4.1. Isolation of microorganisms from soil, screening and selection of antagonists

In order to isolate rhizosphere microorganisms, soil samples were collected from the rhizosphere of the healthy tea plants of different ages growing in different regions of the Terai and Dooars. The collected samples were brought to the laboratory and their pH was noted initially. In all cases, the soils were found to be acidic, ranging from 4.4-5.2. The microorganisms were isolated as described under materials and methods. The numbers of bacterial and fungal colonies were counted in the plates and the microbial population determined in soils from different rhizospheres. Microbial populations, determined as cfu g^{-1} soil, ranged between $12x10^6$- $50x10^8$ cfu g^{-1}. Results revealed that the maximum population was observed in the rhizosphere of 40 years old bushes of the Terai, and minimum in 10 years old bushes of NBU Experimental Garden. The rhizosphere of the very old as well as young tea bushes had much lesser microbial population. Population did not show any consistent difference with the variety. The maximum microbial population was obtained in the hot humid months, which decreased during winter. ((Tables 3 and 4; Plates III and IV)

Table 3: Microbial population and number of isolates from tea rhizosphere

Soil sample	Age (Years)	Microbial population (cfu / g soil)	No. of isolates Bacteria	Fungi
Hansqua Tea Estate, Terai	90	$14x10^7$	10	05
CoochBehar Tea Estate, Dooars	50	$16x10^7$	15	07
Kadommini Tea Estate, Dooars	70	$20x10^7$	18	06
Margaet's Hope Tea Estate, Darjeeling	75	$25x10^8$	12	05
Chandmoni Tea Estate, Terai	80	$10x10^8$	14	10
Tea Research station Nagrakata Doors	40	$15x10^8$	20	12
Tea Experimental Garden, NBU	10	$12x10^6$	10	08

The soil samples from each site were also tested for their pH. The soils were found to be acidic ranging from pH 4.2-5.2 (Table 4)

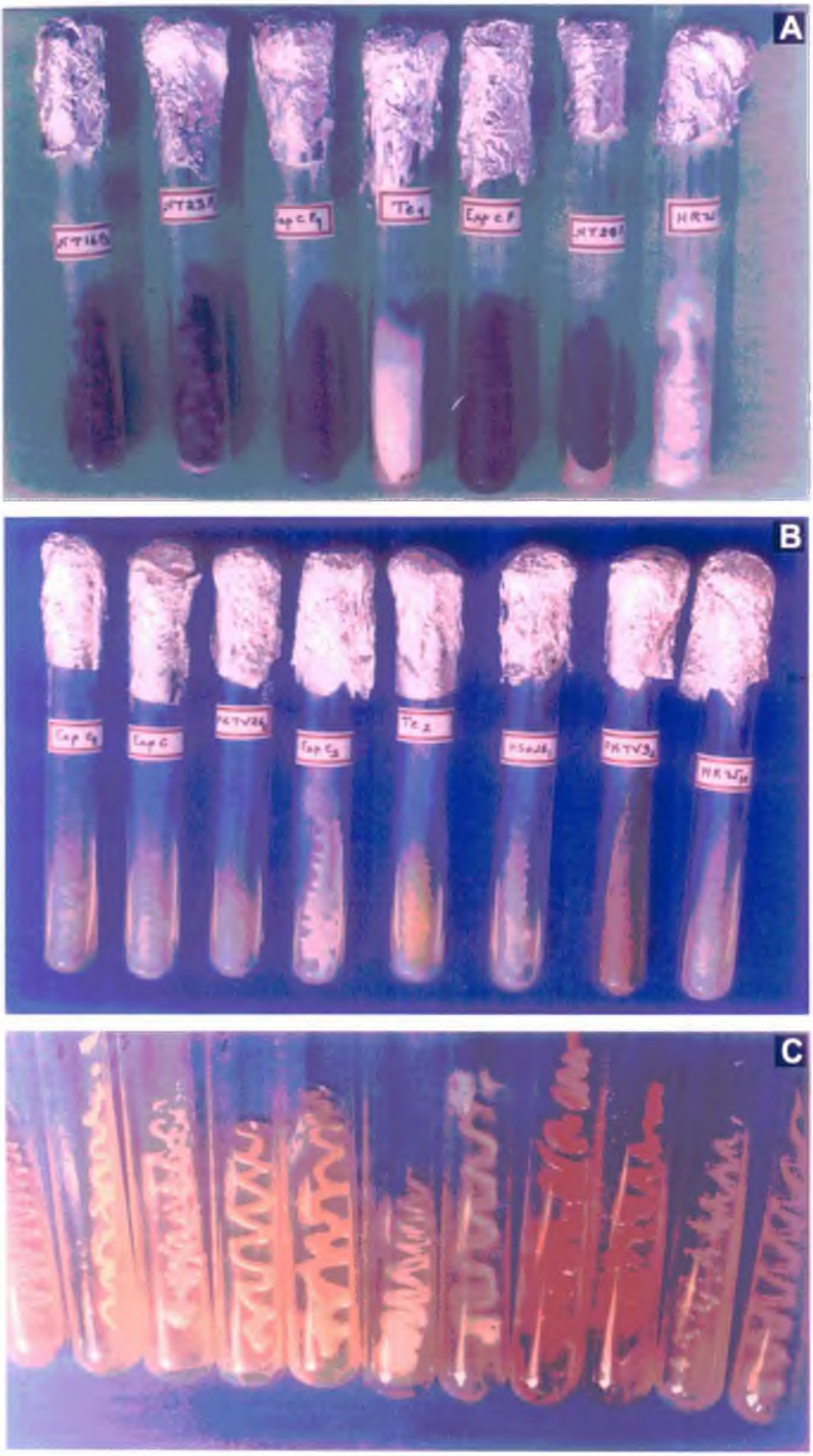


Plate III: Isolated soil microorganisms in test tube: (A) Fungi; (B & C) Bacteria.

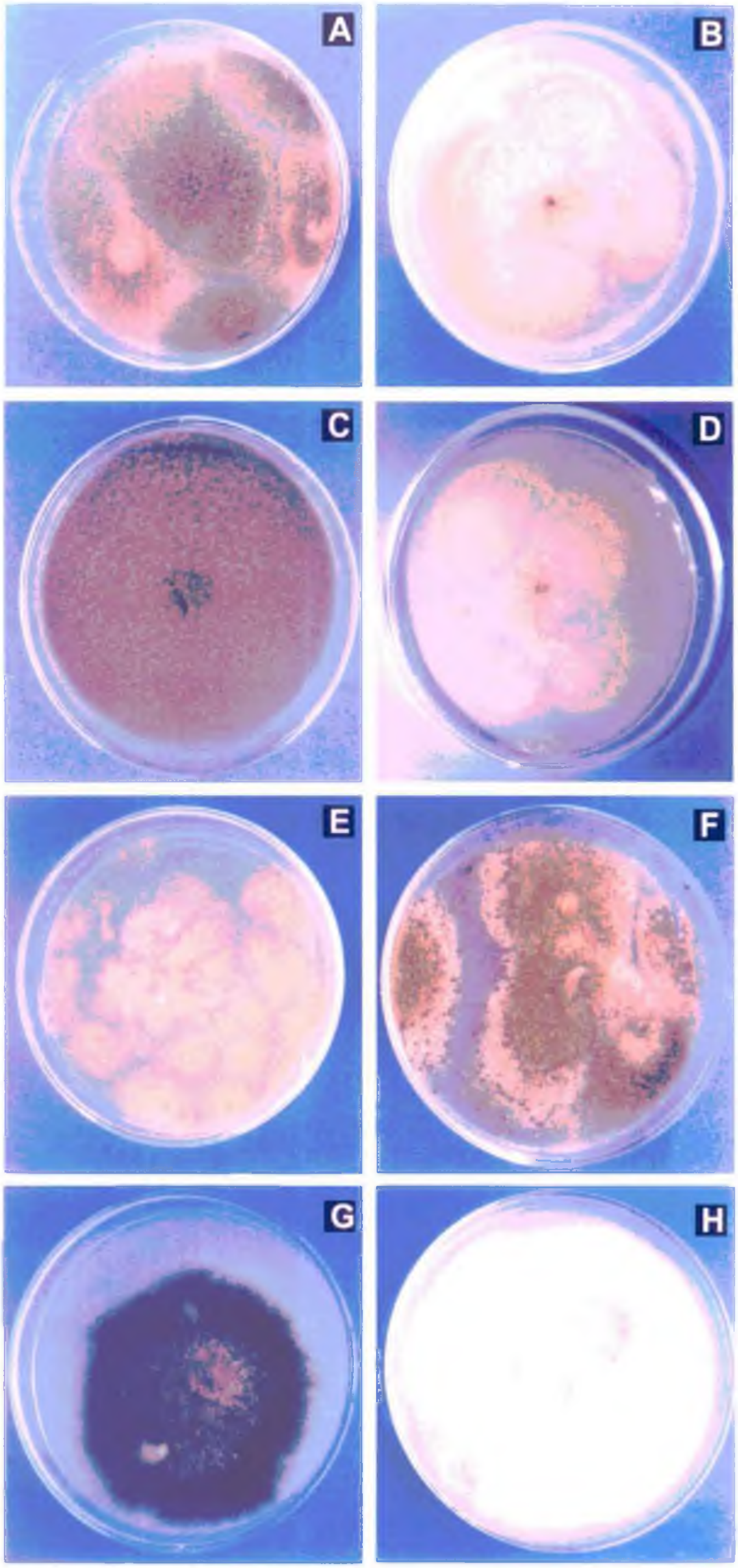


Plate IV(A-H): Fungal isolates obtained from tea rhizosphere grown on PDA.

Table 4: pH of the soil samples collected from different tea plantations

Name of the Garden	Soil pH	Tea variety
Hansqua Tea Estate, Terai	4.3	TV-9
CoochBehar Tea Estate, Dooars	4.8	TV-9, T-17
Kadommini Tea Estate, Dooars	4.9	TV-26
Margaet's Hope Tea Estate, Darjeeling	5.0	BSS-2
Chandmoni Tea Estate, Terai	5.2	TV-18
Nagrakata Tea Research station Dooars	4.9	TV-1,TV-9,TV-16,TV-23, Teenali17/1/54,Jorhat S_3A_1
Tea Experimiental Garden, NBU	4.9	TV-18, HV-39, UP-2,UP-3, UP 26, T-17, T-78,S- 449, K-1/1, CP-1,

The isolated bacteria were studied under microscope after suitable staining. Bacteria were classified into Gram positive and Gram negative and were taken for further biochemical tests. A large number of both Gram positive and negative bacteria were isolated.

All isolated bacteria were tested for their antagonistic activity against certain root pathogens-*Poria hypobrunnea*, *Fomes lamaoensis*, *Sphaerostilbe repens*, *Sclerotium rolfsii* and *Sclerotinia sclerotiorum in vitro* by dual pairing tests. Their interactions were categorized into the type–A: Homogenous; B: Overgrowth; C: Inhibition and D: Cessation at line of contact (Table 5)

Among all tested microorganisms, it was observed that five of the bacterial isolates viz. HRW_9, HRW_{10}, $HSoil_1$, $NT16_4$ and $KT26_2$ showed antagonistic activity against the soil borne pathogens.

Table 5: Interaction of soil isolates and test fungi in pairing test

(a) Bacterial isolates.

Serial No.	Source of Soil	Isolate No.	Antagonistic test performed with		
			Fomes lamaoensis	*Poria hypobrunnea*	*Sphaerostilbe repens*
1	Hansqua Tea Estate	H S1	C	C	C
2		H S 2	B	B	B
3		H S 3	B	B	B
4		H S 4	B	B	B
5		HRW 1	B	B	B
6		HRW 2	B	B	B
7		HRW 3	B	B	B
8		HRW 4	B	B	B
9		HRW 5	B	B	B
10		HRW 6	B	B	B
11		HRW 7	B	B	B
12		HRW 8	B	B	B
13		HRW 9	C	C	C
14		HRW 10	C	C	C
15	Nagrakata Tea Research Station. TV-16	NT16 1	B	B	B
16		NT16 2	B	B	B
17		NT16 3	-	Partial Inhibition	-
18		NT16 4	C	C	C
19		NT16 5	B	B	B
20		NT16 6	B	B	B
21		NT16 7	B	B	B
22		NT16 8	B	B	B
23		NT16 9	B	B	B
24		NT16 10	B	B	B
25		NT16 14	B	B	B
26	S_3A_1	NJ 1	B	B	B
27		NJ 2	B	B	B
28		NJ 3	B	B	B
29		NJ 4	B	B	B
30		NJ 5	B	B	B
31		NJ 6	B	B	B

Serial No.	Source of Soil	Isolate No.	Antagonistic test performed with		
			Fomes lamaoensis	*Poria hypobrunnea*	*Sphaerostilbe repens*
32	T-17/1/154	Te 1	B	B	B
33		Te 2	B	B	B
34		Te 3	-	-	-
35		Te 4	B	B	B
36		Te 5	B	B	B
37		Te 6	B	B	B
38	TV-1	NT1 1	B	B	B
39		NT1 2	B	B	B
40		NT1 3	B	B	B
41	TV-23	NT23 1	B	B	B
42		NT23 2	B	B	B
43		NT23 3	B	B	B
44	TV-9	NT9 1	B	B	B
45		NT9 2	B	B	B
46		NT9 3	B	B	B
47		NT9 4	B	B	B
48		NT9 5	B	B	B
49		NT9 6	B	B	B
50		NT9 7	B	B	B
51		NT9 8	-	-	-
52	TV-28	NT28 1	-	-	-
53		NT28 2	-	-	-
54		NT28 3	B	B	B
55	Chandmoni Tea estate TV-18	TR/Bc 1	B	B	B
56		TR/Bc 2	B	B	B
57		TR/Bc 3	B	B	B
58		TR/Bc 4	B	B	B
59		TR/Bc 5	B	B	B
60		TR/Bc 6	B	B	B
61		TR/Bc 7	B	B	B
62		TR/Bc 8	B	B	B
63		TR/Bc 9	B	B	B
64		TR/Bc 10	B	B	B

Serial No.	Source of Soil	Isolate No.	Antagonistic test performed with		
			Fomes lamaoensis	*Poria hypobrunnea*	*Sphaerostilbe repens*
65	NBU Experimental Garden	TR/B_p1	B	B	B
66		TR/B_p 2	B	B	B
67		TR/B_p 3	B	B	B
68		TR/B_p 4	B	B	B
69		TR/B_p 5	B	B	B
70		TR/B_p 6	B	B	B
71		TR/B_p 7	B	B	B
72		TR/B_p 8	B	B	B
73		TR/B_p 9	B	B	B
74		TR/B_p 10	B	B	B
75	Kadommini Tea Estate TV-26	KT26 1	B	B	B
76		KT26 2	C	C	C
77		KT26 3	B	B	B
78		KT26 4	B	B	B
79		KT26 5	B	B	B
80		KT26 6	B	B	B
81	CoochBehar Tea Estate TV-9	CT9 1	B	B	B
82		CT9 2	B	B	B
83		CT9 3	B	B	B
84	T-17	CT17 1	B	B	B
85		CT17 2	B	B	B
86		CT17 3	B	B	B

(b) Fungal isolates

Serial No.	Source	Isolate No.	Antagonistic test performed with		
			Fomes lamaoensis	*Poria hypobrunnea*	*Sphaerostilbe repens*
1	Nagrakata Tea Research Station. TV-16	NT16F 1	A	A	A
2		NT16F 2	B	B	B
3		NT16F 3	A	A	A
4		NT16F 4	B	B	B
5		NT16F 5	B	B	B
6	TV-9	NT9F1	A	A	A
7		NT9F 2	A	A	A
8		NT9F 3	B	B	B
9		NT9F 4	A	A	A
10		NT9F 5	B	B	B
11	Hansqua Tea Estate	HSF5	A	A	A
12		HRWF1	B	B	B
13		HRW F2	B	B	B
14		HRWF3	B	B	B
15		HRWF4	A	A	A
16		HRWF6	A	A	A

A: Homogenous; B: Overgrowth; C: Inhibition & D: Cessation at line of contact.

