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RESISTENCE ENZYMES IDENTIFICATION FOR THE MANAGEMENT OF WILT DISEASE IN GROUNDNUT

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Abstract

Application of biocontrol agents induced systemic resistance and activated the defense related enzymes viz., PAL, PO, PPO and accumulation of phenol in groundnut plants. Pretreatment with biocontrol agents, enhanced additional the activities of these enzymes upon challenge inoculation with F. oxysporum. The biocontrol agents increased the shoot and root lengths of groundnut in sand tray method.. Plant growth enhancement was more in Tv_1, Pf_1 and Bs_{10} combination followed by Tv_1 and Bs_{10}

Keywords: Groundnut- PAL- PO- PPO

Introduction

Groundnut (*Arachishypogaea*L.) is one of the most important oilseed crops in the world. In India, it is cultivated over an area of 5.31million hectares with the production of 6.93 million tonnes (Source: Directorate of Economics and Statistics, Department of Agriculture and Cooperation, Ministry of Agriculture, Govt. of India). A large number of diseases attack groundnut in India (Mayee and Datar 1988).

Management of the diseases through chemicals and the use of resistant varieties are possible to some extent. But the hazardous impact of agrochemicals on the environment, development of resistant mutants, escalating cost of pesticides and frequent breakdown of resistant varieties strongly demand a sustainable and an alternative management approach to disease.

Biocontrol is an important component of integrated disease management (IDM) that provides disease control while being relatively harmless to humans, non-polluting and bio-degradable, selective in mode of action, difficult for pathogens to develop resistance, unlikely to harm other beneficial microorganisms and generally improves soil health and sustainability of agriculture (Sheo Raj *et al.*, 2004).

Beneficial effects of PGPR that have been documented by many scientists, which include direct plant growth promotion, biological disease management and inducing host resistance (Kloepper, 1992). Knowledge on the molecular mechanisms of the PGPR enables the scientists, to bring the effective technique for managing the diseases. The introduction of

antimicrobial compounds *viz.*, phenazines and phloroglucinals do contribute significantly in the biological control of diseases.

Ecofriendly approach will be always better, it ensures the maximum suppression of the disease without any adverse impact on the ecosystem. All these requirements were considered in the following studies undertaken to develop a suitable ecofriendly management system against wilt of groundnut

Materials and Methods

Formulation of biocontrol agents

Bacteria

The isolate of *P. fluorescensviz.*, Pf_1 and the isolate of *B. subtilisviz.*, Bs_{10} that were found to be more effective *in vitro* were used to prepare talc based formulations. Four hundred ml of 72-h-old bacterial culture in their respective medium with a population of 9 x 10^8cfu/ml were mixed with 1 kg of talc containing 15 g of calcium carbonate and 10 g of CMC. Moisture content of the product was reduced to 20 per cent by shade drying and it was packed in polythene bags for further use (Vidhyasekaran and Muthamilan, 1995).

T.viride

The isolate of *T. virideviz.*, Tv1 was cultured in sterilized molasses yeast medium for 10 days. The fungal biomass and broth containing spore concentration of 1 x 10^7cfu/ml were mixed with talc at 1:2 ratio. The formulation was air dried and packed in polythene covers and used for further study (Jeyarajan*et al.*, 1994).

Induction of systemic resistance in groundnut by biocontrol agents

Groundnut seeds were treated with talc based formulations of effective biocontrol agents singly as., Tv1, Pf1, Bs10 or in combinations as Tv1with Pf1,Tv1with Bs10 andPf1 with Bs10. They were planted in pots containing sterile potting medium. After 30 days the biocontrol agents were applied to the soil and challenge inoculated with the pathogen after two days. The treatments also included soil application of biocontrol agents without challenge inoculation. Leaf samples were collected at 0, 3, 6, 9 and 12 days after pathogen inoculation to assay the change in activities of defense related enzymes *viz.*, PAL, PO, PPO and phenol.

