolving various mechanisms such as antagonism, competition and Induced systemic resistance (ISR)[9-11].

Induced systemic resistance (ISR) triggered by plant growth-promoting fungi (PGPFs) and Plant growth promoting rhizobacteria (PGPR) confers a broad-spectrum resistance that is effective against different types of pathogens[12]. PGPR produce phytohormones that are believed to be related to their ability to stimulate plant growth. Indole-3-acetic acid is a phytohormone which is known to be involved in root initiation, cell division, and cell enlargement[13]. This hormone is very commonly produced by PGPR[14]. Most commonly, IAA-producing PGPR are believed to increase root growth and root length, resulting in greater root surface area which enables the plant to access more nutrients from soil. Cytokinins are a class of phytohormones which are known to promote cell divisions, cell enlargement, and tissue expansion in certain plant parts[13]. Cytokinin is produced by *Pseudomonas fluorescens* isolated from the rhizosphere of the soybean[15].

Compared to the use of individual PGPR strains, mixtures of several strains may result in a more stable rhizosphere community, provide several mechanisms of biological control, and may suppress a broader range of pathogens[16]. Compatible mixtures of certain biocontrol strains with antagonism as the main mechanism of action have provided a greater disease suppression than that used individually[17-22]. In one of the study used PGPR that elicit ISR, indicated that mixtures of PGPR provided synergistic activity against a broader range of pathogens on one host[22]. The aim of the present experimentation is to evaluate the performance of *Trichoderma* and two *Pseudomonas* isolates for their bio control efficacy and ability to induce systemic resistance against *F. udum* causing pigeonpea wilt.

MATERIALS AND METHODS

Bio- control agents

The bio-agents *Trichoderma viride*(Tv-R),*Trichoderma harzianum*(Th- R), *Pseudomonas fluorescens*(RP-46) and *Pseudomonas putida* (RP-56) used in this study were obtained from the collection at the Department of Plant Pathology, University of Agricultural Sciences, Raichur, India and the two isolates, *Trichoderma* spp (ICRISAT-T) and *Trichoderma* spp (GLB- I) were obtained from rhizosphere of pigeonpea from Sick plot (BIL-17), ICRISAT, Hyderabad, India and sick plot, ARS, Kalaburgi, Karnataka, India respectively. The *Trichoderma* isolates were maintained on a *Trichoderma* specific medium and the *Pseudomonas* isolates were maintained on King's B medium[23].

Isolation of the Pathogen

The wilted pigeonpea plant sample collected from the field and the infected tissues from the stem/ collar region were cut into small bits of 1- 2 mm in size, surface sterilized in 1 per cent sodium hypochlorite solution for a minute and then washed three times in sterile distilled water to remove any traces of sodium hypochlorite. They were subsequently blotted dry and plated on sterile Potato Dextrose Agar [PDA] plate and incubated at 26 ± 2ºC and alternate cycles of 12 h light and 12 h darkness. Culture is purified by using single spore isolation and proved Koch's postulate by using susceptible cultivar (ICP 2376). The fungal isolates were preserved in PDA slants for subsequent uses. Total of 111 isolates of *F. udum* were collected from 397 farmers' fields in Karnataka, Madhya Pradesh,

Maharashtra, Tamil Nadu and Telangana states. However, dual culture test and evaluation of bio-control agents under glass house and field condition was carried out using only one local isolate of *F. udum* (FU- 37) from ARS Kalaburgi, Karnataka.

***In-vitro* evaluation of bio-agents by Dual culture test**

Bio-control agents (Fungal and Bacterial isolates) and the *F. udum*(FU- 37) isolate were placed side by side on a single Petri-dish containing solidified PDA. There were three replications for each isolate with one control each of the pathogen and the bio-control agent. They were incubated at 26 ± 2º C and grown for 6-8 days. The diameter of the colony of both the bio-control agent and the pathogen was measured in two directions and the average was calculated. Percent inhibition of growth of the test pathogen was calculated[24]. *In-vitro* evaluation was done three times with three replications.

The percentage of inhibition was estimated using the following formula:

$$I = (C - T)/C * 100$$

Where; I= Percentage of inhibition, C= radial growth of the pathogen in control and T= radial growth of pathogen in treatment.

***In-vitro* evaluation of fungicides by poisoned food technique**

Four non- systemic fungicides namely Capton, Chlorothalonil, Mancozeb and Zineb. four systemic fungicides namely Benomyl, Carbendazim, Thiophanate methyl and Carbendazim 25 % + Mancozeb 50 % were tested against colony growth of *F. udum* isolate FU- 37. Non-systemic fungicides were used at 0.1%, 0.2 % and 0.3 % and systemic fungicides at 0.05%, 0.1% and 0.20% concentrations in autoclaved PDA medium by poisoned food techniques[25]. Five mm diameter agar disc of test fungi was cut from 6 day old culture and placed in the center of Petri plates containing different concentration of fungicides. The plates without fungicides served as control. The inoculated plates were incubated at 26 ± 2°C. The radial growth was recorded after 7 to 8 days of incubation when the fungus covered the plates completely in control. The percent inhibition of the fungus over control was calculated[24].