4.2. Characterization and identification of selected antagonists

Morphological and biochemical characteristics of the selected antagonistic bacteria were studied in the laboratory. (Table 6; Plate VI). Based on their morphology and biochemical tests, these bacteria were tentatively identified in the laboratory and their identity was confirmed at Diagnostic and Advisory Service, CABI Bioscience, U.K. These were identified as;

HRW9	*Ochrobactrum anthropi*	TRS-1
HRW10	*Serratia marcescens*	TRS-2
NT164	*Bacillus megaterium*	TRS-3
HSoil1	*Bacillus pumilus*	TRS-4
KT262	*Paenibacillus lentimorbus*	TRS-5

Among these antagonists, $HSoil_1$ and KT 26_2 i.e. *Bacillus pumilus* and *Paenibacillus lentimorbus* were selected for further investigations. (Plate-V)

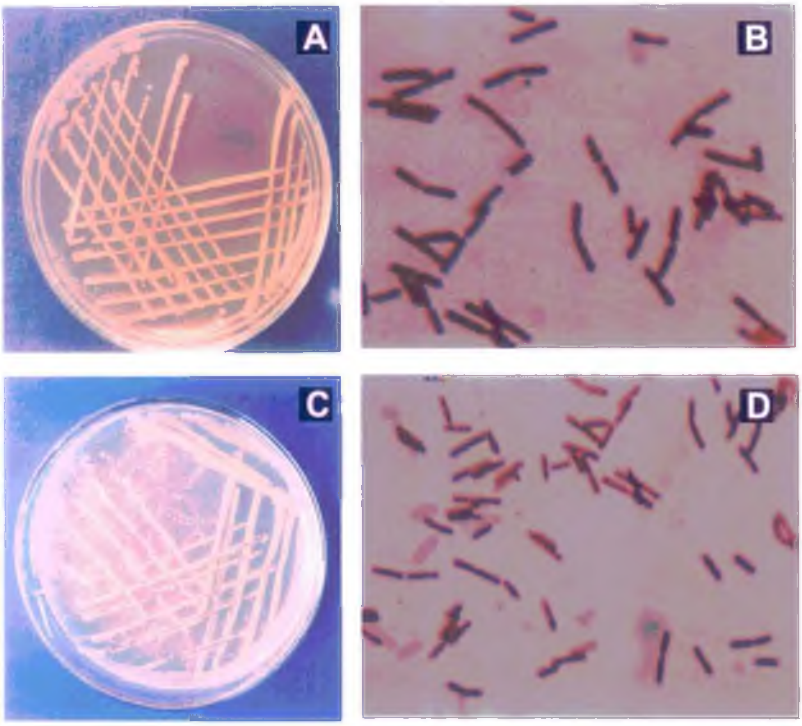


Plate V: Streaks of *B. pumilus* (A) and *P.lentimorbus* (C); *B. pumilus* (B) and *P. lentimorbus* (D) observed under microscope (x 100)

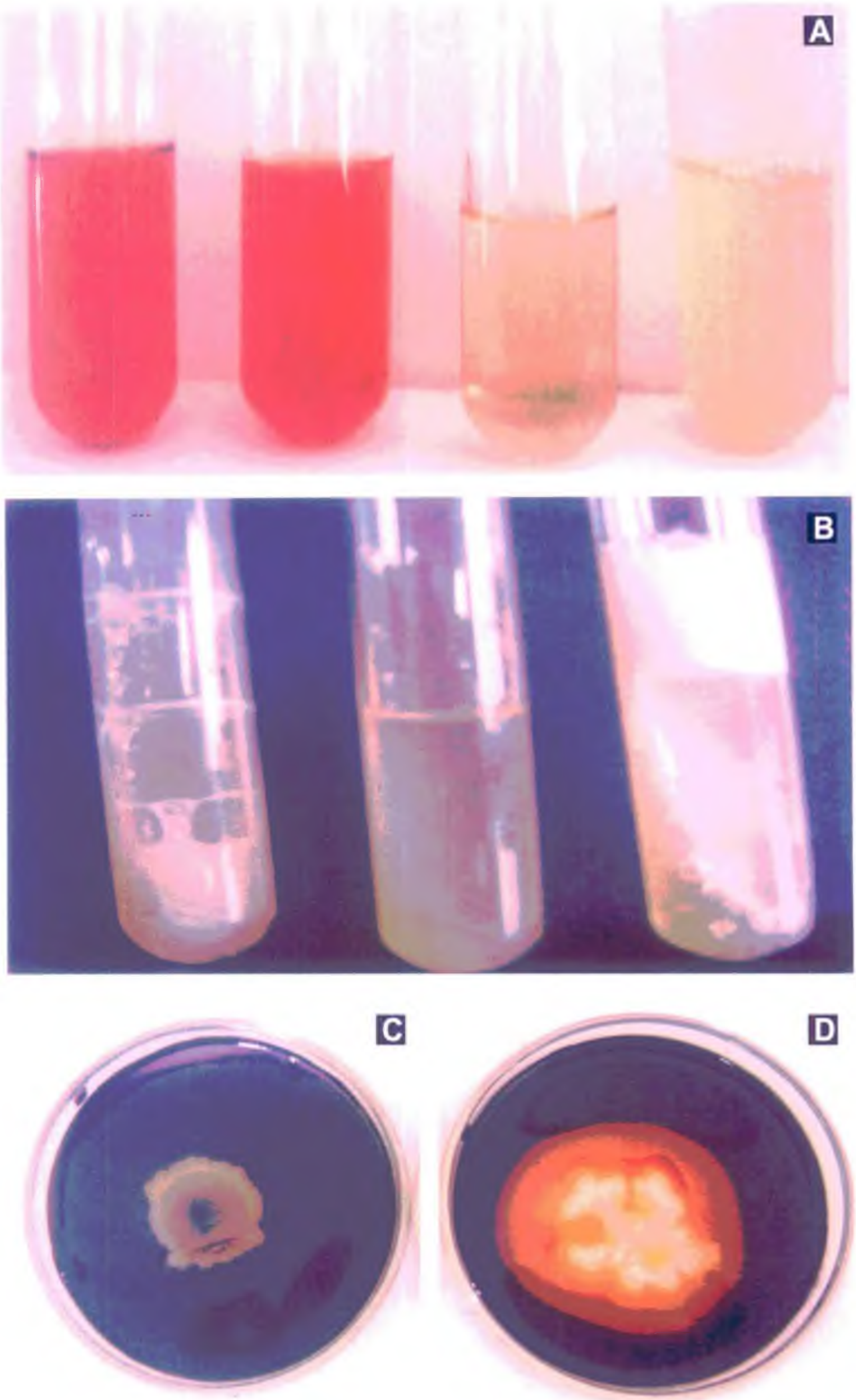


Plate VI (A-D): Biochemical tests of bacterial isolates (A) Carbohydrate digestion; (B) Catalase test; (C & D) Starch hydrolysis

Table 6: Morphological and biochemical characteristics of the selected rhizobacterial isolates.

Characteristics	*B.pumilus*	*P.lentimorbus*
Morphological		
Colony shape	circular	circular
Cell shape	Rod	Rod
Size (µm)	2-3 x 0.7-0.8	2-3 x 0.5-0.6
Capsule/Slime layer	-	-
Motility	+	+
Gram reaction	+	+
Endospore	+	+
Margin	+	+
Surface	wavy	wavy
Pigmentation	white	white
Fluorescein	_	_
Pyrocyanin	_	_
Density	opaque	opaque
Biochemical		
Citrate utilization	+	+
Urea digestion	-	-
V.P.reaction	-	-
Catalase production	+	+
Oxidase production	+	+
Casein hydrolysis	-	-
Starch hydrolysis	-	+
Phenol Red Tartarate	-	-
Nitrate reduction	-	+
Esculine hydrolysis	+	+
Indole production	-	-
H_2S production	+	+
NH_3 production	+	+
Gelatin liquefaction	+	+
Sugar utilization-		
Dextose	+/-	+/-
Lactose	+/ -	+/-
Sucrose	+/ -	+/ -
Glycerol	+/ -	+/ -

+ Positive reaction, - Negative reaction, +/- Weak reaction

4.3. Characterization and identification of fungal isolates

The isolated fungi were allowed to grow in petridishes containing sterile PDA medium for 7 days. Nature of mycelial growth, rate of growth and time of sporulation were observed. For identification, spore suspensions were prepared from individual culture. Drops of spore suspensions were placed on clean, grease free glass-slides, mounted with lacto phenol cotton blue, covered with cover slip and sealed. The slides were then observed under the microscope following which spore characteristics were determined and size of spore measured. On the basis of their morphological characters it was found that most of the fungal isolates belonged to the genera *Fusarium, Aspergillus, Curvularia, Penicillium, Alternaria* and *Colletotrichum* (Table 7).

Isolated fungi were also tested for antagonism against the test pathogens, but none of the tested fungal isolates showed antagonistic effect.

4.4. Cultural conditions affecting the growth of *Poria hypobrunnea*

Since further *in vivo* studies on biocontrol was undertaken against *P.hypobrunnea* causing root and stem rot of tea, initially growth conditions of this fungus was optimized. *In vitro* studies showed that variation in growth occurred depending on different factors like medium, pH, temperature and seasonal changes. The young mycelia were white or hyaline which turned gradually yellowish brown. The mycelial growth was generally submerged but sometimes superficial loose hyphal mat or rare fluffy growth was found depending on the medium. In liquid media, young white submerged mycelia grew at first slowly and then compact mycelia growth formed a plate like structure with horn-beak like edges and white hyphal growth extending a few centimeter on the wall of container. As the days passed, the white mycelial color changed to yellow brown.

4.4.1. Media

P.hypobrunnea was grown in seven different media i.e. Potato dextrose agar (PDA), Potato sucrose agar (PSA), Richard's agar (RA),Yeast extract dextrose agar (YDA) Czapek-Dox agar (CDA), Elliot's agar (EA),Carrot juice agar (CJA).Results revealed that the fungus grew in all media. Maximum growth was recorded in PSA and minimum in EA. (Table 8; Plate VII)

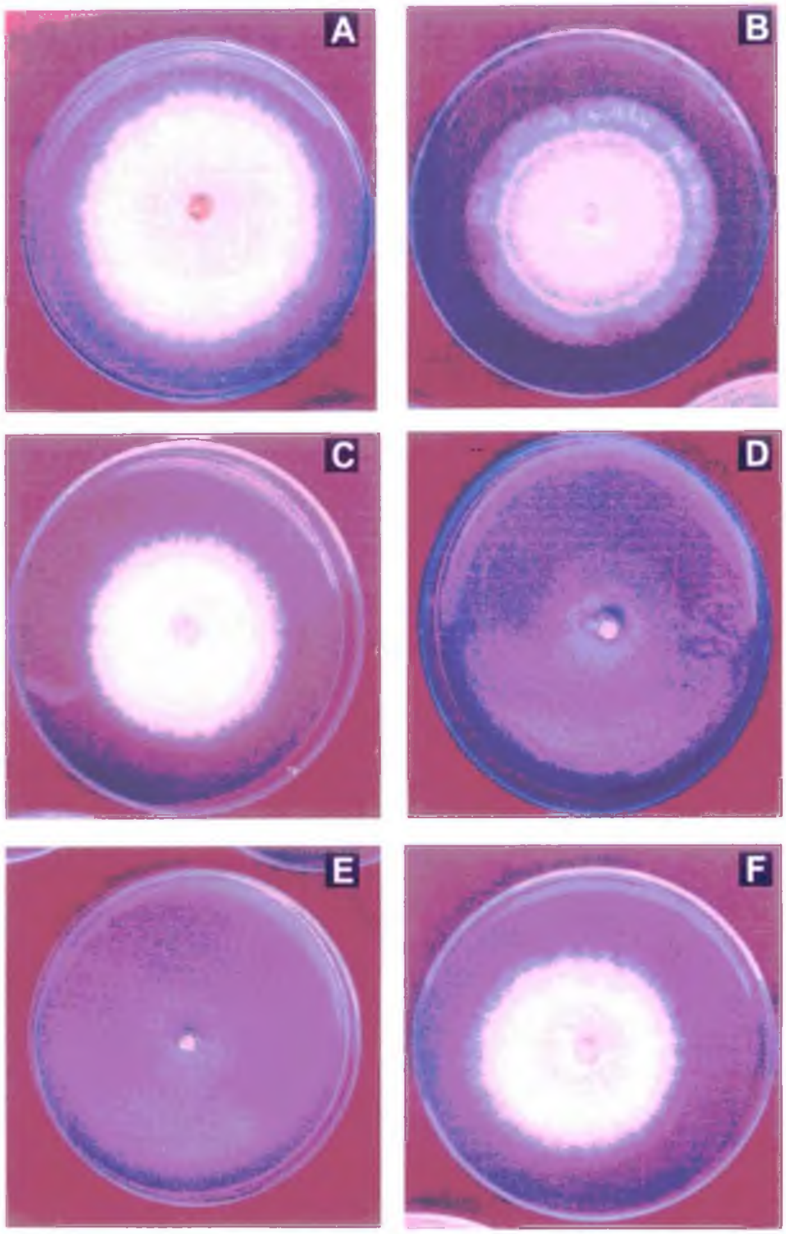


Plate VII (A-F): Growth of *P. hypobrunnea* in different media: (A) Potato sucrose agar; (B) Carrot agar; (C) Yeast dextrose agar; (D) Elliot's agar;(E) Richard's agar and (F) Potato dextrose agar

Table 7: Characterization of fungal isolates collected from tea rhizosphere

Code name	Morphology	Name of organisms
NT9F-1	Conidiophore simple, straight or curved, 1-3 septate, 5μm long, 3-6μm wide, golden brown. obclavate, pigmented conidia formed in long branching chains, ovoid, obpyriform with a short apical beak, smooth walled with 3-8 septa, base broadly round, each portion of lower part with 1-2 longitudinal septa, 18-63 x 7-18 μm.	*Alternaria alternata*
HRWF-1	Conidiophores simple, become geniculated by sympodial elongation, Conidia have borne singly, muriform, beaked, dark, 5-10 transverse septa, and 120-296 x 12-20 μm.	*Alternaria solani*
NT16F-1	Conidiophores hyaline, 0.4-1.0μm long and rough walled, roughened to echinucleate, on large conidiophores a layer of melulae supports the phialides. Conidial head radiating, globose to subglobose, finely roughened to echinulate, 1-3 nucleate 3.5-4.5μm.	*Aspergillus flavus*
NT9F-2	Pigmented conidiophores with clavate vesicles arise from clearly differentiated thick walled foot cells. Strictly columnar conidial heads, conidia globose to subglobose, echinulate, 2.5-3.0μm,	*Aspergillus fumigatus*
HRWF-3	Conidiophores arise from long broad thick walled, mostly brownish; sometimes branched foot cells about 1.5-3.0 mm. Large radiating heads, mostly globose, irregularly roughened 4.0-5.5μm.	*Aspergillus niger*
NT9F-3	Conidiophores Erect, pigmented, geniculated from sympodial elongations, 3-10 septate, / Conidia olive brown, curved, ellipsoidal, 3- septate, rounded at the apex, slightly acuminate at the base, the middle septum below the centre and the third cell strongly curved, 20-30 x 9-15 μm.	*Curvularia lunata*
NT16F-3	Pigmented, erect conidiophores/ conidia predominantly 4-septate, the central cell distinctly geniculated, tapering gradually towards each end, 18-37 x 8-14 μm	*Curvularia geniculatus*
NT9F-4	Simple, lateral phialides short, sparsely branched/ microconidia never in chain, mostly non-septate, ellipsoidal to cylindrical, 5-12 x 2.3-3.5 μm. Macro conidia fusiform, hyaline, smooth walled, moderately curved, and pointed at both ends basal cells pedicellate, 27-46 x 3.0-4.5 μm.	*Fusarium oxysporum*.