Phenylalanine ammonialyase

One g of leaf sample was homogenized in 2 ml of ice cold 0.1 M sodium borate buffer, pH 7.0 and centrifuged at 10,000 rpm for 20 min at 4^{o}C. The supernatant was used to assay the enzyme activity. PAL activity was determined as the rate of conversion of L-phenylalanine to transcinnamic acid at 290 nm (Dickerson *et al.*, 1984). Sample extract of 0.4 ml was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 1 ml of 12 mM L-phenylalanine and incubated for 1 h at 30^{o}C. The reaction was initiated by the addition of L-phenylalanine and stopped with 0.5 ml of 2 N HCl. A blank was maintained by adding L-phenylalanine after the addition of 2 N HCl. The absorbance was read at 290 nm and the results were expressed as nmoltranscinnamic acid/min/g of fresh tissue.

Peroxidase

Activity of peroxidase was determined as detailed by Hammerschmidt*et al.* (1982). One g of leaf sample was homogenized in 1 ml of 0.1 M phosphate buffer pH 7.0 in a pre-cooled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 min. at 4^{o}C. The supernatant was used to assay activities of PO and PPO. 1.5 ml of 0.05 M pyrogallol and 0.1 ml of enzyme extract were taken and added to a cuvette. To initiate the reaction 0.5 ml of 1% H_2O_2 was added. The change in absorbance was recorded at 420 nm at 30 sec interval for three min from zero sec of

incubation at room temperature. The results were expressed as change in absorbance/min/g of fresh tissue.

Polyphenol oxidase

The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer pH 6.5 with 0.1 ml of enzyme extract. To this 0.2 ml of 0.01 M catechol was added to initiate the reaction. The change in absorbance was recorded at 495 nm and the results were expressed as change in absorbance/min/g of fresh tissue (Mayer *et al.*, 1965).

Phenol

Leaf samples were homogenized in 10 ml of 80 per cent methanol and agitated for 15 min. at 70^{o}C. To 1 ml of the extract five ml of distilled water and 250 µl of Folin-ciocalteau reagent (1 N) were added and incubated at 25^{o}C for three min. After that, 1 ml of 20% sodium carbonate was added and mixed well. Then the tubes were placed in boiling water for 1 min and cooled. The absorbance was read at 750 nm and catechol was used as the standard. The total phenol content was expressed in µg of catechol/g of fresh tissue (Zieslin and Benzaken, 1993.

Effect of formulated products on plant vigour

Seeds of groundnut were surface sterilized with 2% sodium hyphochlorite for 30 sec, rinsed in sterile distilled water and soaked overnight in sterile distilled water. The seeds were treated with formulated products and the seedling vigour index was assessed by the standard sand tray method (ISTA, 1993 or 1999). The vigour index was calculated using the formula of Abdul Baki and Anderson (1973).

Vigour index = seedling length x germination %

Results and Discussion

Induced systemic resistance

P.fluorescens could act as strong elicitor of plant defense reaction (M'piga*etal*.,1997). Induced resistance by *P. fluorescens* is associated with accumulation of pathogenesis related proteins (Viswanathan and Samiyappan, 1999). Application of fluorescent pseudomonads strengthen the cell wall structures resulting in restriction of pathogen invasion in plant tissue (Benhamou*et al*.,2000; Chen *et al*.,2000). Saravanan*et al*. (2004) reported that increased level of PO and PAL in roots treated with *P. fluorecens* against Fusarium wilt of banana. In our investigation enhanced activities of defense related enzymes were observed in groundnut plants in response to the application of biocontrol agents against *F. oxysporum* indicating the induction of systemic resistance.

Phenylalanine ammonia lyase

The present study revealed that all the biocontrol agents induced the plants to synthesize more of PAL. The maximum PAL activity was observed on sixth day in plants treated with Tv_1 and BS_{10} when challenged with the pathogen.The product of PAL activity in transcinnamic acid which is an immediate precursor for the biosynthesis of SA, a signal molecule in SAR (Klessing and Malany, 1994). PAL activity could be induced during plant-pathogen interactions. (Ramanathan*et al*., 2000; Bharathi*et al*., 2004; Kandan*et al*., 2002; Harish, 2005). Induction of PAL by florescent pseudomonads was reported in cucumber against *P.aphanidermatum*(Chen *et al*.,2000). When turmeric plants were sprayed with *P. fluorescens* , PAL activity got enhanced (Kavitha, 2004).