Seedling vigour

Seedling vigour of the *Pseudomonas* spp. and *Trichoderma* spp. treated seeds was determined by the standard roll towel method (ISTA in 2005). Four replicates of 50 treated seeds were placed at equi-distance on the paper towel and covered with another pre-soaked paper towel, rolled up along with polythene wrapping to prevent drying of the towels. The rolled towels were then incubated in an incubation chamber for 8 days. Paper towels were unrolled after incubation period and number of germinated seeds were counted and represented in percentage. Seedling vigour was analysed using the method of Abdul-Baki and Anderson[26]. The length of the root and shoot of individual seedlings were measured with different treatment combination to assess the vigour. The vigour index (VI) was calculated using the formula VI = (Mean root length+ Mean shoot length) x % germination.

Induction of defense mechanisms

Two each of *Trichoderma* and *Psuedomonas* isolates were used in the induction of defense reactions in two pigeonpea genotypes, namely BSMR- 736 (Moderately resistant) and ICP 2376 (Susceptible). Fungus treated (*Trichoderma*) and bacterized (*Pseudomonas*) seeds were sown in polythene covers filled with sterilized river bed sand. Eight-day-old seedlings were transplanted in plastic pots @ 10 seedlings per pot. Ten days after transplanting, soil application with *Trichoderma* suspension (3×10^3 spores/ml) and bacterial suspension (10 mL of suspension containing 10^8 cfu mL"[1]) was done. Bacterized plants were divided into two treatments. In the first treatment, bacterized plants were challenge inoculated with *F. udum* (50 mL of microconidial suspension containing 6×10^6 conidia/ ml per pot) at one day after soil application of fungal and bacterial suspension and in the second treatment, bacterized plants were not challenged with the pathogen. Plants without prior treatment of *Trichoderma* and *Pseudomonas* were inoculated with the pathogen. The plants neither treated with bacterial suspension nor challenged by the pathogen were kept as control. Three replications were maintained in each treatment; each replicate consisted of 10 pots and in each pot six plants were maintained. The experiments were conducted

using randomized block design on a greenhouse bench. The humidity in the greenhouse was maintained at RH of 70%. The temperature was adjusted to 25- 27æ%C (day) / 20- 22æ%C (night).

Sample collection for biochemical analysis

Plants were carefully uprooted without causing any damage to root tissues at different time intervals (0, 3, 6, 9 and 12 days after the pathogen inoculation). Six plants were sampled from each replication of the treatment separately (treatments were mentioned in the experimental design) and were maintained separately for biochemical analysis. Fresh roots were washed in running tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized root tissues were stored in deep freezer ("80$^{æ\%}$ C) until used for biochemical analysis.

Assay of PO

Root samples (1 g) were homogenized in 2 mL of 0.1 M phosphate buffer, pH 7.0 at 4 æ%C. The homogenate was centrifuged at 16 000 g at 4 æ%C for 15 min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 mL of 0.05 m pyrogallol, 0.5 mL of enzyme extract and 0.5 mL of 1% H_2O_2. The reaction mixture was incubated at room temperature (28±2æ%C). The changes in absorbance at 470 nM were recorded at 30 s intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min"1 mg"1 protein[27].

Assay of PPO

PPO activity was determined as per the procedure[28]. Root samples (1 g) were homogenized in 2 mL of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16 000 g for 15 min at 4 æ%C. The supernatant was used as the enzyme source. The reaction mixture consisted of 200 µL of the enzyme extract and 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200µL of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 420 nM min"^{1}mg"^{1} protein.

Estimation of PAL activity

Root samples (1 g) were homogenized in 3 mL of ice cold 0.1 M sodium borate buffer, pH 7.0 containing 1.4 m M of 2-mercaptoethanol and 0.1 g of insoluble polyvenylpyrrolidone. The extract was filtered through cheese cloth and the filtrate was centrifuged at 16000g for 15 min. The supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nM[29]. Sample containing 0.4 mL of enzyme extract was incubated with 0.5 mL of 0.1 M borate buffer, pH 8.8 and 0.5 mL of 12 mM L-phenylalanine in the same buffer for 30 min at 30æ%C. The amount of trans-cinnamic acid synthesized was calculated using its extinction coeffi- cient of 9630 m"^{1} [29]. Enzyme activity was expressed as nmol trans-cinnamic acid min"^{1} mg"^{1}protein.

Evaluation of bio-control agents under glass house condition

Efficacy of those bacterial and fungal isolates (*Pseudomonas* spp. and *Trichoderma* spp.) causing enhanced seedling growth and inhibition to *F. udum in vitro* were selected and tested for their ability to reduce pigeonpea wilt under glass house conditions with different treatment combinations by using root-dip-inoculation technique. The disease incidence is calculated using the following formula.