Code name	Morphology	Name of organisms
HRWF-6	Short, branched conidiophores 8-16 x 2-4 μm / microconidia abundant, chlamydospores borne singly, sometimes in pairs, in terminal, lateral, hyaline, smooth walled 6-10 μm.	*Fusarium solani*
TR/F1-1	Conidiophores 100-200 μm long in compact columns /globose to subglobose, smooth walled, sometimes finely roughened 3.0-3.5 μm.	*Penicillium frequentans.*
TR/F1-2	Conidiophores typically two-stage branched, phialides cylindrical, tip distinctly tapering. / Conidia form deep crusts which appear silky, strongly ellipsoidal, smooth-walled, 4.5-6.5 μm.	*Penicillium oxalicum*
TR/F1-3	Very dense ramification of the conidiophores, conspicuously roughened stripe / Subglobose, finely echinate, long 2.5-3.0 μm.	*Penicillium simplicissimu m*
TR/F1-4	Long conidiophores. Grayish green, conidia globose to subglobose, slightly roughened, mostly 3.5-4.0 μm.	*Penicillium sp.*
NT16F-4	Conidiophores are hyaline, short obpyriform to cylindrical producing aseptate, hyaline, oval, enteroblastic conidia.	*Macrophomin a phaseolina*
TR/F1-4	Sporangiophores pale to dark brown, straight, mostly 1.5 mm tall, 20-25 μm wide, each sporangiophores bear globose to subglobose multispored columellate sporangium, biconical to oval, ridged, mostly 4 nucleate 7-12 x 6-8.5 μm.	*Rhizopus stolonifer*
TR/F2-1	Conidia appearing scantily on solitary phialides but normally in orange sporodochia, basal stromatic cushion covered with dense layer of cylindrical, slightly tapering phialides upto 20μm long. Cylindrical with a roughed apex, slightly truncated base, hyaline, filled with granular cytoplasm forming orange-red shiny masses, 9-24 x 3-4.5μm.	*Colletotrichum gloeosporoides*
TR/F2-2	Conidiophores are hyaline to brown, septate, branched at the base, smooth, formed from the upper cells of the fructification. Conidia hyaline, unicellular, falcate or lunate or cylindrical, more or less guttulate, muticate or with the apex prolonged into a simple cellular appendage, produced from phialides.	*Colletotrichum* sp
NT16F-5	Oblong, sometimes two-celled, 4.0-8.5 x 2.0-3.0 μm.	*Phoma exigua*

Table 8: Effect of different media on mycelia growth of *P.hypobrunnea*

Media	Diameter of growth (cm)			
	Incubation period (days)			
	3	6	9	12
PDA	1.2±0.05	3.5±0.11	6.3±0.14	8.2±0.08
PSA	1.3±0.11	4.2±0.26	7.1±0.18	9.0±0.00
RA	0.7±0.05	2.4±0.05	3.8±0.11	5.4±0.05
YDA	1.1±0.08	3.3±0.09	5.5±0.26	7.9±0.05
CDA	0.9±0.26	2.6±0.11	4.8±0.29	6.0±0.13
EA	0.8±0.05	2.3±0.11	4.5±0.09	5.8±0.14
CJA	1.1±0.11	3.0±0.17	5.9±0.05	7.8±0.08

± Standard error; Temperature 30±1^{0}C;

4.4.2. Incubation period

P. hypobrunnea was grown in Richard's medium (RM) for a period of 25 days, mycelial growth was recorded after 3, 6, 9, 12, 15, 18, 21, 24 and 25 days of growth and incubated at 30±2^{0}C.Maximum growth was recorded after 15 days of incubation after which it declined. (Fig. 1)

4.4.3. pH

pH of the medium plays an important role in the growth of all microorganisms. To determine the effect of pH, buffer systems have to be used to stabilize the pH. Initially buffer solution with pH values ranging from 4.0 to 8.0 were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of 0.03M. The pH was finally adjusted usingN/10 HCL or N/10 NaOH in each case. Richard's medium and phosphate buffer were sterilized separately by autoclaving for 15 min. at 15 lbs. pressure. Equal parts of the buffer and medium were mixed before use in Laminar Flow Bench. After mixing flasks were inoculated and incubated for 15 days after which dry wt. was taken .Results revealed that growth of *P. hypobrunnea* was optimum in the range pH 4-7. Maximum growth was observed at pH 5.0-5.5 (Fig. 1)

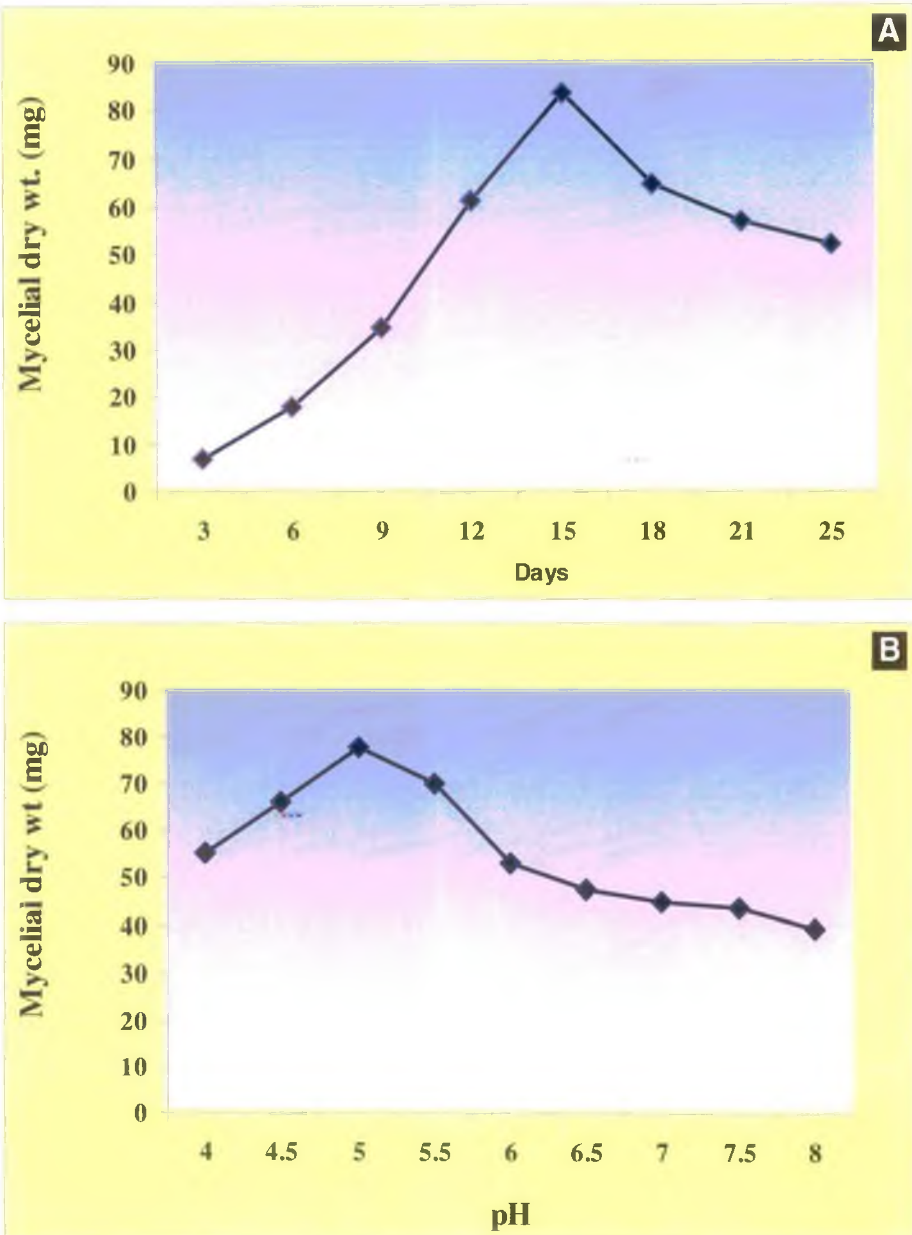


Fig. 1: Effect of incubation period (A) and pH (B) on mycelial growth of *P. hypobrunnea.*

4.4.4. Carbon source

Like the pH of the medium the growth of the fungus is greatly influenced by available nutrients. The availability of the fungi to grow in different media depends on the their capability to utilize the available nutrients, of which carbohydrates are the major ones .All carbohydrates are not utilized by the fungus in the same rate and so the growth rate varies with different carbon sources. In the present investigation, seven different carbon sources (fructose, dextrose, mannitol, maltose, sucrose, starch and lactose) were tested for their effect on the growth of *P.hypobrunnea*. These were added separately to the basal medium. Richard's medium without sugar was used as the basal medium which served as control set. Data was recorded after 15 days of incubation. Results given in table revealed maximum growth using fructose as the carbon source while minimum growth was observed in mannitol. (Table 9; Fig. 2)

Table 9: Effect of different carbon sources on mycelial growth of *P.hypobruunea*

Carbon sources	**Dry mycelial wt. (mg)**			
	Expt.1	**Expt.2**	**Expt.3**	**Mean**
Fructose	54.3	55.4	56.2	53.30±0.55
Dextrose	42.7	41.3	45.1	43.03±1.08
Mannitol	20.1	21.3	20.6	20.66±0.35
Sucrose	48.6	47.3	47.1	47.66±0.47
Starch	32.3	33.1	30.6	32.00±0.74
Maltose	30.1	27.5	28.3	28.63±0.77
Lactose	38.2	39.5	40.0	39.23±0.54
Control	5.0	7.0	5.0	5.66±0.66

± Standard error; Temperature 30±2^{0}C

4.4.5. Nitrogen source

The availability of nitrogen for growth of the organism depends to a great degree on the form in which it is supplied. Hence the most suitable nitrogen source for any particular microorganism can only be determined by testing a number of sources including both inorganic and organic. The effect of inorganic nitrogen sources (potassium nitrate, sodium nitrate, ammonium sulphate, calcium nitrate) as well as complex organic sources (urea, peptone, casein, yeast extract and beef extract) on the mycelial growth of *P. hypobrunnea* was tested. A basal medium without any nitrogen source was considered as control.

After 15 days of incubation data was recorded and result revealed maximum growth in ammonium sulphate followed by potassium nitrate in inorganic nitrogen source while maximum growth in peptone and minimum in casein among organic nitrogen sources. (Table 10; Fig. 2)

Table 10: Effect of different nitrogen sources on mycelial growth of *P. hypobrunnea*

Nitrogen source	**Dry weight of fungal mycelium (mg)**			
	Expt.1	**Expt.2**	**Expt.3**	**Mean**
Inorganic				
Potassium nitrate	20.4	25.7	24.3	23.46±1.59
Sodium nitrate	15.3	16.2	16.0	15.83±0.27
Ammonium sulphate	28.9	27.3	27.5	27.90±0.50
Calcium nitrate	12.9	15.6	14.5	14.33± 0.79
Organic				
Urea	-	-	-	-
Peptone	520.2	531.3	528.3	526.60±3.22
Casein	100.2	117.3	115.8	111.11±5.47
Yeast extract	420.3	426.4	427.3	424.67±2.20
Beef extract	342.6	355.6	348.3	348.83±3.77
Control	8.0	6.0	5.0	6.33±0.88

± Standard error; Temperature 30±2^{0}C; Incubation period 15 days

4.5. Cultural characteristic of bacterial antagonists

To determine the effect of different factors on growth of *B. pumilus* and *P. lentimorbus* both bacteria were grown in different pH, temperature and medium

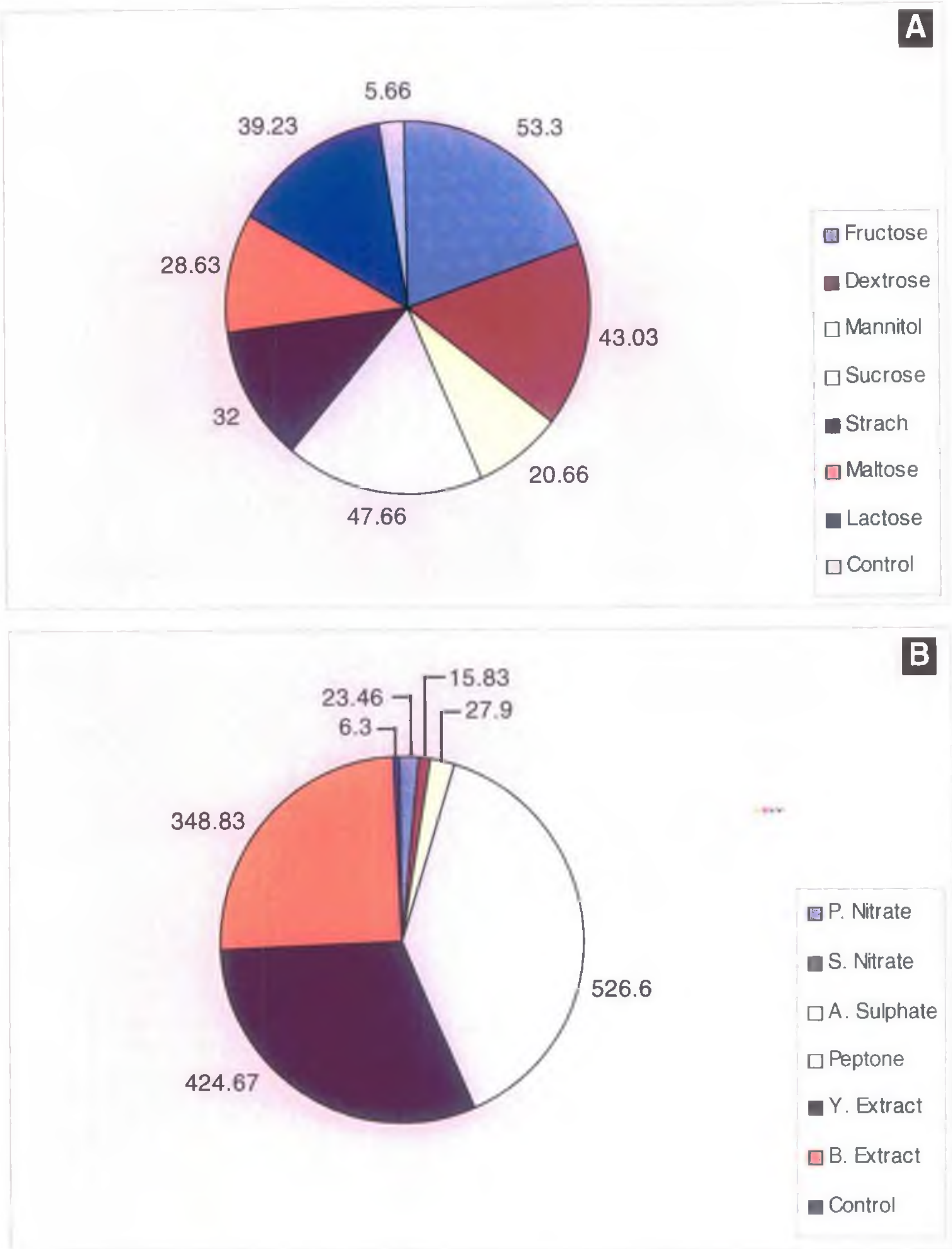


Fig. 2: Effect of Carbon sources (A) and Nitrogen sources (B) on mycelial growth of *P. hypobrunnea.*

Table11: Effect of antagonistic rhizobacterial isolates on mycelial growth of test pathogens.

Fungal Pathogen	*B. pumilus*	*P.lentimorbus*
	MDW* (g)	MDW*(g)
P.hypobrunnea	0.101	0.120
F.lamaoensis	0.125	0.140
S.repens	0.387	0.400
S.rolfsii	0.105	0.130
S.sclerotirum	0.130	0.150

*Average mycelial dry weight of three replicates; after 7 days of growth in PDB.