Table 1. Induction of phenylalanine ammonialyase in groundnut treated with biocontrol agents

SI. No	Treatments	Change in absorbance / min /g of fresh tissue				
		0 DAI	3DAI	6DAI	9DAI	12DAI
1	Tv_1	257^{cd}	259^{gh}	264^{gh}	253^{f}	225^{f}
2	Tv_1+ pathogen	259^{cd}	263^{cd}	272^{de}	255^{c}	253^{c}
3	Pf_1	252^{d}	254^{h}	258^{h}	241^{g}	214^{g}
4	Pf_1 + pathogen	253^{d}	285^{e}	301^{d}	271^{d}	225^{f}
5	Bs_{10}	263^{bc}	266^{fg}	272^{fg}	257^{f}	246^{e}
6	Bs_{10} + pathogen	264^{bc}	326^{bc}	333^{b}	301^{b}	289^{c}
7	Tv_1 +Pf_1	254^{d}	256^{h}	261^{h}	252^{f}	223^{f}
8	Tv_1 +Pf_1+pathogen	256^{cd}	297^{d}	316^{de}	294^{f}	260^{c}
9	Tv_1 +Bs_{10}	282^{a}	284^{e}	289^{e}	298^{bc}	280^{d}
10	Tv_1 + Bs_{10}+pathogen	284^{a}	344^{a}	345^{a}	338^{a}	330^{a}
11	Pf_1+ Bs_{10}	269^{b}	272^{f}	278^{f}	262^{d}	239^{e}
12	Pf_1+ Bs_{10}+ pathogen	271^{b}	334^{b}	311^{c}	300^{bc}	309^{b}
13	Healthy control	180.2^{e}	182.9^{i}	185^{i}	183^{i}	182^{i}
14	Control (Pathogen alone)	183.8^{e}	269^{f}	258^{h}	230^{h}	196^{h}

Means in columns followed by the same letter are not significantly different ($P< 0.05$) according to DMRT.

Table 2. Induction of phenols in groundnut treated with biocontrol agents

Sl. No.	Treatments	µg of catechol /g of fresh tissue				
		0 DAI	3DAI	6DAI	9DAI	12DAI
1	Tv_1	489^{e}	501^{i}	518^{gh}	492^{ef}	475^{ef}
2	Tv_1+ pathogen	501^{d}	661^{c}	690^{c}	679^{b}	619^{b}
3	Pf_1	522^{b}	524^{gh}	531^{gh}	484^{gf}	444^{i}
4	Pf_1 + pathogen	529^{ab}	587^{d}	618^{d}	517^{d}	509^{d}
5	Bs_{10}	478^{e}	482^{j}	484^{i}	468^{i}	460^{gh}
6	Bs_{10} + pathogen	484^{e}	528^{gh}	539^{f}	511^{c}	499^{d}
7	Tv_1 +Pf_1	508^{cd}	514^{hi}	520^{gh}	483^{fg}	452^{gh}
8	Tv_1 +Pf_1+pathogen	513^{c}	537^{fg}	558^{fg}	499^{e}	478^{de}
9	Tv_1 +Bs_{10}	532^{ab}	547^{ef}	581^{e}	482^{fg}	462^{fg}
10	Tv_1 + Bs_{10}+pathogen	541^{a}	699^{a}	729^{a}	697^{a}	601^{c}
11	Pf_1+ Bs_{10}	502^{cd}	504^{i}	508^{h}	475^{gh}	453^{gh}
12	Pf_1+ Bs_{10}+ pathogen	504^{cd}	678^{b}	711^{b}	669^{b}	638^{a}
13	Healthy control	450^{f}	451^{k}	456^{j}	426^{j}	431^{j}
14	Control (Pathogen alone)	458^{f}	479^{j}	515^{h}	470^{hi}	452^{hi}

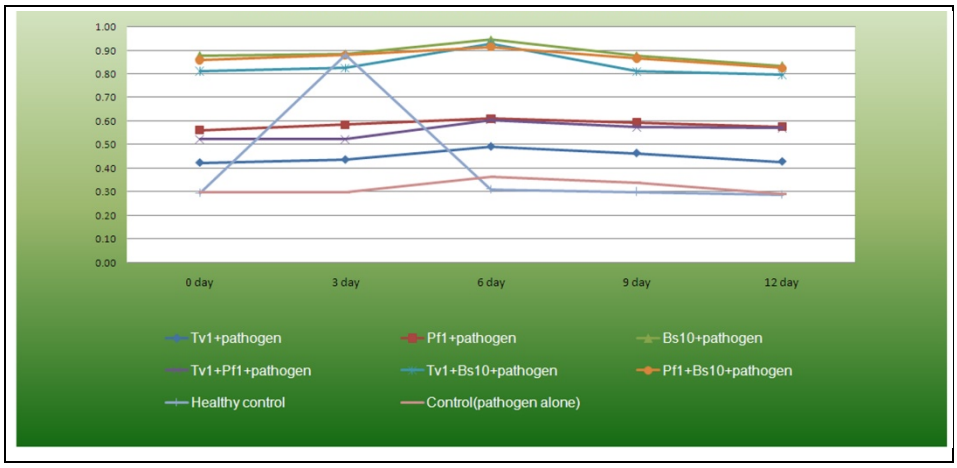
Means in columns followed by the same letter are not significantly different ($P< 0.05$) according to DMRT