Wilt incidence (%) = Number of diseased seedlings/ Total number of seedlings ×100

Evaluation of bio-control agents under field condition

The isolates of *Trichoderma* and *Pseuomonasa* having maximum inhibition of growth of *F. udum* under laboratory conditions were chosen for field evaluation and consortium of *Trichoderma*(*T. viride* + *T. harzianum*) and *Pseudomonas* (*P. fluorescence* + *P. putida*) preparations were used for pigeonpea seed treatment(Cv. BSMR- 736) @ 4 g/kg seed. The bio-control agents were multiplied on talc based formulations then enriched in farm yard manure and applied to the soil along with the seed treatments. The seeds were placed in a clean container, sprinkled with water until they were wet, adequate quantity of the bio-control agent available in a talc based dry powder form[30] was added and the container was agitated thoroughly until the seeds were uniformly coated. Farm yard manure (FYM) to be used for soil application was enriched with *Trichoderma*(*T. viride* + *T. harzianum*) or *Pseudomonas* (*P. fluorescence* + *P. putida*) consortium. The enrichment process involved adding of 2 kg of the respective bio-control agent

to 1 ton of FYM and incubating the mixture at ambient temperature under shade for a period of 15 days prior to planting pigeonpea. Optimum moisture content (15%) was maintained by adding water as needed during this period. The bio-control agent enriched FYM was added at 15 tons/ha after enrichment.

Field trials were conducted in *Fusarium udum* wilt sick plot at the farm of Agricultural Research Station (ARS), Kalaburgi (Karnataka) for two growing seasons in 2013/14 and 2014/15. The experiment was set up in a randomized block design (RBD) with three replications of seven treatments each with a plot size of 2.5 ×1.8 m. The treatments used included *Trichoderma* consortium and *Pseudomonas* consortium treated seeds sown after soil application of *Trichoderma* consortium and *Pseudomonas* consortium enriched FYM applied at 15 tons/ha. Carbendazim @ 0.3 per cent was used for soil drenching. A non-treated control was also included in the trials. All recommended agronomic practices for the region were followed to raise a good crop. The germination percentage was noted 15 days after planting and the incidence of wilt was monitored periodically and the terminal incidence was recorded 120 days after planting. The wilt incidence was calculated by counting the total number of diseased plants in $1m^2$ which was then divided by the total number of plants in the area and expressed as percentage.

Statistical analysis

Data were statistically analyzed using the standard procedures for completely randomize design, complete randomized block and split designs [31]. The averages were compared at 1% and 5% level using least significant differences (L.S.D)[32].

RESULTS AND DISCUSSION

Evaluation of bio-control agents under laboratory conditions

Four isolates of *Trichoderma* spp and two isolates of *Pseudomonas* spp were evaluated for their efficacy as antagonists against *F. udum* (FU-37), the cause of vascular wilt of pigeonpea using dual culture technique. All isolates were found to cause significant reduction in fungal growth as compared to the control.

The per cent inhibition of *F. udum* ranged from 46.52 to 70.84 per cent. Among tested fungal antagonists, the maximum inhibition of *F. udum* growth was observed in *T. harzianum* (Th-R) bioagents as compared to other bio-control agents and inhibited maximum fungal growth (74.52 %) of *F. udum* followed by *Trichoderma* spp (ICRISAT-T) (72.23 %). *T. viride* (TV-R) and *Trichoderma* spp (GLB) with 70.84% and 67.91% respectively. In bacterial bioagents *P. fluorescens* (RP- 46) inhibited to the extent of 50.28 per cent. Least inhibition was recorded with 46.52 per cent in *P. putida* (RP- 56)[Table. 1]. *In- vitro* evaluation of antagonistic microorganisms against *F. udum* recorded maximum inhibition of *F. udum* against *T. viride* (87.03 %) and *T. harzianum* (85.40 %), *P. fluorescens* (81.87 %)[33]. Evaluation of six bioagents against *F. udum* though dual culture technique and recorded highest inhibition from *G. virens* (Pantnagar) and *T. viride* (Coimbatore) [34].