4.5.1. pH

B.pumilus and *P.lentimorbus* grew best at pH 6.0 and did not grow well below pH 4.0 and above pH 8.0. Results are given in Fig. 3

4.5.2. Temperature

The growth of *B.pumilus* and *P.lentimorbus* were observed at different temperatures ranging from 20 to 50^{0}C.NB medium was inoculated with the bacteria and the flasks were incubated at 20, 25, 30, 35, 40, 45 and 50^{0}C.Both the bacteria grew well within these ranges of temperature but grew best at 35^{0}C. (Fig. 3)

4.5.3. Media

Six different media (PDB, NB, KB, LB, NSB and GYP) were selected to assess the growth of the rhizobacteria and it was recorded that Nutrient Broth is the best medium for growth of *B.pumilus* and *P. lentimorbus*. (Table 12)

Table 12: Effect of media on the growth of *B.pumilus* and *P. lentimorbus*

Name of medium	Cfu/ml	
	*B.pumilus**	*P.lentimorbus**
PDA	3.31 x 10^7	1.81X10^6
NB	6.02X10^{13}	3.38X10^{12}
KB	1.90X10^{11}	2.60X10^9
LB	5.88X10^8	1.38X10^8
NSA	1.81X10^6	1.81X10^6
GYP	2.60X10^9	3.31X10^7

Average of three replicates; Incubation period-4 days

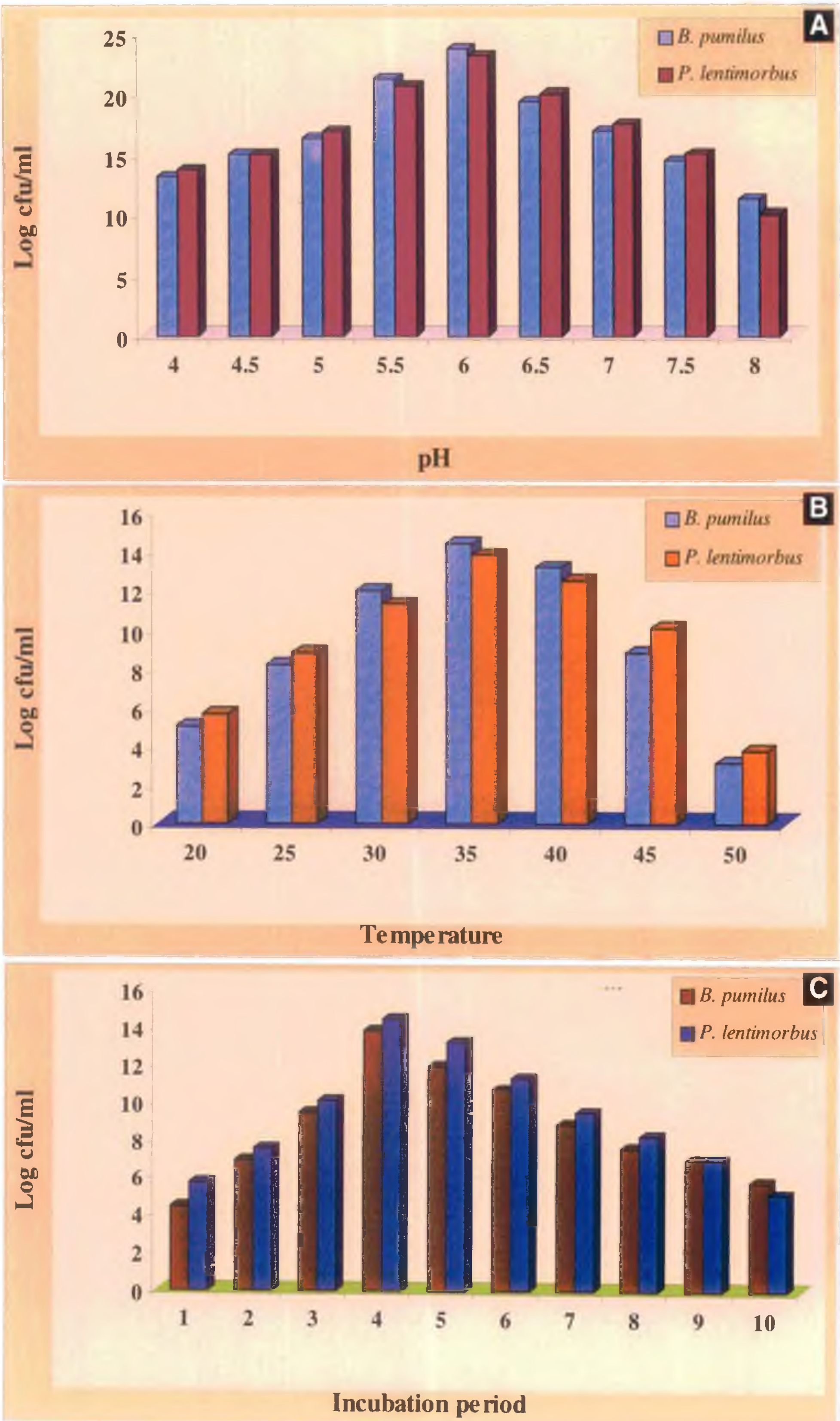


Fig. 3: Effect of pH (A), temperature (B) and incubation period (C) on growth of *B. pumilus* and *P. lentimorbus.*

4.5.4. Incubation period

B.pumilus and *P.lentimorbus* were grown in Nutrient Broth (NB) for a period of 10 days with growth being recorded after 1, 2, 3, 4, 5, 6, 7, 8, 9 and10 days of growth at temperature of 35±2^{0}C. Maximum growth was recorded after 4 days and then growth gradually decreased. (Fig. 3)

4.5.5. Antibiotic sensitivity

Antibiotic sensitivity test were performed to know the strength of rhizobacteria against particular antibiotic. Antibiotics were mixed at rate of 15µg/ml in Nutrient Agar medium; Results showed that *B.pumilus* and *P.lentimorbus* were highly sensitive to amoxicillin, streptomycin, gentamycin and norfloxacin. (Table 13)

Table13: Antibiotic sensitivity of the rhizobacterial isolates

Antibiotics	*B.pumilus*	*P.lentimorbus*
Amoxicillin	HS	HS
Penicillin	PS	PS
Chloramphenical	HS	HS
Streptomycin	HS	HS
Gentamycin	HS	HS
Norfloxacin	HS	HS
Tetracycline	HS	MS
Kanamycin	MS	MS

PS-Partially sensitive; HS-Highly sensitive; MS-Moderately sensitive

4.6. *In vitro* tests of antagonists against test fungi

Antagonistic activity of *B.pumilus* and *P.lentimorbus* were confirmed by dual culture method both in solid and liquid media.

4.6.1. Solid media

In case of solid medium tests, diameter of fungal growth alone or with antagonistic bacteria and zone of inhibition were noted. The results revealed that both the bacteria inhibited *F.lamaoensis* most, followed by *P.hypobrunnea*, *S.repens*, *S.sclerotiorum*, and *S. rolfsii* and percentage of inhibition was maximum in *P.hypobrunnea*. (Table 14, Plates VIII and IX)

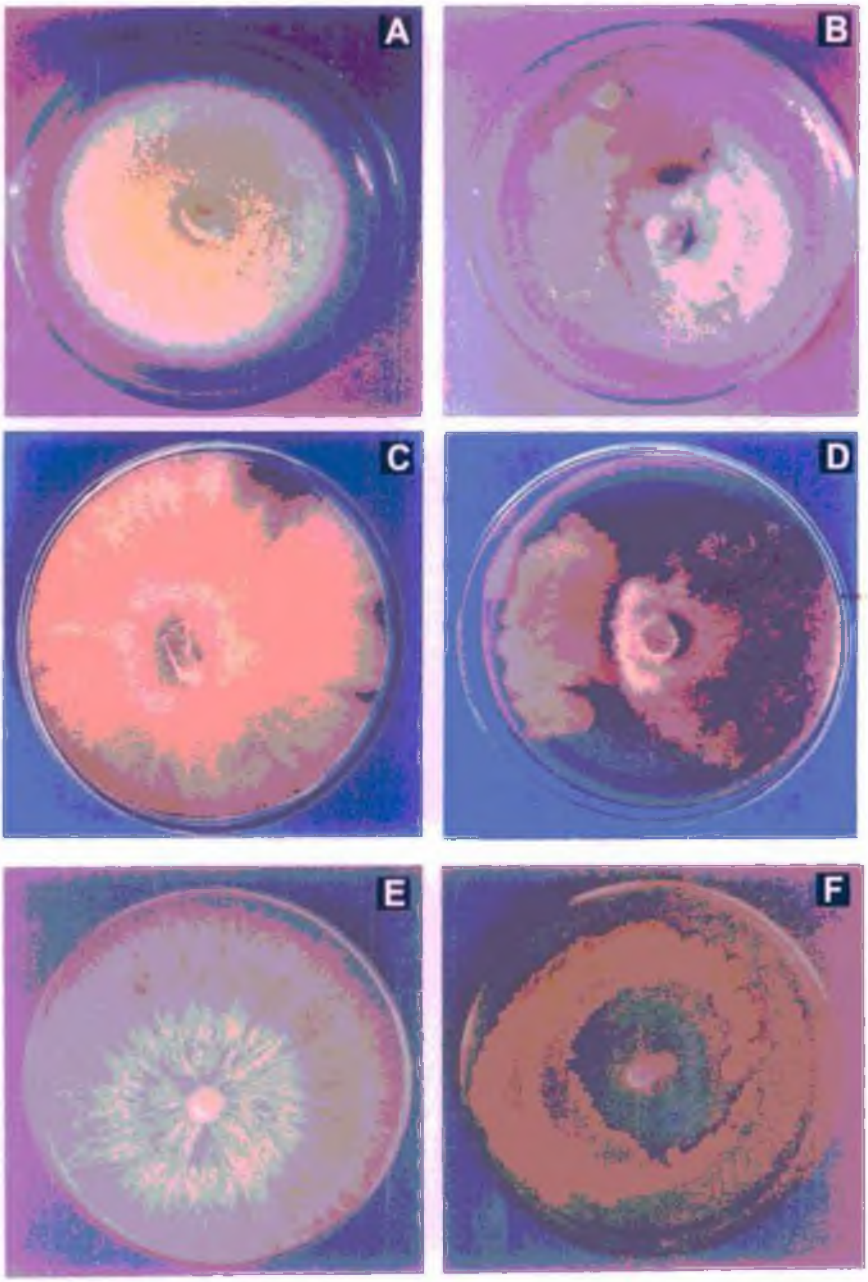


Plate VIII (A-F): Antagonistic activity of *B. pumilus* against *P. hypobrunnea* (B); *F. lamaoensis* (D); *S. rolfsii* (F); A,C and E are respective controls

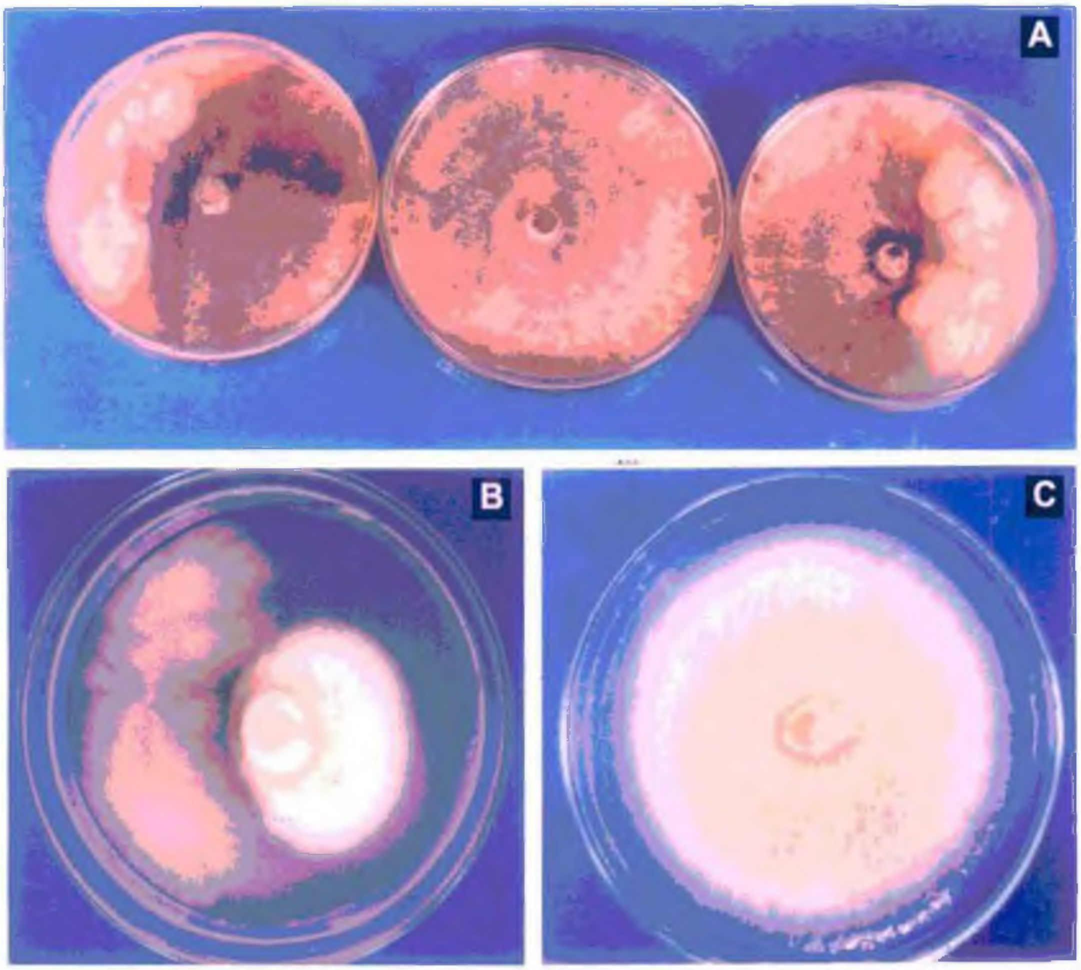


Plate IX: Pairing test of *P. lentimorbus* against (A) *F. lamaoensis* and (B) *P. hypobrunnea* (C) Control *P. hypobrunnea.*

Table 14: Effect of rhizobacterial isolates against the test pathogen in solid medium

Test pathogen	Dia. of inhibition Zone (cm)		% of inhibition	
	B.pumilus	*P.lentimorbus*	*B.pumilus*	*P.lentimorbus*
P.hypobrunnea	1.4 ± 0.03	1.1 ± 0.04	50.33	48.11
F.lamaoensis	1.6 ± 0.08	1.3 ± 0.05	43.67	42.44
S.repens	1.0 ± 0.01	0.9 ± 0.02	33.22	28.89
S.rolfsii	0.6 ± 0.05	0.3 ± 0.04	22.71	21.33
S.sclerotiorum	0.9 ± 0.06	0.8 ± 0.07	27.78	24.71

± Standard error; Average of three replicates; Temperature 30±2^{0}C; after 7 days of incubation

4.6.2. Liquid media

The bacteria were also tested for their inhibitory activity against the test fungi in liquid medium. After 7 days of growth, mycelia were harvested and dried and mycelial dry weigh was taken. Lowest mycelial dry weight was recorded in *P. hypobrunnea* and percentage of reduction was less in *S. repens* as compared to others (Table 12; Fig. 4).

4.7. Effect of application of rhizobacteria on the growth of plants

4.7.1. Chickpea and Mungbean

Before applying to perennial plants experiments were conducted on some annual plants i.e. chickpea and mungbean. Seeds of chickpea and mungbean were bacterized and sown. After that, germination percentage and vigour index of the plants were calculated. Both the bacterial isolates enhanced the germination percentage and vigour index markedly as compared to non-inoculated control seeds. In both seeds, *B.pumilus* was more effective than *P.lentimorbus* (Table 15; Plate X).

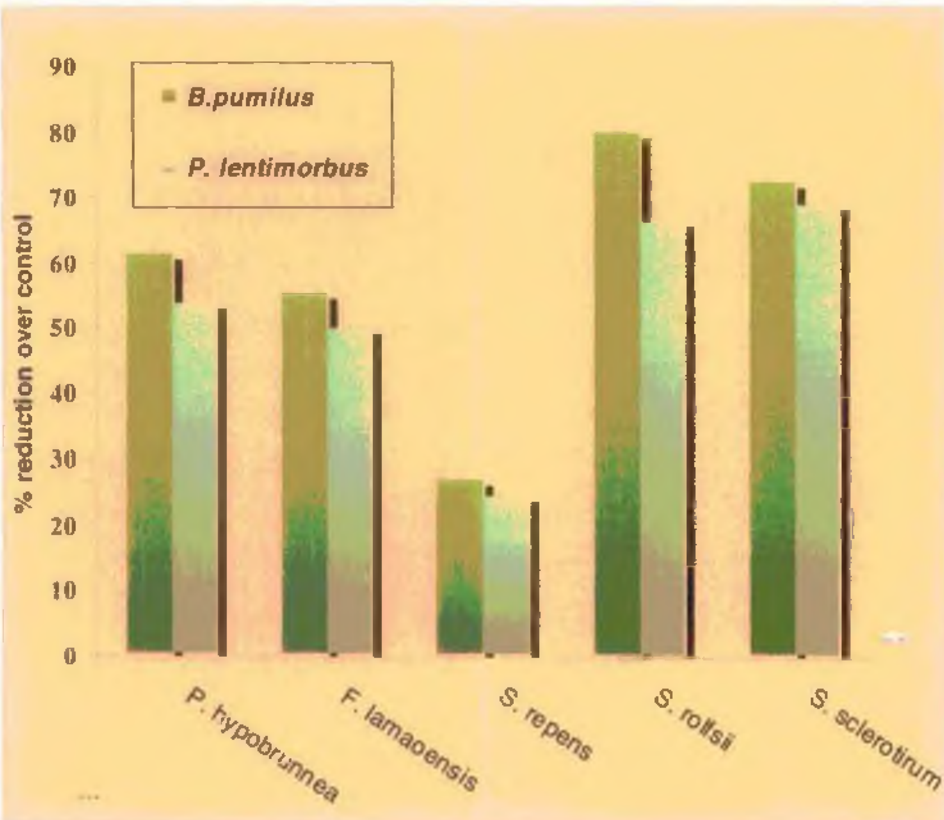


Fig. 4: Reduction of mycelial growth of *P. hypobrunnea* by antagonistic bacteria.