Peroxidase

Peroxidase is a key enzyme in the biosynthesis of lignin (Bruce and West, 1989). Increased activity of PO has been elicited by fluorescent pseudomonads in rice sheath blight (Nandakumar*et al*., 2001; Radjacommare*et al*.,2002),blackgram root rot (Karthikeyanh*et al*., 2003) and groundnut late leaf spot (Meena*et al*.,2000) against plant pathogens.-In the present

study, groundnut plants treated with the combination of $Tv_1$and BS_{10} when challenged with the pathogen showed increased activity of PO.

Fig1: Induction of peroxidase in groundnut treated with biocontrol Agents



Accumulation of PO has been correlated with ISR in several crops (Ramamoorthy and Samiyappan, 2001). Isolates of *Pseudomonas* systemically induced resistance against Fusariumwilt of chickpea and suppressed the disease by 34.45 per cent when compared to control (Saikia*et al.*,2005). Mandal (2009) reported that exogenous application of SA could induce resistance against *F. o.* f. sp. *lycopersici* in tomato.

Table 3. Induction of peroxidase in groundnut treated with biocontrol agents

Sl. No.	Treatments	Change in absorbance / min /g of fresh tissue				
		0 DAI	3DAI	6DAI	9DAI	12DAI
1	Tv_1	0.419^{fg}	0.430^{f}	0.487^{ef}	0.455^{ef}	0.432^{g}
2	Tv_1+ pathogen	0.424^{f}	0.437^{f}	0.493^{e}	0.465^{e}	0.429^{g}
3	Pf_1	0.560^{d}	0.565^{de}	0.593^{d}	0.577^{cd}	0.552^{f}
4	Pf_1 + pathogen	0.562^{d}	0.586^{d}	0.611^{d}	0.595^{c}	0.557^{e}
5	Bs_{10}	0.813^{b}	0.849^{b}	0.914^{bc}	0.810^{b}	0.797^{d}
6	Bs_{10} + pathogen	0.859^{a}	0.883^{a}	0.929^{bc}	0.866^{a}	0.825^{c}
7	Tv_1 +Pf_1	0.514^{e}	0.543^{e}	0.599^{d}	0.568^{d}	0.553^{df}
8	Tv_1 +Pf_1+pathogen	0.525^{e}	0.549^{e}	0.606^{d}	$0.575c^{d}$	0.572^{ef}
9	Tv_1 +Bs_{10}	0.747^{c}	0.826^{c}	0.895^{c}	0.798^{b}	0.786^{b}
10	Tv_1 + Bs_{10}+pathogen	0.879^{a}	0.886^{a}	0.946^{a}	0.878^{a}	0.835^{bc}
11	Pf_1+ Bs_{10}	0.831^{b}	0.879^{a}	0.893^{c}	0.864^{a}	0.855^{ab}
12	Pf_1+ Bs_{10}+ pathogen	0.874^{a}	0.882^{a}	0.912^{bc}	0.866^{a}	0.871^{c}
13	Healthy control	0.296^{h}	0.301^{g}	0.312^{g}	0.302^{g}	0.290^{h}
14	Control (Pathogen alone)	0.298^{gh}	0.317^{fg}	0.365^{f}	0.338^{f}	0.292^{h}

Means in columns followed by the same letter are not significantly different ($P< 0.05$) according to DMRT.

Polyphenoloxidase

PPO is a copper containing enzyme which usually accumulates on wounding in plants. Induction of PPO activity has been correlated with a resistance response (Velazhahan and Vidhyasekaran,1994). Expression of new PPO isoform was observed in *P. fluorescens*

Pf_1 treated tomato plants challenged with *F. o.* f. sp. *lycopersici*(Ramamoorthy*et al*., 2002). Harish (2005) reported higher induction of PPO enzymes in plant growth promoting bacteria treated banana plants.