Evaluation of fungicides under laboratory conditions

In contact fungicides, Mancozeb and capton recorded maximum inhibition (> 75%) of mycelial growth at 0.20 and 0.30 per cent and chlorothalonil showed 62.50 per cent inhibition at 0.10 per cent concentration, more than 65 per cent inhibition at 0.2 and 0.3 per cent concentrations. In systemic fungicides, carbendazim 25 per cent + mancozeb 50 per cent showed 100 per cent inhibition at all concentrations (0.05, 0.10 and 0.20 %). Benomyl, carbendazim, thiophanate methyl showed 100 per cent inhibition at 0.2 per cent concentration and more than 90 per cent inhibition was recorded in 0.05 and 0.1 per cent concentrations of benomyl and carbendazim (Table. 2). The fungicides suppressed *Fusarium* by altering and inhibiting cell metabolism and these biochemical alterations may lead to inhibition in fungal growth[35]. Carbendazim may directly inhibit conidial germination and sporulation of *F. oxysporum*[36] as well as colonization [37]. *In- vitro* evaluation of different fungicides against *F. udum* and reported carbendazim and thiram fungicides were quite effective in inhibiting the growth of the fungus at 1000 and 2000ppm, which gave 93.8 and 91.3 per cent inhibition, respectively[38]. Similarly, efficacy of different fungicides tested against *F. udum* by using poisoned food technique *in vitro* revealed

that carbendazim inhibited the growth of pathogen at all concentrations(100, 250 and 500 ppm)[39].

Seedling vigour

In the moderately resistant cultivar (CV. BSMR- 736), *P. fluorescens* (RP- 46) + *P. putida* (RP- 56) treated seeds showed highest germination (95.34 %), mean root length (20.63 cm), shoot length (7.56 cm) and vigour index of 2688.40, which differed significantly from all other isolates. Whereas in susceptible cultivar (CV. ICP 2376) also the same combined isolates *P. fluorescens* (RP-46) + *P. putida* (RP-56) treated seeds showed highest germination (93.67 %), mean root length (16.36 cm), shoot length (7.1 cm) and vigour index (2193.67) which differed significantly from all other isolates (Table. 3). Highest vigour index was shown by the combined isolates of *P. fluorescens* (RP- 46) + *P. putida* (RP- 56) and as far as germination and vigour index is concerned all the isolates differed significantly in both the cultivars (BSMR- 736 and ICP 2376). These findings are in confirmation with the earlier workers [24] concluded that germination and vigour index were considered as indices of systemic induction of resistance and observed that the indigenous isolate of *P. fluorescens* (RP- 56) showed highest induction of resistance resulting in highest seed germination and vigour indices in chilli seeds against *F. solani*. Similarly, other researchers also observed the increased ISR, vigour index, germination by plant growth promoting rhizobacteria (PGPR) and *Trichoderma* spp[40].

Induction of defense mechanisms

Major defense related enzymes focussed in the present study were peroxidase (PO), polyphenol oxidase (PPO), phenyalanine ammonia lyase (PAL). The increased activity of all these enzymes is possible when any biocontrol agent having the capacity to suppress the disease is applied through a reliable established method, so that it has consistent performance for a longer time period. Peroxidases are used primarily for the synthesis of secondary metabolites and are known to be induced by various types of stresses including pathogen infection [41]. Peroxidases have been implicated in a number of physiological functions that may contribute to resistance phenol oxidation, lignification and in the deposition of phenolic material into plant cell walls during resistant interaction [42]. Both PAL and PPO play important roles in biosynthesis of phenolics, phytoalexins and lignin, the three key factors responsible for disease resistance [43]. Phenylalanine ammonialyase catalyzes the conversion of phenylalanine to trans cinnamic acid, a key intermediate in the synthesis of salicylic acid. Enhanced PAL and PPO activity was reported in tomato infected by *Fusarium oxysporum* [40]. Present study revealed that higher accumulation of PO was observed in moderately resistant cultivar (BSMR-736) than susceptible cultivar (ICP 2376). The treatment *P. fluorescens* (RP- 46) + *F. udum* (FU-37) showed maximum PO activity (0.96 change in absorbance at 470 nm/ min/mg protein) followed by *P. fluorescens* (RP-46) + *P. putida* (RP- 56) + *F. udum* (FU-37) which were significantly different from all other treatments. Accumulation of PO started three day after challenge inoculation. The maximum accumulation was observed on 6th day after challenge inoculation and the level of

Table 1. Efficacy of bio-agents against *F. udum* of pigeonpea under dual culture

Bioagents	Percent inhibition over control
Pseudomonas fluorescens(RP- 46)	50.28* (45.18)**
Pseudomonas putida (RP- 56)	46.52 (43.03)
Trichoderma viride (Tv-R)	70.84 (57.35)
Trichoderma harzianum (Th-R)	74.52 (59.71)
Trichoderma spp (ICRISAT-T)	72.23 (58.23)
Trichoderma spp (GLB-I)	67.91 (55.52)
Control	0.00 (0.00)
SEM±	0.90
C. D at 0.01%	2.79

Note: * Original values; ** Arcsine transformed values

expression of the enzyme declined in subsequent days. Plants inoculated with pathogen alone had comparatively less PO activity but compared to control, the activity was higher (Figure 1). Increased activity of cell wall bound peroxidases has been elicited in different plants such as cucumber [44], rice [45], tomato [46] and tobacco [47] due to pathogen infection. PO1 isoform was prominently expressed in *P. ûuorescens* isolate Pf1-treated root tissues against *F. oxysporum* f. sp. *lycopersici* [40].