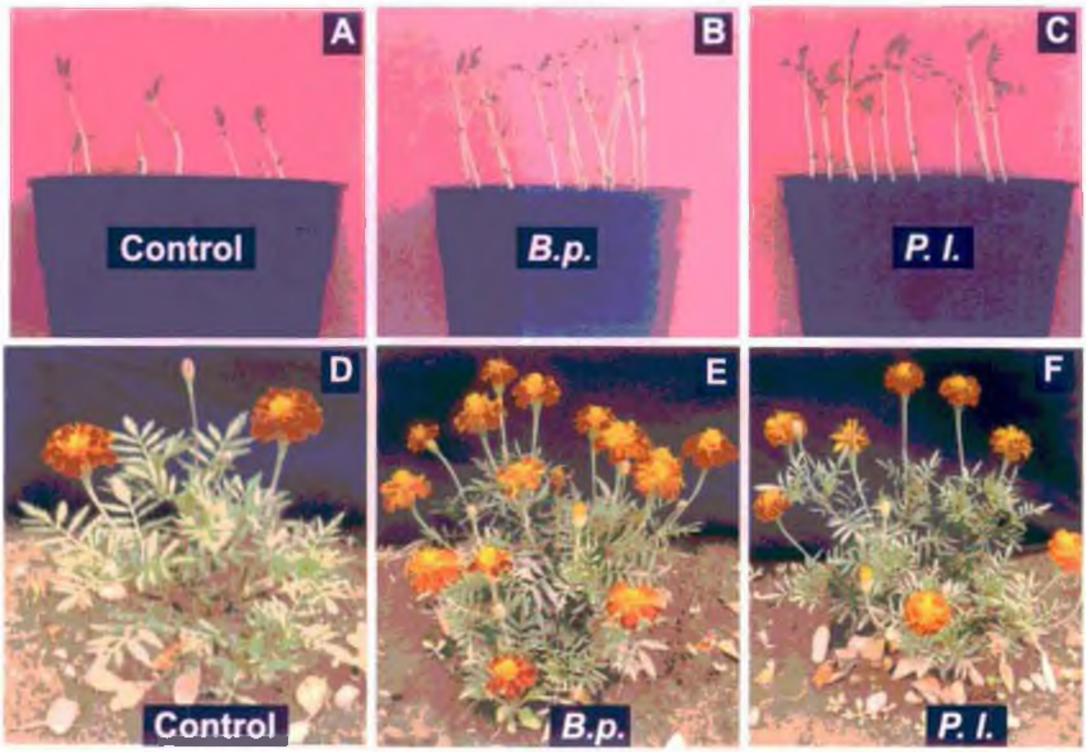


Plate X : Effect of *Bacillus pumilus* (B.p.) and *Paenibacillus lentimorbus* (P.l.) on chickpea (A-C) and marigold (D-F) plants.

Table15: Effect of seed bacterisation with rhizobacteria on vigour index of seedling.

Treatment	% germination	Mean root length+ Mean shoot length (cm)	Vigour Index
Cicer arientinum			
Control	80	4.2	409.2
B.pumilus	98	5.5	472.5
P.lentimorbus	93	5.2	433.3
Vigna radiata			
Control	84	6.3	629.9
B.pumilus	97	8.1	810.0
P.lentimorbus	96	7.9	750.2

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants.

4.7.2. Marigold

Besides chickpea and mungbean, effect of the PGPR on growth and flowering of marigold, a garden plant, was also tested. The bacterial suspensions of both the bacteria enhanced height and number of flower in marigold plants compared to untreated control (Table 16; Plate X)

Table 16: Effect of bacterial soil drench on Marigold

Treatment	After 30 days	
	% Increase in height (cm)	% Increase in flower no.
Control	40.5	79.3
B.pumilus	492.4	692.4
P.lentimorbus	468.7	574.7

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants.

4.7.3. Tea

4.7.3.I. Seedling

As the selected bacteria enhanced the growth promotion in annual plants these rhizobacteria were applied to perennial plant i.e. in tea seedlings. The growth promotion of different varieties of tea seedling was observed in terms of increase in height of seedlings, number of shoots and number of leaves. It was observed that treatment with both the bacteria increased the rate of growth of seedling in relation to untreated control. Percentage increases in height of plants as well as number of leaves after two and four months of application of bacteria to the soil were calculated. The results showed that both *B.pumilus* and *P.lentimorbus* efficiently promoted growth in tea plants irrespective of their variety. (Tables 17 and 18; Plate XI; Fig. 5)

Table17: Effect of rhizobacteria on growth of tea seedlings

Tea varieties	Treatment	2 months after treatment			
		Height of seedling (cm)		No. of Leaves of seedling	
		Initial	Final	Initial	Final
CP-1	Control	12.7±0.7	15.5±1.2	7±0.6	9±0.5
	B. pumilus	12.7±0.9	20.8±1.5	8±0.5	18±1.2
	P.lentimorbus	12.7±0.6	19.6±1.4	8±0.5	15±1.2
TV-20	Control	14.0±1.2	15.2±1.3	8±0.4	12±0.9
	B. pumilus	14.0±1.1	23.1±2.1	8±0.4	14±1.1
	P.lentimorbus	14.0±1.4	22.4±2.0	9±0.6	15±1.3
T-17	Control	12.7±1.0	14.0±0.9	7±0.5	8±0.6
	B.pumilus	12.7±0.9	19.1±1.1	7±0.3	12±0.8
	P.lentimorbus	12.7±1.0	19.8±1.0	7±0.7	13±1.2
K-1/1	Control	15.2±1.7	17.0±1.4	8±0.6	10±0.8
	B. pumilus	15.2±1.6	19.8±1.6	8±0.4	13±1.1
	P.lentimorbus	15.2±1.1	19.3±1.9	9±0.6	13±1.0
UP-3	Control	14.0±1.0	15.5±1.6	8±0.5	10±0.9
	B.pumilus	14.0±0.7	21.1±2.7	7±0.5	11±1.1
	P.lentimorbus	14.0±0.9	20.1±2.5	8±0.6	12±1.0

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded 2 months following the bacterial inoculation; ± S.E.

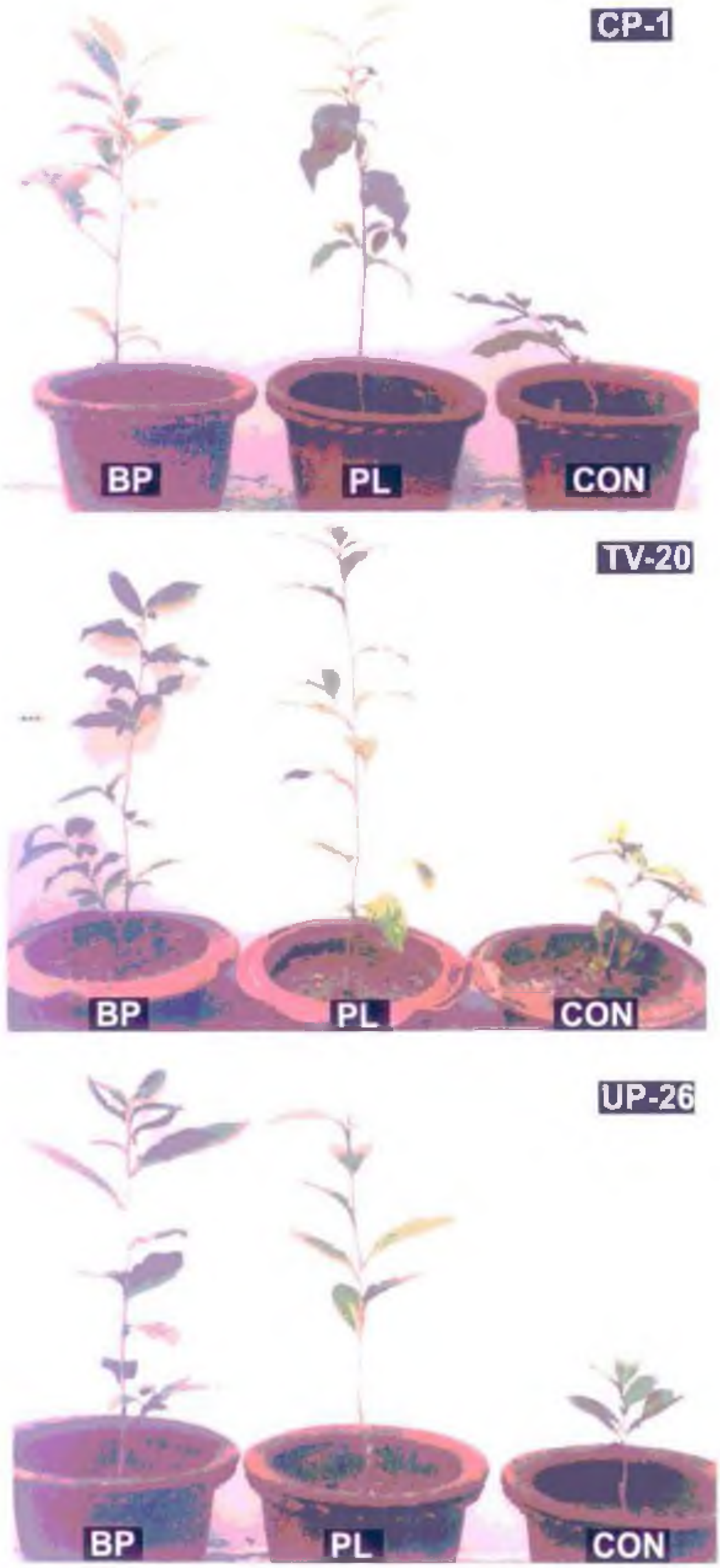
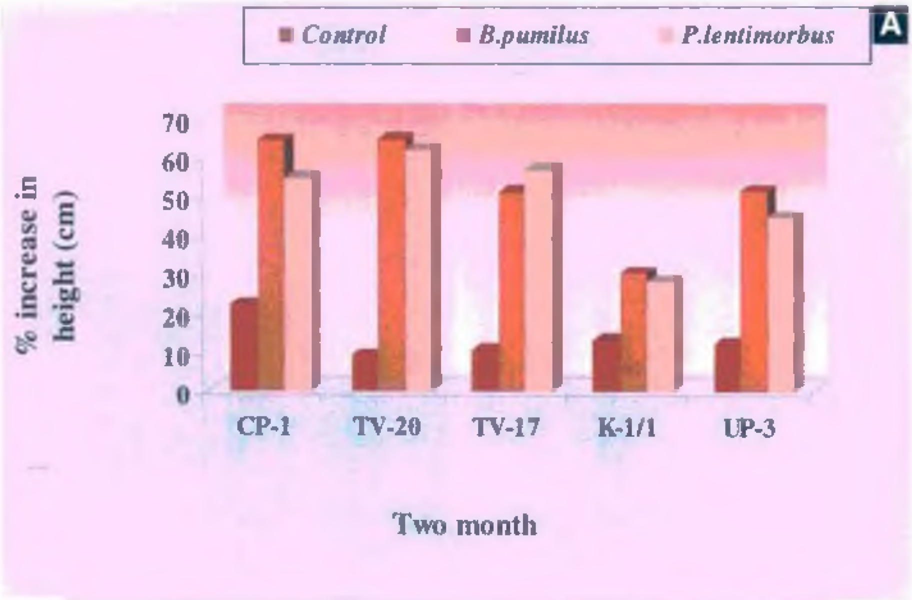


Plate XI: Growth promotion of different varieties of tea seedling following inoculation with *B. pumilus* (BP) and *P. hypobrunnea* (PL).



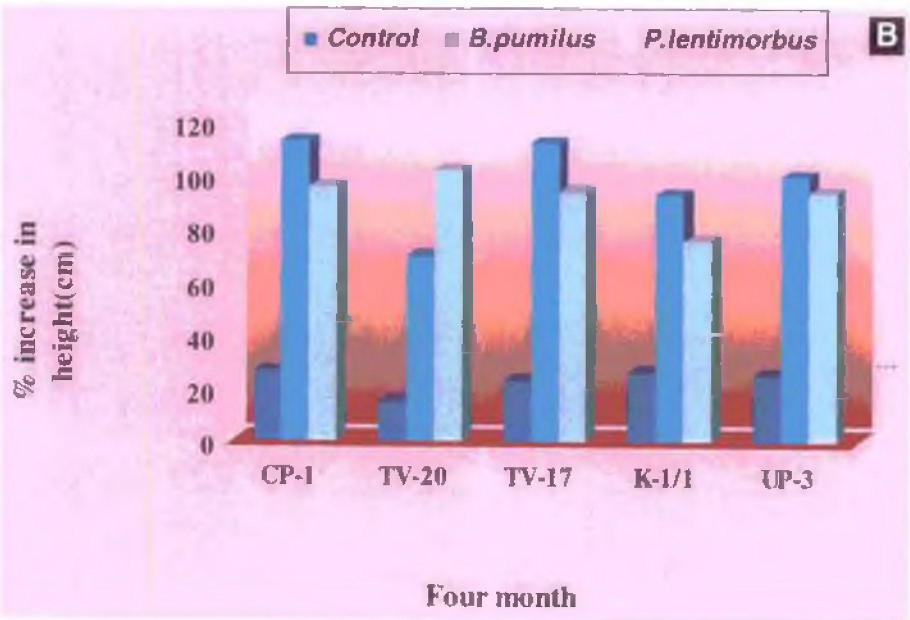


Fig. 5: Effect of PGPRs on growth of tea seedlings of different varieties.

Table 18: Effect of rhizobacteria on leaf development of tea seedlings

Tea Variety	Treatment	% Increase in no. of leaves	
		2 months*	4 months*
CP 1	Control	28.6± 1.71	75.0± 1.95
	B.pumilus	125.0± 1.75	175.0±1.20
	P.lentimorbus	87.5± 2.11	167.0±1.00
TV-20	Control	50.0±1.92	51.0± 2.05
	B.pumilus	75.0±1.82	133.3±1.65
	P.lentimorbus	66.6± 2.11	123.0±1.65
T-17	Control	14.3 ±1.46	52.0± 1.73
	B.pumilus	65.5±0.78	130.3 ± 1.43
	P.lentimorbus	84.6 ±3.22	142.0± 4.20
K-1/1	Control	25.0± 1.73	60.5± 2.02
	B.pumilus	62.5 ± 2.31	168.4± 4.56
	P.lentimorbus	44.4 ± 6.01	178.5 ±1.00
UP-26	Control	25.0± 2.31	60.0± 2.31
	B.pumilus	57.1± 2.31	165.7± 3.32
	P.lentimorbus	50.0 ±1.64	128.4±1.20

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants; ± S.E; Results were recorded 2 and 4 months following the bacterial inoculation.

4.7.3.2. Two years old plants

Bacterial suspensions of *B.pumilus* and *P.lentimorbus* were also applied to the rhizosphere of two years old potted plants at a regular interval of 20-25 days under same environment and physical conditions. Growth promotion of different varieties by individual bacteria was noted as compared to untreated control. The results showed that both *B. pumilus* and *P. lentimorbus* significantly increased height, lateral branches and leaves in all tested varieties. (Table 19; Plate XII; Fig. 6)

4.7.3.3. Tea bushes

In order to further confirm the ability of the bacteria to promote growth, aqueous suspensions of these bacteria were made and sprayed on the tea bushes after pruning. It was observed that spraying with the suspension led to significantly better growth of the shoot. More luxuriant growth was obtained. It was also observed that certain infections which normally occur in natural conditions were also not present following spraying with the bacterial suspension (Plate XIII).

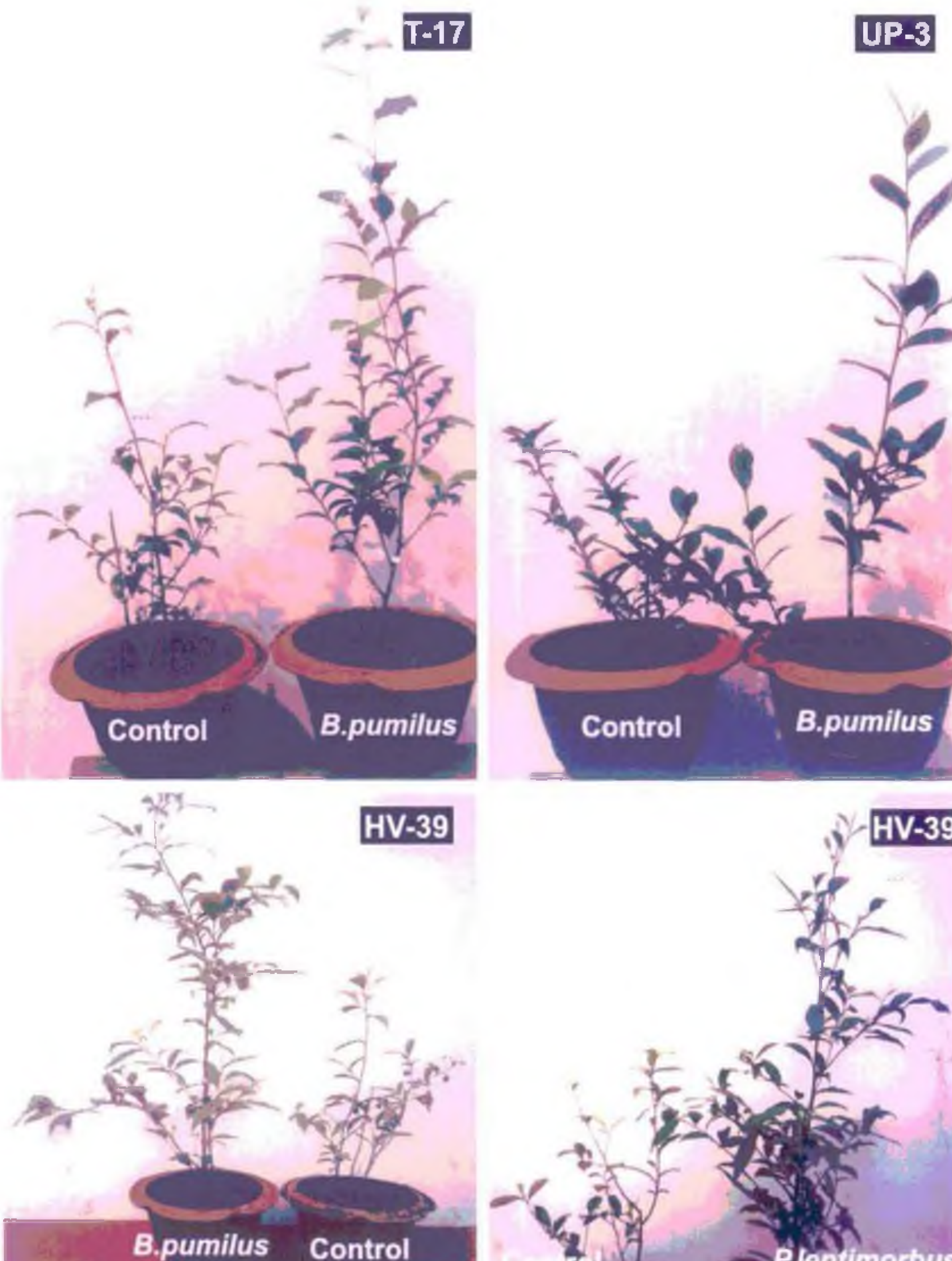


Plate XII: Effect PGPRs on growth of two year old potted tea plants of different varieties.