Fig2: Induction of Poly phenoloxidase in groundnut treated with biocontrol Agents

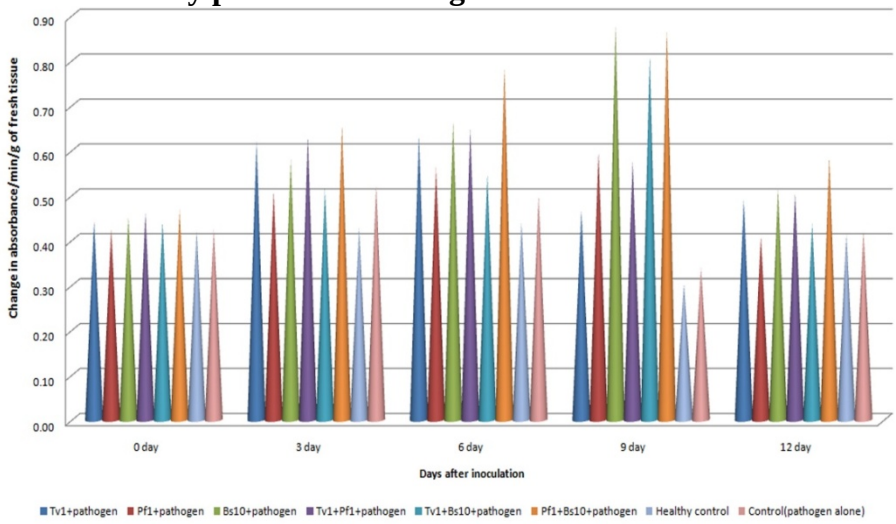


Fig.3.Induction of Phenols in groundnut treated with biocontrol agents

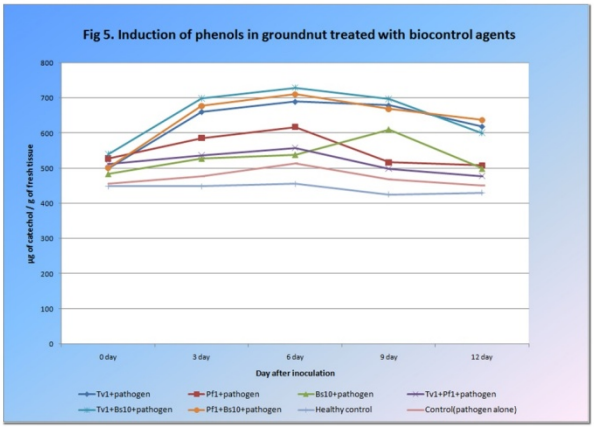
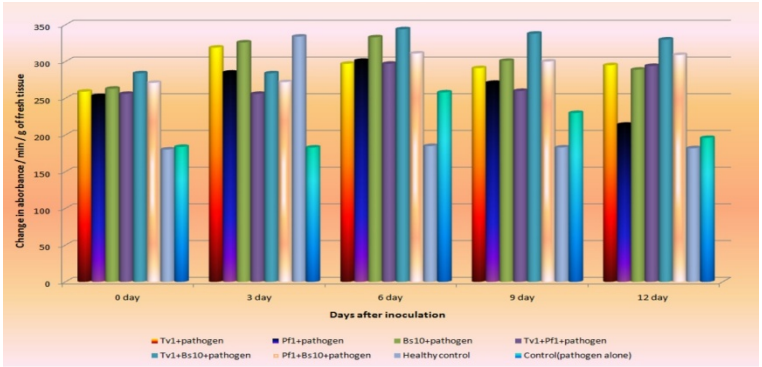


Fig 4. Induction of Phenyl alanine ammonia lyase in groundnut treated with biocontrol agents



Ramamorothy and Samiyappan (2001) observed accelerated PPO activity in chilli plants treated with *P. fluorescens*when challenge inoculated with *C. capsici*. Kavitha (2004) and Kamalakannan (2004) observed that application of *P. fluorescens* and its combination with *B. subtilis* significantly increased PPO against *P.aphanidermatum* and *M. phaseolina*respectively