A similar pattern of increased activity of PPO was observed in moderately resistant cultivar rather than susceptible cultivar. Here also the PPO activity was maximum on 6th day after challenge inoculation and expression level was reduced afterwards. The same *P. fluorescens* (RP- 46) + *F. udum* (FU-37) treatment recorded 1.21 and 0.98 change in absorbance at 420 nm/ min/mg protein) which significantly differed from all other treatments in BSMR- 736 and ICP- 2376 respectively. In moderately resistant cultivar (BSMR 736) the lower activity of PPO was recorded in *T. harzianum* (Th-R) + *F. udum* (FU-37) as compared to *F. udum* alone treated plants. Where as in susceptible cultivar the lower PPO activity was recorded in *T. viride* (Tv-R) + *T. harzianum* (Th- R) + *F. udum* (FU-37) interactions as compared to *F. udum* alone treated plants (Figure1).

PAL is the key enzyme in inducing synthesis of Salicylic Acid (SA) which induces systemic resistance in many plants. PAL plays an important role in the biosynthesis of phenolics and phytoalexins [48]. PAL activity showed increased trend 6th day after challenge inoculation. Similar to

Table 2. *In vitro* evaluation of fungicides against *F.udum*

	Non systemic fungicides			
Fungicides	Per cent inhibition at different concentrations			
	0.1%	0.2%	0.3%	Mean
Capton 50 % WP	67.23 (55.10)	76.38 (60.96)	80.00 (63.47)	74.54
Chlorothalonil 75 % WP	62.50 (52.27)	65.37 (53.98)	67.87 (55.50)	65.25
Mancozeb 75% WP	70.56 (57.17)	77.59 (61.78)	87.78 (69.57)	78.64
Zineb 70% WP	22.31 (28.20)	31.57 (34.21)	37.23* (37.62)**	30.37
	Systemic fungicides			
Fungicides	Per cent inhibition at different concentrations			
	0.05%	0.10%	0.20%	Mean
Benomyl 50 % WP	93.34 (75.07)	93.34 (75.07)	100 (90.05)	95.56
Carbendazim 50 % WP	93.34 (74.07)	100 (90.05)	100 (90.05)	97.78
Thiophanate methyl 70 % WP (46.47)	53.67 (78.05)	90.46 (90.05)	100	81.38
Carbendazim 25 % + Mancozeb 50 % 75 % WP	100 (90.05)	100 (90.05)	100 (90.05)	100.00
Control	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
	SEM±		C. D at 0.01%	
F	0.25		0.95	
C	0.14		0.55	
F x C	0.44		1.65	

Note: * Original values;** Arcsine transformed values; F- Fungicide & C- Concentration

PO and PPO, the PAL activity also maximum in *P. fluorescens* (RP- 46) + *F. udum* (FU-37) treatment ((31.26 nmol transcinnamic acid/hr/mg protein) but in *T. harzianum* (Th-R) + *F. udum* (FU-37), the PAL activity was lower compared to *F. udum* alone treated plants and in healthy control the activity was recorded up to 19.91 transcinnamic acid/hr/mg protein(Figure 1).

Similarly, roots collected from *P. fluorescens* treated seedlings induced early and enhanced level of PAL, PO and PPO in tomato plants challenged with *F. o* f. sp. *lycopersici* [40]. Induction of high PO, PPO and phenolic activity was noticed in tomato against *Fusarium* wilt pathogen, *F. o* f.sp. *lycopersici* when treated with *T. harzianum* [49]. Increased level of defense related enzymes, *viz.*, PAL, PO and PPO was found in co-inoculation of plant growth promoting rhizobacteria, *Rhizobium* and challenge inoculation with *F. udum* of pigeonpea [50]. Increased activity of defense related enzymes mainly PO, PAL, total phenol and â 1,3 glucanase was noticed due to application of *P. fluorescens* isolates in chilli plants challenge inoculated with *F. solani* causing wilt of chilli [51].

Evaluation of bio-control agents under glass house condition

In moderately resistant cultivar (CV. BSMR- 736), least wilt incidence (8.34 %) was recorded in *P. fluorescens* (RP- 46) treatment followed by *P. fluorescens* (RP- 46) + *P. putida* (RP- 56) with mean incidence (13.89 %). Whereas in susceptible cultivar (Cv. ICP 2376) also least wilt incidence (29.17 %) was recorded in *P. fluorescens* (RP- 46) treatment followed by *P. fluorescens* (RP- 46) + *P. putida* (RP- 56) with mean incidence of

Table 3. Seedling vigour of bioagents treated seeds by standard roll towel method.