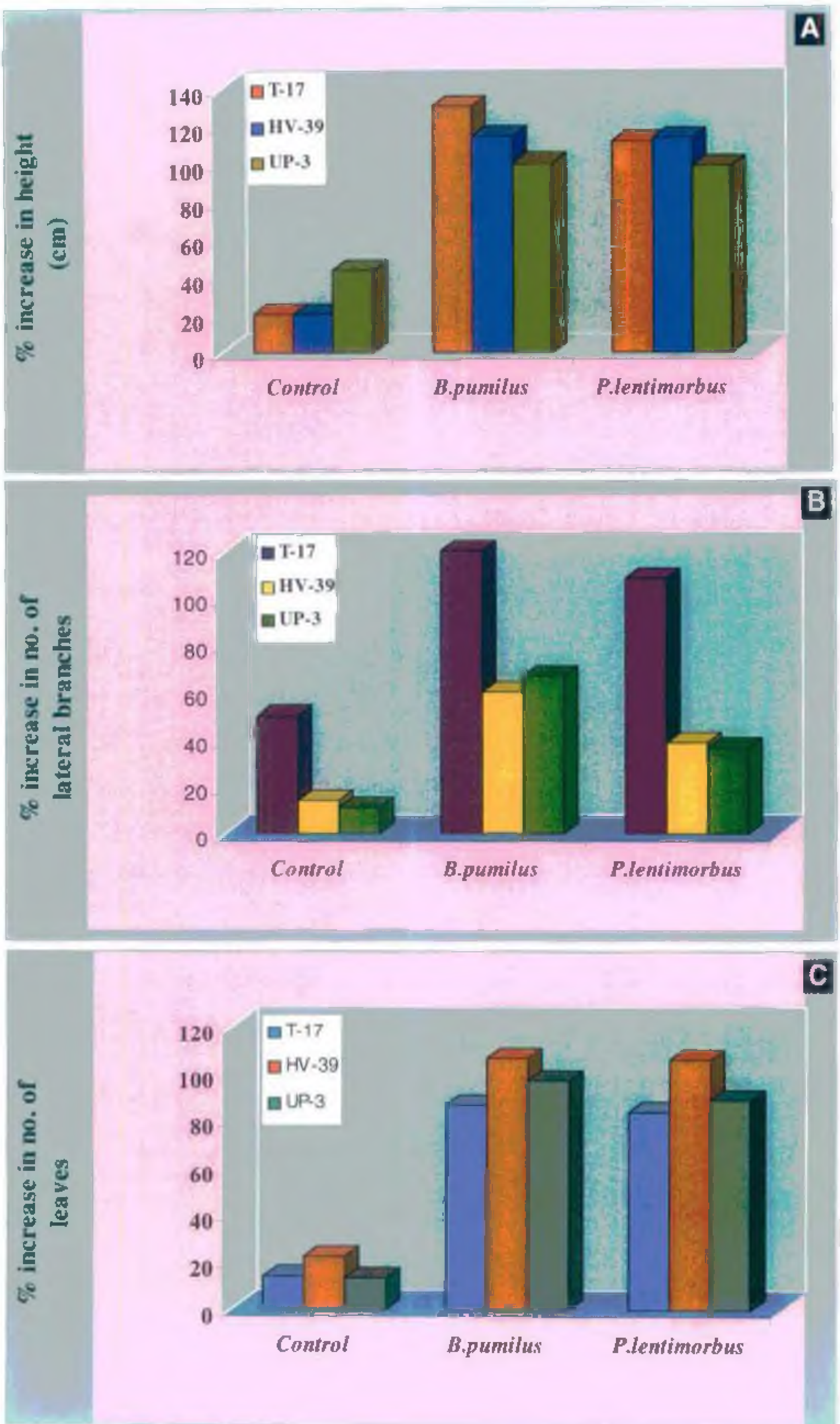


Fig. 6: Effect of PGPRs on two year old tea plants of three varieties (2 months after application).

Plate XIII: Foliar application of *B.pumilus* on potted plants and tea bushes. Control-(A) Potted plant and (C & D) Tea bush; (B) Potted plant sprayed with *B.pumilus*; (E & F) Tea bush sprayed with *B.pumilus*.

Table 19: Growth of 2 year old tea plants after treatment with bacterial isolates

Tea Variety	Treatment	Height of Plants (cm)		No. of lateral Branches		No. of leaves	
		Initial	After 2 months	Initial	After 2 months	Initial	After 2 months
T-17	Control	31.2±2.1	37.6±2.3	10±0.6	15±1.0	43±2.3	49±2.8
	B.pumilus	34.3±1.8	78.7±1.8	10±0.5	22±1.2	44±1.8	82±3.4
	P.lentimorbus	33.0±2.1	70.0±3.1	12±0.4	25±0.9	43±1.7	79±3.2
HV-39	Control	37.1±1.8	44.7±2.5	14±0.7	16±0.8	60±3.1	73±2.9
	B.pumilus	35.6±2.0	76.2±3.2	10±0.4	16±0.7	33±1.9	68±2.5
	P.lentimorbus	38.6±2.2	82.5±3.5	13±0.6	18±0.9	37±1.8	76±3.1
UP-3	Control	31.2±1.7	44.7±1.9	9±0.2	10±0.4	45±1.9	51±2.3
	B.pumilus	40.4±1.6	81.2±3.6	9±0.6	15±0.8	32±1.2	63±2.5
	P.lentimorbus	38.6±1.3	76.7±3.1	11±0.7	15±0.7	35±1.4	66±2.3

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded 2 months following the bacterial inoculation; ± S.E.

4.8. Effect of co-inoculation with bacteria on growth of tea seedlings

Since it was proved that both the bacteria had the power to promote plant growth activity, it was decided to co-inoculate the bacteria to determine if they show any synergistic growth promoting activity. Accordingly, bacteria grown in broth were applied individually as well as in different combinations to the young seedlings of 6 varieties. It was observed that in relation to control, tea plants subjected to all treatments showed increased growth rate. It was further observed that percentage increase in height and number of leaves was greater when two bacteria were applied together. (Table 20 and 21; Plate XIV)

Table 20: Effect of joint application of bacteria in soil on growth of tea seedlings

Treatment	2 months after application					
	% Increase in height of seedling*			% Increase in no. of leaves*		
	T-17	K-1/1	T-78	T-17	K-1/1	T-78
Control	10.0	13.0	16.7	14.3	20.0	25.0
B.pumilus	50.0	33.8	50.0	53.0	33.0	50.0
P.lentimorbus	43.3	37.5	41.1	49.7	32.2	48.4
B.pumilus+P.lentimorbus	62.5	44.3	47.0	64.6	48.5	52.3

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded 2 months following the bacterial inoculation.

Plate XIV: Effect of coinoculation with PGPRs on growth of tea seedlings of two varieties (P.l. - *P.lentimorbus*; B.p - *B.pumilus*).

Table 21: Effect of bacterial consortia on the growth of tea seedling (after 10 months)

Treatment	% increment in shoot length		
	T-17	K-1/1	T-78
Control	85.7	94.6	53.2
B.pumilus	213.8	220.8	91.6
P.lentimorbus	207.1	209.3	78.8
B.pumilus+P.lentimorbus	360.3	345.5	122.5

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded 10 months following the bacterial inoculation.

4.9. *In vitro* determination of mechanism of action of selected antagonists

The growth promotion of plants may be achieved by the ability of bacteria to fixor sequester iron, facilitate phosphorus uptake or produce phytohormones (IAA), HCN, volatiles and chitinase that trigger responses in a growing plants. To determine the mechanisms of action of two selected rhizobacteria following experiments were conducted. Results are summarized below.

4.9.1. IAA production

IAA production by the selected rhizobacteria was assessed for their ability to produce Indole Acetic Acid (IAA) by growing them in Tripticase Soya agar supplemented with tryptophane (0.1 mM). *B. pumilus* recorded the IAA production of 42mg/L and *P.lentimorbus* was found to produce 46 mg/L.

4.9.2. Phosphate solubilization

Formation of clear zone around the colony grown in Pikovskaya's medium is an indication of phosphate solubilisation by rhizobacteria. In Pikovskaya's medium both *B.pumilus* and *P. lentimorbus* produced clear zone of diameter 2.65cm and 2.15cm after 5 days of incubation which indicated that both the isolates could solubilise the insoluble phosphate into simpler substances.

4.9.3. Siderophore production

To assess the siderophore production by the antagonistic rhizobacteria bacteria, these were inoculated into Chrome Azurol Sugar agar plates and were incubated for 10-15 days. The appearance of yellow halo region was observed around both *B. pumilus* and *P. lentimorbus* which indicated the both bacterial isolates were able to chelate Fe^{3+} from Chrome Azurol Sugar. The diameter of halo region was 1.6

cm and 1.5 cm for *B. pumilus* and *P. lentimorbus* respectively after 12 days of incubation.

Production of siderophore was further confirmed by Fe^{+} chelate test. Both *B.pumilus* and *P.lentimorbus* showed reduction in their ability to inhibit the mycelial growth of *Poria hypobrunnea* with increasing concentration on iron supplemented in the medium. The inhibition was maximum in case of 150µg/ml and minimum in case of 600µg/ml iron concentration.

4.9.4 HCN production

To determine the ability of *B.pumilus* and *P.lentimorbus* to produce Hydrocyanic acid (HCN) the bacteria were grown in medium amended with glycine.Filter paper strip soaked in picric acid was placed on the inner side of the lid of each petriplate and sealed properly with paraflim.Results were observed after 4-7 days. Both *B.pumilus* and *P.lentimorbus* were found to be non-cyanogenic in nature. This suggests that compound other than HCN may be associated in the inhibition of *P. hypobrunnea* in dual plate inverted chamber.

4.9.5. Chitinase production

To determine chitinase production by the antagonistic rhizobacteria bacteria spot inoculation was made in the 5 % colloidal chitin amended minimal medium and incubated at 28^{0}C for 6-7 days. It was observed that no extracellular chitinase was secreted by *B.pumilus* and *P.lentimorbus* even when grown on chitin amended media.

4.9.6. Volatile production

Volatile compound production by the antagonistic rhizobacteria was assessed by the inhibition of the mycelial growth of the test pathogen in comparison to the mycelial growth in control plate containing only the pathogen as described under materials and methods. Results showed that both the antagonists produced volatile compounds. The maximum inhibition of *F.lamaoensis* was exhibited by *B.pumilus* where as the maximum inhibition of *P.hypobrunnea* was exhibited by *P. lentimorbus*

Further, when the effect of age of bacteria on the growth inhibition of pathogen was compared maximum inhibition was observed on the 4th day of incubation and one day old inocula as compared to same age, 2 and 3days old inocula (Table 22 and 23; Plate XV).

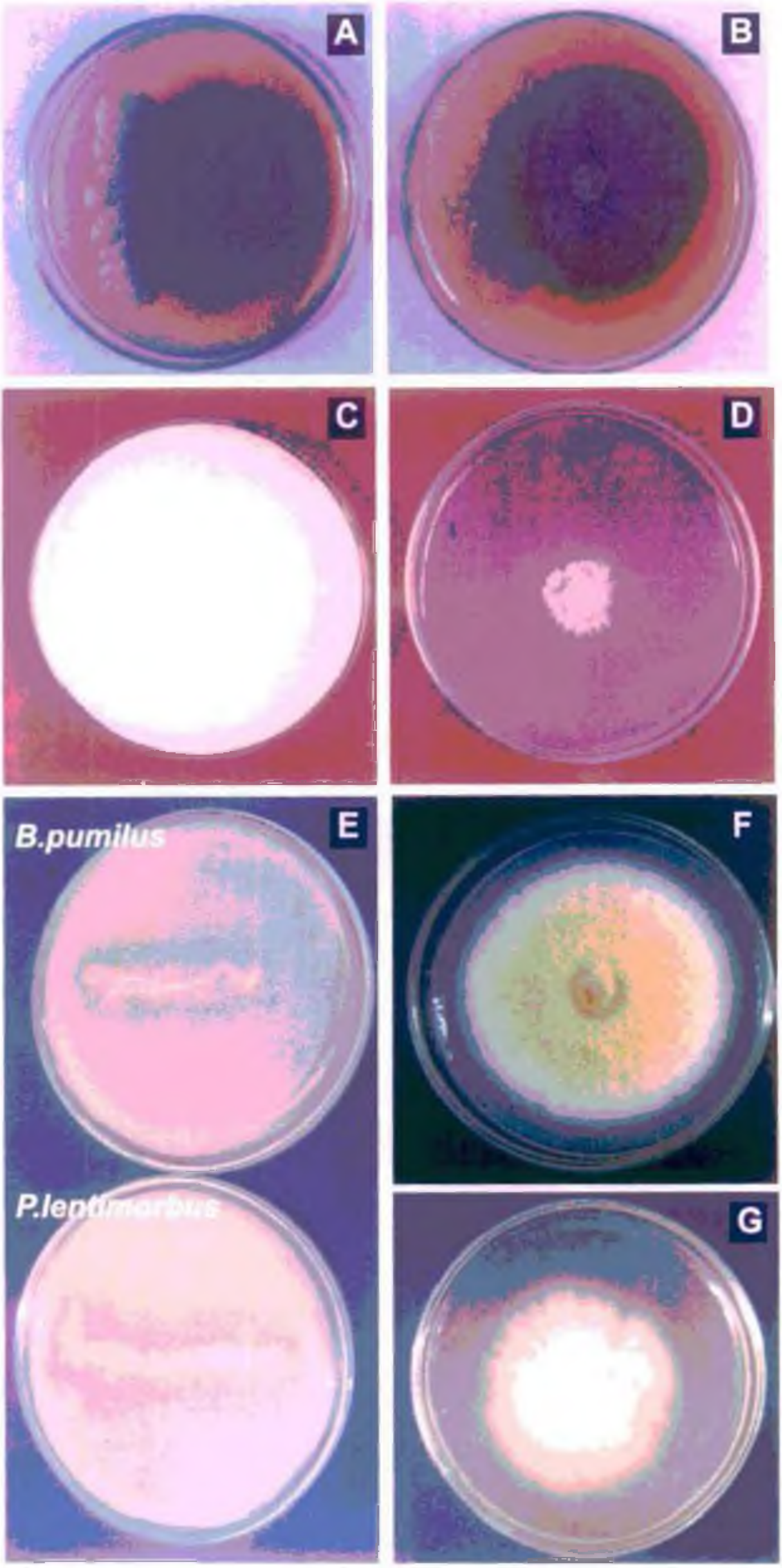


Plate XV(A-G): *In vtro* PGPR activities of *B.pumilus* and *P.lentimorbus*. Siderophore production of *B.pumilus* (A) and *P.lentimorbus* (B); Radial growth of *S.rolfsii* (D) and *P.hypobrunnea* (G) inhibited by volatiles from *B.pumilus;* C and F-respective controls; E-Phosphate solubilization by two bacteria.