Table 4. Induction of polyphenoloxidase in groundnut treated with biocontrol agents

Sl. No.	Treatments	Change in absorbance / minute /gram of fresh tissue				
		0 DAI	3DAI	6DAI	9DAI	12DAI
1	Tv_1	0.427^{ef}	0.431^{gh}	0.443^{f}	0.380^{k}	0.330^{h}
2	Tv_1+ pathogen	0.442^{cd}	0.619^{b}	0.633^{c}	0.524^{d}	0.489^{c}
3	Pf_1	0.420^{g}	0.424^{h}	0.436^{f}	0.376^{k}	0.339^{h}
4	Pf_1 + pathogen	0.423^{fg}	0.508^{d}	0.562^{d}	0.457^{f}	0.408^{ef}
5	Bs_{10}	0.448^{bc}	0.450^{f}	0.452^{f}	0.403^{ij}	0.377^{g}
6	Bs_{10} + pathogen	0.451^{bc}	0.580^{c}	0.664^{b}	0.633^{b}	0.513^{b}
7	Tv_1 +Pf_1	0.446^{bc}	0.443^{gh}	0.448^{f}	0.436^{gh}	0.400^{f}
8	Tv_1 +Pf_1+pathogen	0.459^{ab}	$0.630.^{b}$	0.647^{bc}	0.566^{c}	0.504^{b}
9	Tv_1 +Bs_{10}	0.436^{de}	0.444^{fg}	0.449^{f}	0.429^{hi}	0.399^{f}
10	Tv_1 + Bs_{10}+pathogen	0.468^{a}	0.654^{a}	0.783^{a}	0.671^{a}	0.584^{a}
11	Pf_1+ Bs_{10}	0.440^{cd}	0.475^{e}	0.484^{e}	0.461^{f}	0.428^{d}
12	Pf_1+ Bs_{10}+ pathogen	0.441^{cd}	0.513^{d}	0.546^{d}	0.483^{e}	0.437^{d}
13	Healthy control	0.422^{g}	0.427^{h}	0.439^{f}	0.415^{ij}	0.413^{ef}
14	Control (Pathogen alone)	0.424^{fg}	0.522^{d}	0.528^{e}	0.447^{fg}	0.419^{e}

Means in columns followed by the same letter are not significantly different ($P< 0.05$) according to DMRT.

Plant growth promotion

In the present investigation, application of biocontrol agents increased the shoot and root lengths of groundnut in sand tray method.. Plant growth enhancement was more in Tv_1, Pf_1 and Bs_{10}combination followed by Tv_1 and Bs_{10}. Similar results have been documented in many crops by earlier workers.

Table 5. Effect of antagonistson growth parameters of groundnut seedling

S.No	Treatments	Germination (%)	Shoot length (cm)	Root length (cm)	Vigour index
1	Tv_1	90.0	11.7	7.3	1710.0
2	Pf_1	90.6	11.0	7.5	1676.1
3	Bs_{10}	95.3	12.2	11.3	2239.6
4	Tv_1 + Pf_1	90.0	12.0	10.0	2205.0
5	Tv_1 + Bs_{10}	95.3	14.5	18.9	2944.8
6	Pf_1+ Bs_{10}	87.5	10.7	9.0	1723.8
7	Tv_1 + Pf_1+ Bs_{10}	95.3	18.5	21.9	3850.1
8	Control	85.6	9.5	6.0	1326.8
CD		5.21	0.74	0.78	140.04

Application of *B. subtilis* F2B24 increased the growth and yield in peanut (Backmann*et al*.,1994). Manjula and Podile (2001) reported that use of *B. subtilis* Af_1 promoted seed germination and biomass of groundnut and pigeonpea even at high pathogen pressure. Bharathi*et al.* (2004) reported that *P. fluorescens* (Pf_1) and *B. subtilis* increased the seed germination and seedling vigour of chillies. Similarly, promotion of plant growth by *P. fluorescens*, *Bacillus* spp. and *Trichoderma* spp. has been documented by various workers. (Chang *et al*.,1986; Schipper*set al*.,1987; Rabindran*et al*., 2005).

Table 6. Effect of antagonistson growth parameters of groundnut seedling

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5	$Tv_1 + Bs_{10}$	95.3	14.5	18.9	2944.8
6	$Pf_1 + Bs_{10}$	87.5	10.7	9.0	1723.8
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