Treatments	Germination (%)		Mean Root Length (cm)		Mean Shoot Length (cm)		Vigour Index	
	BSMR - 736	ICP -2376	BSMR - 736	ICP - 2376	BSMR -736	ICP - 2376	BSMR - 736	ICP - 2376
Trichoderma viride (Tv- R)	93.34 * (75.07)**	86.34 (68.34)	13.67	11.53	6.05	7.05	1840.85	1413.00
Trichoderma harzianum (Th-R)	88.67 (70.36)	82.39 (65.18)	15.24	13.00	5.69	6.00	1855.20	1503.00
Pseudomonas fluorescens (RP- 46)	85.34 (67.52)	88.67 (70.36)	18.94	14.25	6.47	5.74	2168.89	1863.77
Pseudomonas putida (RP- 56)	91.67 (73.26)	84.67 (66.98)	17.82	9.17	7.05	5.62	2280.36	1147.51
T. viride (Tv-R) + *T. harzianum* (Th- R)	92.87 (74.33)	91.67 (73.26)	14.38	13.35	7.23	6.47	2002.83	1682.38
P. fluorescens (RP-46) + *P. putida* (RP- 56)	95.34 (77.56)	93.6 (75.46)	20.63	16.36	7.56	7.10	2688.4	2193.67
Control	81.44 (64.43)	62.00 (63.95)	10.70	6.11	3.91	3.91	1188.28	715.84
SEM±	1.32	1.30	0.55	4.10	0.16	0.24	53.30	69.18
C. D at 0.01 %	4.08	4.01	1.69	12.43	0.50	0.75	161.68	209.85

Note: * Original values; ** Arcsine transformed values; BSMR- 736 (Moderately resistant) & ICP- 2376 (Susceptible) cultivars.

42.06 per cent (Table. 4). Similar study conducted by other workers also by biological control of pigeonpea wilt under glasshouse condition and found *T. viride* and *T. harzianum* isolate- C as effective bicontrol agents[52]. Evaluation of 20 isolates of fluorescent pseudomonads and *Bacillus* spp. in the laboratory and glasshouse condition and six isolates were considered as potential for the biocontrol of the disease on the basis of antibiotic sensitivity, antifungal activity [53]. Efficacy of *T. viride*, carbendazim, Rhizobium, *T. viride* + carbendazim, *T. viride* + *Rhizobium*, carbendazim + *Rhizobium* and *T. viride* + *Rhizobium* + carbendazim against *F. udum* in a pot experiment and observed that all treatments significantly reduced the wilt incidence over the control (73.30 %) except *Rhizobium* alone (64.40 %) [54]. I*n- vitro* efficacy of eleven *P. fluorescens* strains (I1, I2, I3, I4, I5, I6, I7, I8, I9, I10 and I11) in controlling wilt of pigeonpea and recorded seed treatment with *P. flurescens* strains I10 from pigeonpea plants resulted in the lowest (16.66 %) incidence of the disease [55].

Evaluation of bio-control agents under field condition

Trials to evaluate the ability of the bio-control agents to control vascular wilt under *Fusarium* wilt sick plot were set up using moderately resistant cultivar(Cv. BSMR- 736) during 2013/14 & 2014/15 using *Trichoderma* and *Pseudomonas* consortium applied as seed treatments alone and in combination with a soil treatment. Chemical soil drenching with Carbendazim, commonly used by growers in the region for control of *F. udum* were also included in the trial. In 2013/14 *Kharif* percent wilt incidence was found to be significantly reduced, showing a nearly 35.18- 80.16 % reduction in wilt incidence in the treatments overall when compared to the non-treated control (Table 5). Whereas in 2014/15 *Kharif* percent wilt incidence was found to be significantly reduced from 38.09- 83.13 per cent when compared to the non-treated control thereby emphasizing the contribution of seed and soil treatments towards yield enhancement.

In *Kharif* season 2013/14 soil drenching with 0.2% carbendazim fungicide recorded significantly lowest mean wilt incidence (7.06 %) with highest yield (1723.96 kg / ha). Among the bio-control agents seed treatment + soil application of PGPR consortium, recorded wilt incidence of 10.31% and yield of 1594.79 kg per ha (Table. 5).

Table 4. Efficacy of bioagents against *Fusarium* wilt of pigeonpea under glasshouse conditions

Treatments	Per cent wilt incidence	
	BSMR- 736	ICP- 2376
Trichoderma viride (Tv- R) + *Fusarium udum* (FU-37)	22.23*	83.33
	(28.14)**	(60.94)
Trichoderma. harzianum (Th-R) + *F. udum* (FU-37)	20.05	94.44
	(26.62)	(76.41)
Pseudomonas fluorescens (RP- 46) + *F. udum* (FU- 37)	8.34	29.17
	(16.79)	(32.70)
Pseudomonas putida (RP- 56) + *F. udum* (FU-37)	27.78	77.78
	(31.82)	(61.91)
T. viride (Tv-R) + *T. harzianum* (Th- R) + *F. udum* (FU-37)	23.09	73.02
	(28.74)	(58.73)
P. fluorescens (RP- 46) + *P. putida* (RP- 56) + *F. udum* (FU-37)	13.89	42.06
	(21.89)	(40.45)
F. udum (FU-37)	38.69	100.00
	(38.48)	(90.05)
Control	0.00	0.00
	(0.00)	(0.00)
SEM± C. D at 0.01 %	5.32	8.48
	16.14	25.73

Note: * Original values; ** Arcsine transformed values; BSMR- 736 (Moderately resistant) & ICP- 2376 (Susceptible) cultivars.