Table22: Effect of volatile compounds on growth inhibition of test pathogens

Pathogens	*B.pumilus*		*P.lentimorbus*	
	RMG (cm)	GI (%)	RMG (cm)	GI (%)
P.hypobrunnea	3.3	63.33	3.2	64.44
F.lamaoensis	3.0	66.76	3.9	56.66
S.repens	4.3	52.22	4.7	47.77
S.rolfsii	5.2	44.22	5.5	38..89
S.sclerotirum	5.0	44.45	5.2	42.21
Control	9.0	-	9.0	-

RMG-Radial mycelial growth; GI- Growth inhibition; Average of three replicates; 1 day old inocula; 4 day of incubation.

Table 23: Growth inhibition (%) of *P.hypobrunnea* by volatile compounds released by *B.pumilus*

Antagonist	Grwoth inhibition (%)				
	Days	Same age	1 day old	2 day old	3 day old
B.pumilus	1	16.25	10.70	00.00	00.00
	2	30.45	40.23	37.61	41.26
	3	39.71	52.55	40.25	46.83
	4	47.78	63.33	45.56	49.63

Results of all the above tests showed positive reaction; negative reactions were obtained incase of HCN production and chitinase production. (Table 24; Plate XV)

Table 24: PGPR activity of selected rhizobacteria

Mechanism of action	*B.pumilus*	*P.lentimorbus*
IAA production	+	+
Phosphate solubilisation	+	+
Siderophore production	+	+
Volatile production	+	+
HCN production	-	-
Chitinase production	-	-

Average of three replicates; + Positive reaction; - Negative reaction.

4.10. Bioassy of active principle from *B.pumilus* and *P.lentimorbus* against test fungi

4.10.1. Cell free culture filtrate

Since the bacteria inhibited the growth of the pathogens both in solid and liquid medium, it was decided to determine whether the culture filtrates could inhibit the growth of the pathogens. Cell free culture filtrate of both the bacterial strains *B.pumilus* and *P.lentimorbus* were prepared and sterilized either by autoclaving (15 lbs. for 15 min.) or by passing through sterilized G-5 filter. These were then mixed with the medium in different proportions and the test pathogen (*P. hypobrunnea)* was inoculated into it. Results revealed that both autoclaved and cold-sterilized cell free culture filtrates significantly restricted the growth of *P. hypobrunnea in vitro*. As the ratio of culture filtrate in broth increased, a marked decrease in mycelial growth of the pathogen was recorded. (Table 25)

The active principle present in the culture filtrate was found to be heat stable. Autoclaved culture filtrate retained full inhibitory activity against the mycelial growth of the pathogens. There were no significant differences in the percentage of inhibition of pathogen growth due to the autoclaved culture filtrate and cold sterilized culture filtrate.

Table 25: Effect of Autoclaved culture filtrate (ACF) on the mycelial growth of *Poria* h*ypobrunnea*

Treatment	**Mycelial dry wt. (mg)**	
	B.pumilus	***P.lentimorbus***
PDB	285.1	289.2
ACF	0 (100)	0 (100)
ACF+10%PDB	040.4 (85.8)	043.3 (85.0)
ACF+30%PDB	090.2 (68.4)	101.0 (65.1)
ACF+50% PDB	124.7 (56.3)	138.6 (52.1)

PDB- Potato Dextrose Broth; Figures in parenthesis indicate percentage (%) of inhibition over control

4.10.2. Solvent extraction

Results of previous experiments showed that both the rhizobacteria secreted antifungal substances into the medium. For the characterization of these antifungal metabolites, the cell-free culture filtrates were extracted separately with five solvents- benzene, hexane, chloroform, diethylether, petroleum ether or ethylacetate. The solvent extracts were dried in vacuum and were finally dissolved in methanol as described earlier.

4.10.2.1. Bioassay of solvent extracts from *B.pumilus* and *P.lentimorbus*

The different solvent extracts were bioassayed against *P.hypobrunnea* to determine the fraction containing the active principle.

4.10.2.1.1. Spore germination

For spore germination bioassay a drop of each solvent extract was placed on clean, grease-free glass slide and allowed to evaporate following which drop of spore suspension of *P.hypobrunnea* was placed over it. Percentage spore germination was determined after 24 h. For control set drop of methanol was used. Results showed spores germination of most of the test pathogens totally inhibited by diethylether fraction of *B. pumilus* and benzene fraction of *P.lentimorbus*. Partial inhibitions were found in other fraction. (Table 26; Fig. 7)

Table 26: Effect of solvent extracts from culture filtrate of *P.lentimorbus* on spore germination of different fungal spores.

Fungal species	% spore germination						
	Control	fr.1	fr.2	fr.3	fr.4	fr.5	fr.6
C.lunata	97.9 ±4.1	03.5 ±0.3	08.3 ±0.6	01.5 ±0.8	12.4 ±0.9	13.2 ±1.2	10.7 ±0.6
F.oxysporum	98.3 ±2.7	04.6 ±0.4	08.7 ±0.5	01.0 ±0.2	13.4 ±0.8	11.2 ±0.6	14.4 ±1.1
A.niger	98.4 ±1.2	04.2 ±0.2	09.5 ±1.2	00.0	14.8 ±1.1	13.2 ±1.2	15.7 ±1.1
S.repens	98.0 ±1.2	06.3 ±0.5	15.4 ±1.3	02.5 ±0.1	17.3 ± 1.7	18.7 ±1.9	23.8 ±2.2
S.rolfsii	98.4 ±2.4	07.2 ±0.2	17.7 ±1.5	03.3 ±0.2	15.8 ±1.4	20.5 ±2.0	21.2 ±2.1
F.lamaoensis	97.6 ±2.3	05.3 ±0.8	08.1 ±0.7	00.0	08.3 ±0.8	09.4 ±0.7	11.5 ±0.8
P.hypobrunnea	97.2 ±4.5	06.4 ±0.6	09.8 ±0.5	00.0	07.9 ±0.7	09.6 ±0.5	12.3 ±0.7

Fr.1 – Diethyl ether extract; Fr.2- Hexane extract; Fr.3- Benzene extract; Fr.4- Petroleum ether; Fr.5- Ethyacetate extract; Fr.6– Chloroform extract; [A] Average of 200 spores/treatment; [B] Average of 50 germ lings/treatment.

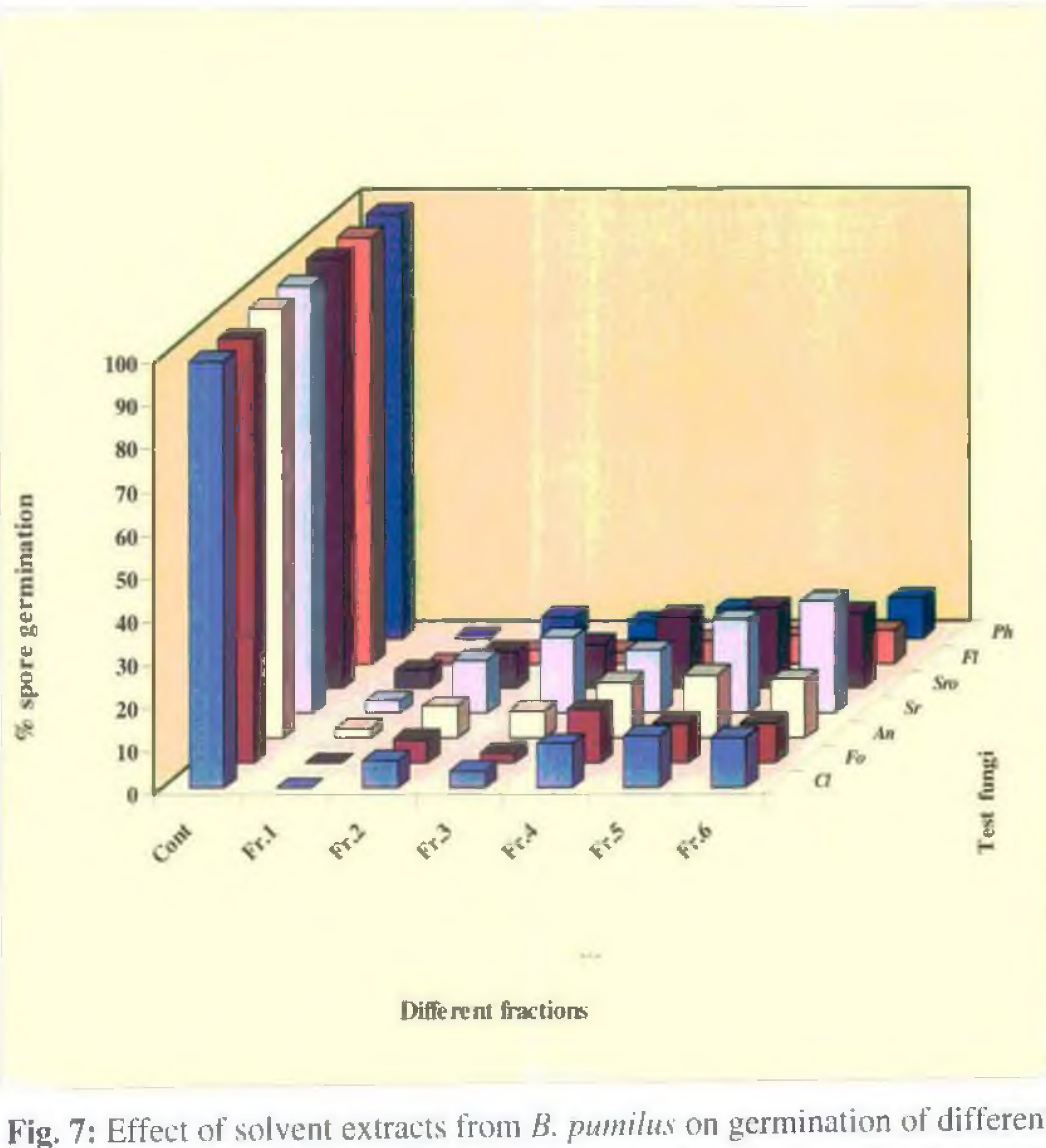


Fig. 7: Effect of solvent extracts from *B. pumilus* on germination of different fungal spores; Cl - *C. lunata*; Fo - *F. oxysporum*; An - *A. niger*; Sr - *S. repens*; Sro - *S. rolfsii*; Fl - *F. lamaoensis* and Ph - *P. hypobrunnea*. Fr.1 – Diethyl ether extract; Fr.2- Hexane extract; Fr.3- Benzene extract; Fr.4- Petroleum ether; Fr.5- Ethyacetate extract and Fr.6– Chloroform extract.

4.10.2.1.2. Radial growth

The effect of the different extracts on radial growth of *P. hypobrunnea* was tested on solid medium. After 4 days of growth in the control sets the mycelia completely covered the petridishes while varying degrees of inhibition in mycelial growth was noted in the different extracts. (Table 27; Plates XVI & XVII) In the diethyl ether fraction of *B. pumilus* maximum (82.7%) inhibition was recorded whereas maximum inhibition (79.1%) showed by benzene fraction of *P. lentimorbus*.

Table 27: Effect of solvent extract of *B.pumilus* against *P.hypobrunnea*

Solvent	*B.pumilus*		*P.lentimorbus*	
	Mycelia Diameter (cm)	**Reduction of mycelial growth (%)**	**Mycelia Diameter (cm)**	**Reduction of mycelialgrowth(%)**
Control	7.0	—	7.0	
Benzene	1.6 ± 0.2	71.6 ± 2.4	1.7 ± 0.4	79.1 ± 3.2
Hexane	2.0 ± 0.4	75.4 ± 1.7	2.5 ± 0.5	64.2 ± 1.6
Diethyl ether	1.4 ± 0.1	82.7 ± 3.8	1.9 ± 0.7	75.5 ± 2.8
Petroleum ether	2.4 ± 0.3	65.7 ± 1.8	2.1 ± 0.4	70.0 ± 3.6
Ethyl acetate	2.6 ± 0.2	62.9 ± 3.4	2.8 ± 0.5	60.0 ± 3.1
Chloroform	2.2 ± 3.2	68.6 ± 1.5	2.7 ± 0.3	61.4 ± 1.8

Average of three replicates; Cork Borer size-0.6 cm; Petri dish size -7.0 cm.

4.11. Optimization of active principle production by antagonists

Results of the previous experiments have established that bacteria were antagonistic to *P.hypobrunnea* both *in vitro* and *in vivo*. So, another experiment was conducted to optimize the active principle produced by antagonists. The production of antifungal metabolites by bacteria in culture is influenced by a number of factors including available carbon and nitrogen sources, pH of the medium, temperature, size of inoculums and time period. Maximum production of the antifungal principle will be at the optimum combinations. Considering the above, it was decided to study the effect of the important factors on production of active principle by *B.pumilus*. Incubation period, pH, different media and carbon sources were considered.

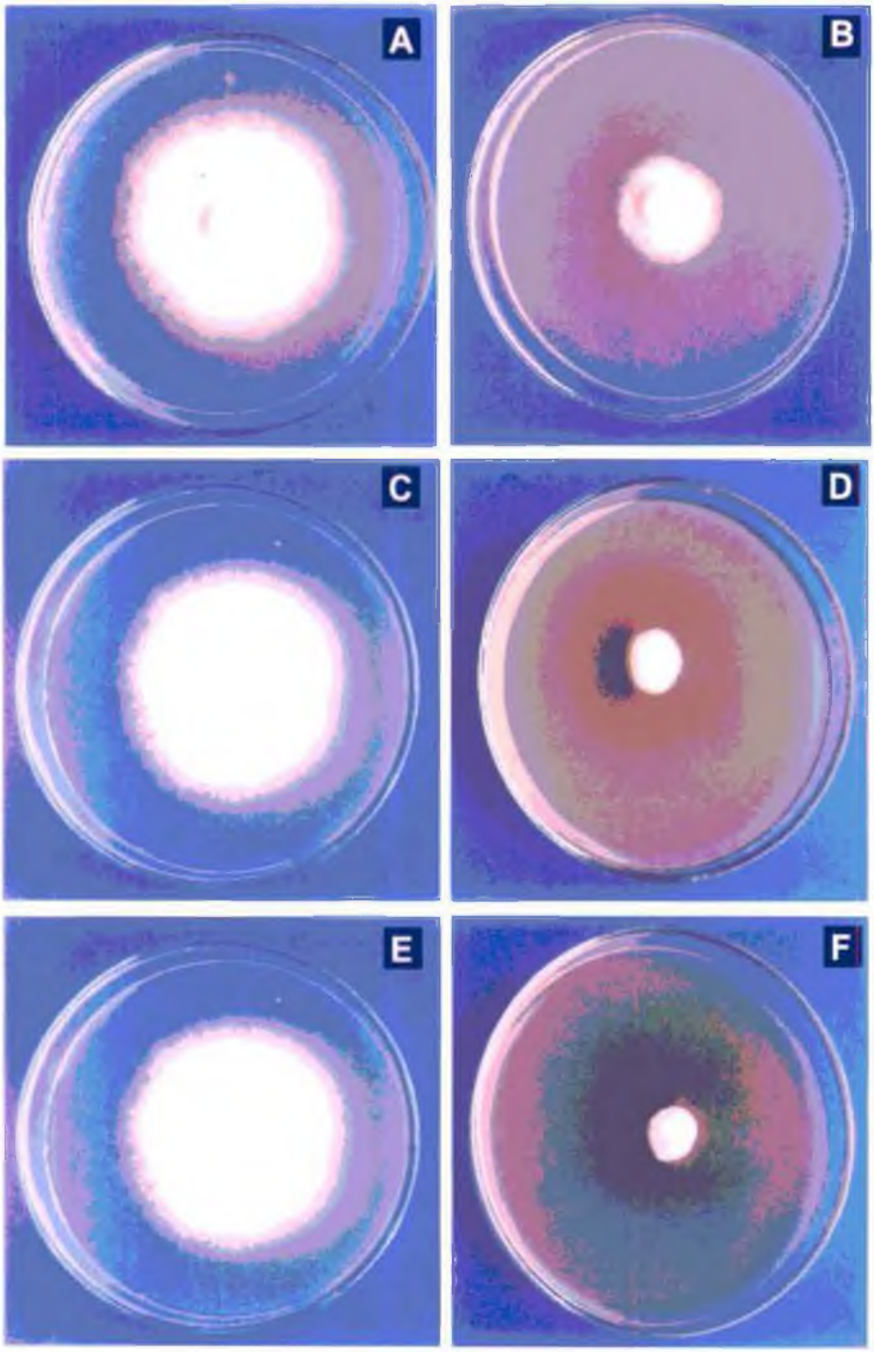


Plate XVI: Radial growth bioassay of solvent extracts from *B.pumilus*.Hexane (B); Benzene (D); Di-ethylether (F); Respective controls - A,C and E.