Whereas in *Kharif* season 2014/15 with same treatments combinations, observed that again in the same treatment *viz*., soil drenching with 0.2% carbendazim fungicide recorded significantly lowest mean wilt incidence (5.30 %) with highest yield (1653.13 kg/ ha). Effective control of wilt of pigeonpea by soil drenching with 0.1% carbendazim but their approach was preventive not curative and they applied pre decided soil drenching at 30 days after sowing without considering wilt appearance[56]. Plant growth promotion activity of *Trchoderma* is well established [57- 59] and researchers have reported significant yield enhancement [60]. Root colonization by *Trchoderma* strains frequently enhances root growth, development, and crop productivity, resistance to abiotic stresses and the uptake and use of nutrients [61]. Seed treatment @ 4g per kg seeds + soil application of PGPR (*P. fluorescens and P. putida*) consortium @ 25 kg per ha in FYM @ 50 kg per ha, recorded the wilt incidence (7.28%) and yield (1540.63 kg/ ha) among bio-control agents. Soil application of *P. fluorescens* formulation was effective against the wilt [62]. Numerous strains of *P. fluorescens* and *B. subtilis* have been found suppressive against soil borne fungal pathogens. In the present study used strains of PGPR *P. flourescens* and *P. putida* consortium found to be an efficient plant growth promoter. Its application resulted to significantly greater production of dry matter and yield of pigeonpea. The possible mechanism involved in the suppression may be the competition and rhizosphere colonization[63]. Antibiosis is the other mechanism by which the biocontrol bacteria would have suppressed *F. udum*. Antibiotics such as

Table 5. Management of *Fusarium* wilt of pigeonpea during *Kharif* 2013-14 conducted at ARS, Kalaburgi

Treatments	Per cent wilt incidence		Yield (Kg/ha)	
	2013/14	2014/15	2013/14	2014/15
T_1:Seed treatment with *Trichoderma* spp @ 4 g per kg seed	18.41* (25.42)**	15.33 (23.06)	960.42	904.17
T_2:Seed treatment with *Pseudomonas* spp @ 4 g per kg seed	17.76 (24.94)	11.06 (19.43)	969.79	912.50
T_3:Seed treatment @ 4g per kg seed with *Trichoderma* spp+ soil application of consortium of *T. viride* @ 2.5 kg per ha and *T. harzianum* @ 2.5 kg per ha enriched with 2.5 tones FYM	13.63 (21.68)	10.90 (19.28)	1353.13	1183.33
T_4 :Seed treatment @ 4g per kg seed with *Pseudomonas* spp+ soil application of consortium of *P. fluorescense* @ 2.5 kg per ha and *P. putida* @ 2.5 kg per ha enriched with 2.5 tones FYM	10.31 (18.74)	7.28 (15.66)	1594.79	1540.63
T_5:Soil application of consortium of *P. fluorescense* @ 2.5 kg per ha and *P. putida* @ 2.5 kg per ha enriched with 2.5 tones FYM	23.09 (28.74)	19.46 (26.19)	947.92	935.42
T_6:Soil drenching with carbendazim @ 0.3 per cent	7.06 (15.42)	5.30 (13.32)	1723.96	1653.13
T_7:Control	35.62 (36.66)	31.43 (34.12)	564.51	553.33
SEM± C. D at 0.05%	2.15 6.64	5.03 15.50	119.42 362.23	104.36 316.54

Note: * Original values; ** Arcsine transformed values; cropping season- I (2013/ 14) & Cropping season- II (2014-15)

pholoroglucinols[64] and pyrolnintrin[65] produced by *P. fluorescens* and agrocin-84 [66], bulbiformin [67] etc. by *B. subtilis* have been reported to be fungicidal in nature. In present study *Trichoderma* spp consortium was found less effective in controlling wilt of pigeonpea as compared to *Pseudomonas* spp consortium. Similar relative effectiveness of the biocontrol agents have been reported against wilt of chickpea and pigeonpea [68- 69].

The success of dual application could be attributed to establishment and rapid build-up of bio-control agents in the soil which would assist in reducing the infectivity of soil borne inoculum or suppression of the pathogen[70] enabled by the soil application while the seed treatment would prevent proliferation of the seed borne inoculum. This is particularly relevant for soil borne pathogens such as *F. udum* where drenching the soil with chemicals is not only deleterious to the environment but is also practically not feasible due to high cost as pigeonpea is generally cultivated under rainfed condition without much investment

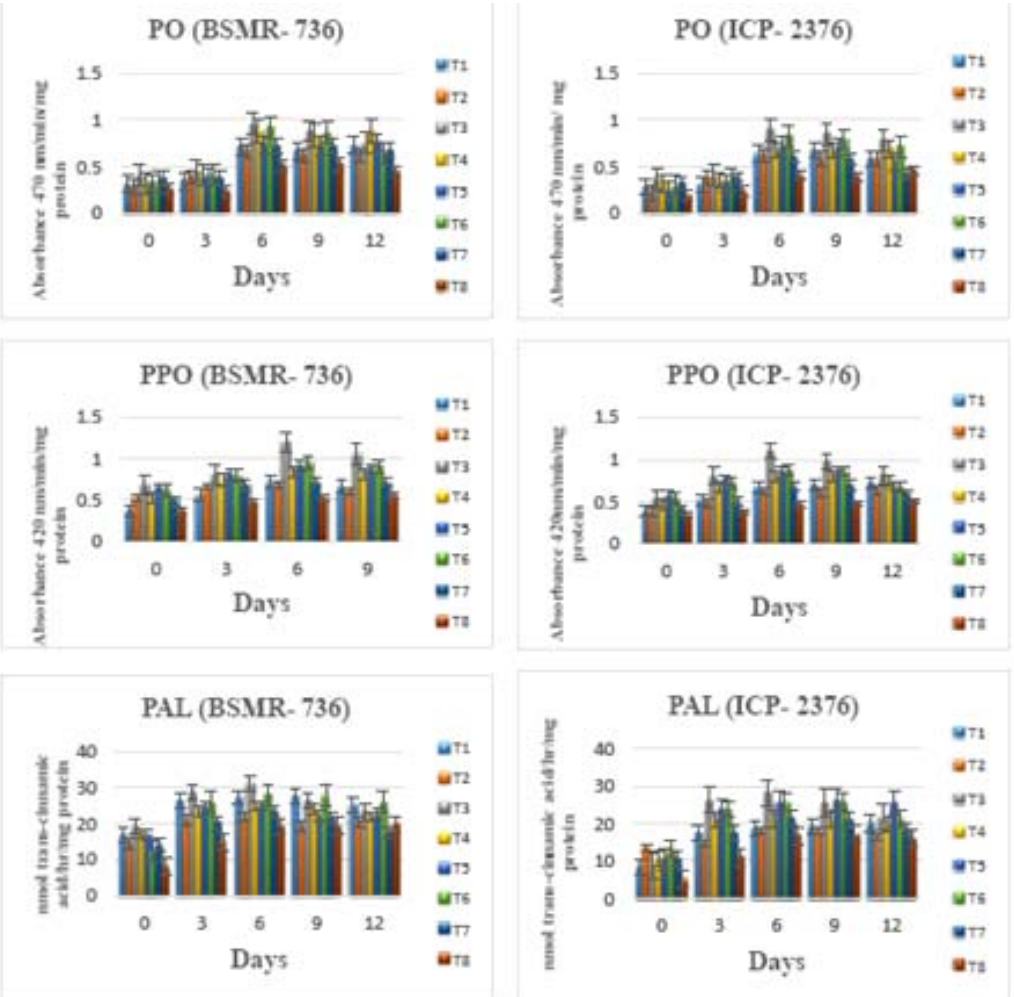

Fig.1. Changes in PO, PPO and PAL activity induced by *Psuedomonas* and *Trichoderma* bio agents in Pigeonpea roots challenged with or without the pathogen *F. udun*,.T1 = *Trichoderma viride* (Tv - R)+ *Fusarium udum* (FU-37); T2= *T. harzianum* (Th-R) + *F. udum* (FU- 37); T3= *Pseudomonas fluorescencens* (RP-46)+ *F. udum* (FU-37); T4= *P.putida*(RP-56)+*F. udum* (FU-37); T5= *T. viride*(Tv-R)+ *T.harzianum* (Th-R)+ *F. udum* (FU-37); T6= *P.fluoresciens* (RP-46)+ *P.putida* (RP-56)+ *F. udum*(FU-37); T7= *P.fluorescens*(RP-46)+ *P.putida*(RP-56)+ *F.udum*(FU-37) alone; T8=Un-inoculated.

for crop production as well as protection. Considering the fact that the cost of seed treatment chemicals under Indian conditions is almost three times that of the indigenously produced bio-control agents such as those used in this study, the use of two different methods and added amounts of inoculants will not significantly affect the cost of production. Moreover, prolonged use of the bio-control agents will increase their rhizosphere population [5, 30] and thereby enhancing the disease suppressive characteristics of the soil. Therefore, this study not only reports the availability of isolates of *Trichoderma* and *Pseudomonas* that can effectively control vascular wilt of pigeonpea but also promote the plant growth and induces resistance in plants.

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