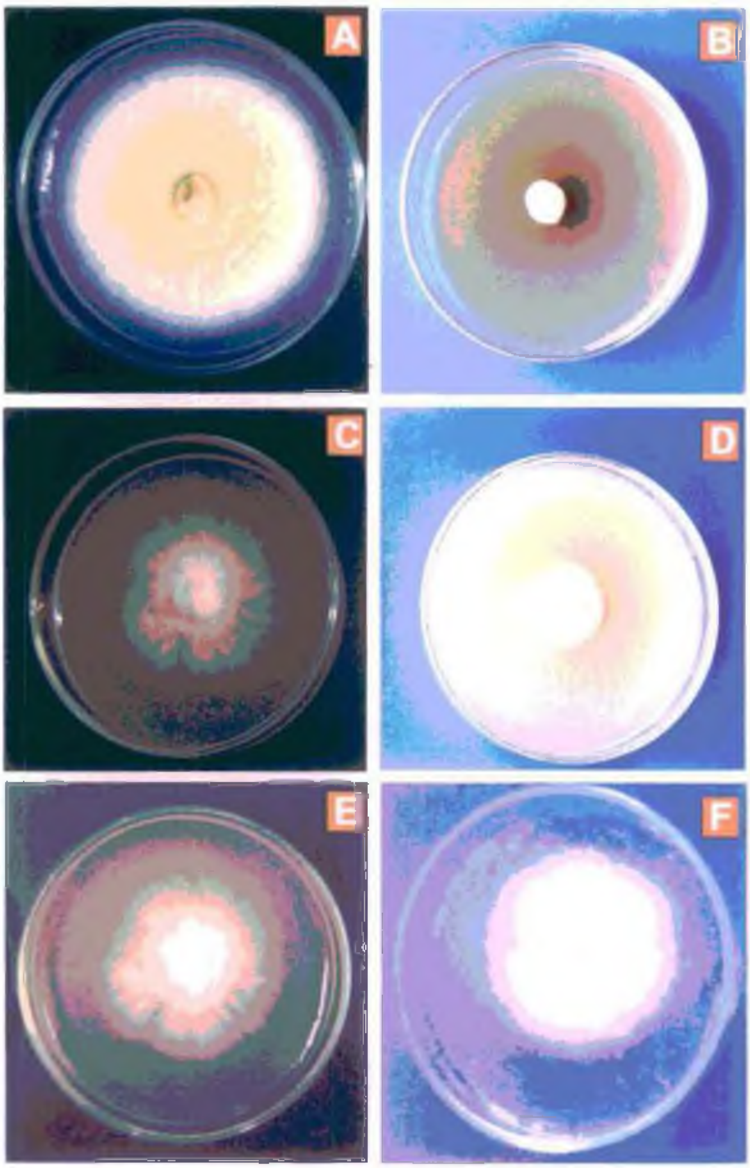


Plate XVII: Radial growth bioassay of solvent extracts from *P.lentimorbus*. Control (A); Benzene (B); Hexane (C); Diethyl ether (D); Ethyl acetate (E) and Chloroform (F).

4.11.1. Effect of incubation period

To determine the optimum incubation period for the production of antifungal compound by *B. pumilus*, nutrient broth (50 ml/250 ml flask) was inoculated with 0.5 ml inoculums and kept for 7 days at 35°C in shaking condition. The culture filtrate was collected at intervals of 24h upto 168h. The culture filtrates were extracted with diethyl ether as described earlier. The extracts were bioassayed by radial growth inhibition and agar cup methods. The results revealed that maximum production of antifungal compound was at 96h of incubation as evidenced by minimum radial growth (17mm) and maximum inhibition zone (25mm). Production of the metabolite increased from 24h to 96h and there was a steady decline after 96h. Results, therefore, indicate that 96h is the optimum incubation period for production of antifungal metabolite by *B .pumilus.* (Table 28; Fig. 8)

Table 28: Effect of different incubation time on production of antifungal compound by *B.pumilus*

Growth period of *B.pumilus* (h) [a]	Inhibition zone in Agar cup assay (mm) [b]
24	19.5 ± 1.5
48	21.5 ± 1.6
72	22.8 ± 1.4
96	25.0 ± 1.4
120	23.3 ± 1.2
140	18.6 ± 2.2
168	16.4 ± 1.4

Average of three replicate/ treatment; [a] *B. pumilus* grown in Nutrient Broth
[b] Growth measured after 4 days; Petridish size –7 cm; Cork borer size- 0.6cm

4.11.2. Effect of different media

Four different media (Nutrient Broth, Nutrient Sucrose Broth, Glucose Yeast Peptone Broth and Luria Broth) were considered to select the most suitable one for production of antifungal compound. After 96h of growth in the different media the bacterial culture filtrates were collected and extracted as before. Activities of the extracts were tested by radial growth inhibition and agar cup bioassays. It was observed that among the tested media, Nutrient Sucrose Broth was the most effective followed by Glucose Yeast Peptone Broth. (Table 29)

Table29: Effect of different media on production of antifungal compound of *B.pumilus*

Media	**Radial growth of *P.hypobrunnea*(mm)[a]**	**Inhibition zone in Agar cup assay(mm)[a]**
Nutrient Sucrose Broth	17.3 ± 2.1	28.4 ± 2.3
Nutrient Broth	22.2 ± 1.6	24.3 ± 2.2
Glucose Yeast Peptone Broth	19.7 ± 1.3	26.6 ± 1.5
King's B	26.1 ± 2.4	23.6 ± 1.7

Average of three replicate/ treatment; [a]Growth measured after 4 days; Petri dish size – 7 cm; Cork borer size- 0.6cm

4.11.3. Effect of carbon sources

Since nutrient sucrose broth was most effective among all four tested media, it was selected as the basal medium for further studies. To determine the effect of carbon sources on production of antifungal compound, the original carbon source of the medium (sucrose) was replaced by fructose, maltose, dextrose, starch, galactose, or mannitol. In all cases the medium was supplemented with 0.1% w/v C- source to maintain the same concentration as in the original medium. Productions of antifungal metabolites in the above cases were tested by radial growth inhibition and agar cup bioassay techniques. Both bioassays confirmed maximum production of antifungal compound in the medium containing sucrose as carbon source, followed by dextrose. (Table 30)

Table 30: Effect of different carbon sources on the production of antifungal compound by *B.pumilus*

Carbon sources[a]	**Radial growth of *P.hypobrunnea*(mm)[b]**	**Inhibition zone in Agar cup assay(mm)[b]**
Dextrose	23.7 ± 1.9	23.0 ± 2.1
Fructose	25.6 ± 2.0	21.4 ± 2.0
Maltose	27.3 ± 1.9	20.6 ± 2.3
Sucrose	16.3 ± 1.1	25.1 ± 1.7
Starch	27.1 ± 1.6	19.7 ± 2.1
Galactose	28.2 ± 1.7	19.1 ± 1.1
Mannitol	29.3 ± 2.1	18.8 ± 1.3

Average of three replicate/ treatment; [a]Basal medium Nutrient Sucrose Broth.
[b] Growth measured after 4 days; Petridish size – 7 cm; Cork borer size- 0.6cm.

4.11.4. Effect of different pH

As pH of the medium is known to influence the production of metabolites by microorganisms, it was one of the factors considered here. For this nutrient sucrose nroth was adjusted to different pH (5.5, 6.0, 6.5, 7.0, and 7.5) with HCl or NaOH. Culture filtrates obtained from each pH was extracted separately with diethyl ether and tested for their activity. Results revealed that 6.0 was the optimum pH for production of antifungal compound from *B. pumilus*. (Fig. 8)

4.12. Effect of *B.pumilus* on disease development in tea

As the bacteria inhibited the growth of the pathogens *in vitro* studies were conducted to determine the effectiveness of these bacteria in controlling root rot diseases. For this experiment, an important root rot diseases caused by *Poria hypobrunnea* was selected. It was observed that *B. pumilus* was successful in reducing intensity of root rot disease. (Table 31; Plate XVIII)

Table 31: Effect of *B. pumilus* on development Poria root rot disease of tea

Varieties	Treatment	Root rot index Days after inoculation 15	30	45
HV-39	*Poria hypobrunnea*	1.55	3.10	5.80
	Poria hypobrunnea+ B.pumilus	0.25	1.15	2.45
UP-3	*Poria hypobrunnea*	1.35	2.45	4.85
	Poria hypobrunnea+ B.pumilus	0.35	2.45	4.85
T-17	*Poria hypobrunnea*	1.10	2.50	4.75
	Poria hypobrunnea+ B.pumilus	0.40	0.95	2.10

Age of the plant 2yr.; Average of 10 separate inoculated plants; Rot index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering and 20-30% of roots turn brown; 3- leaves withered and 50% roots affected; 4- shoot tips also withering; 60-70% roots affected; 5- shoots withered with defoliation of lower withered leaves, 80% roots affected; 6- whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

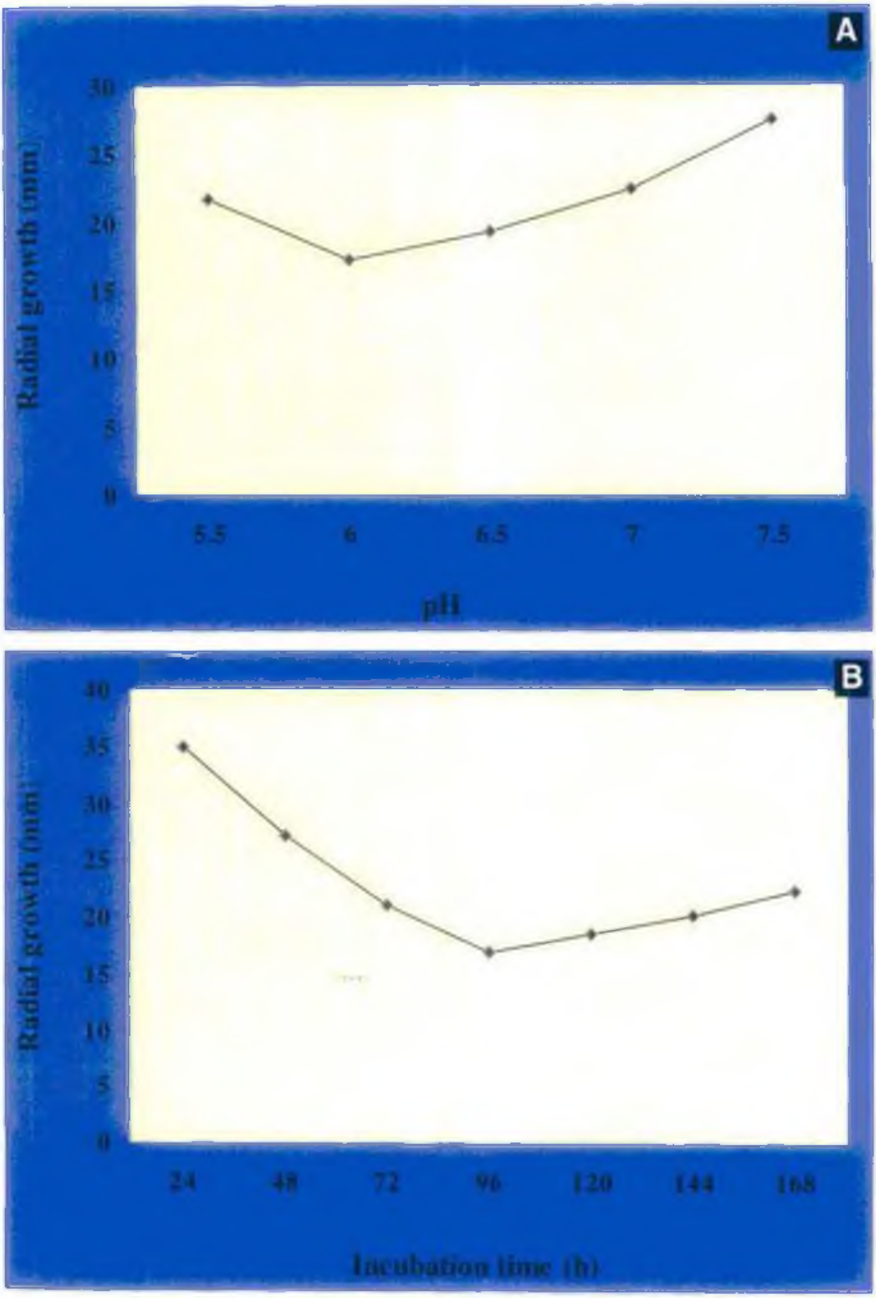


Fig. 8: Optimization of active principle production by *B. pumilus* - A: pH; B: Incubation time.

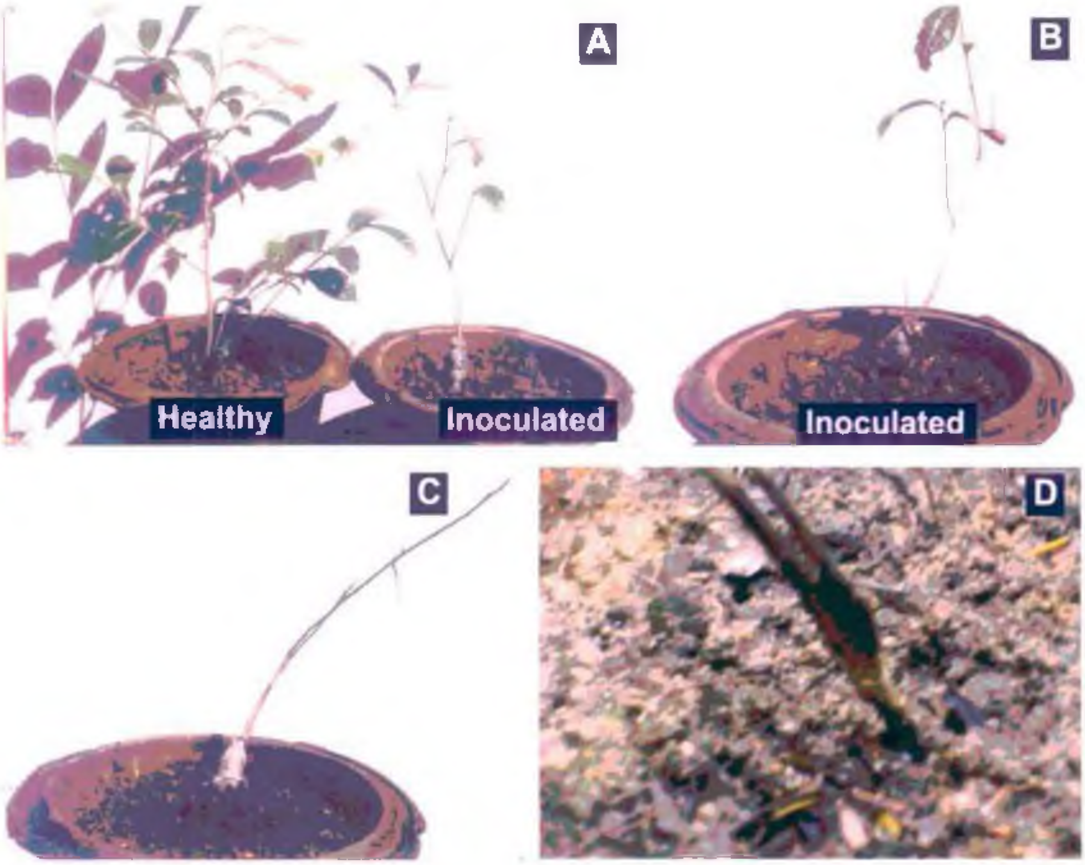


Plate XVIII (A-D): Disease symptoms in tea plants inoculated with *P.hypobrunnea.*

4.13. Biochemical changes in Tea leaves induced by application of *B.pumilus*

Since plant growth promotion could also be due to induction of biochemical responses within the host, experiments were conducted to assess the effect of *B.pumilus* on biochemical components of tea leaves. Polyphenols are major constituents of tea leaves and hence phenol contents were determined. In order to determine the effect on photosynthetic apparatus changes in chlorophyll content were also analyzed. Besides, catechins the flavonoid flavour component of tea leaves are extremely important and changes in these were also analyzed by HPLC. Activities of some of the enzymes which are involved in phenol metabolism as well as in defense- i.e., peroxidase, phenylalanine ammonia lyase, chitinase, β-1, 3 glucanase were also determined.

4.13.1. Chlorophyll

The quantitive analysis revealed that all treatments led to an increase in chlorophyll content both total Chlorophyll as well as Chl-a and Chl-b. (Table 32)

Table 32: Effect of *B.pumilus* on chlorophyll contents of tea leaves

Treatment	Varieties	Total Chlorophyll (mg/gm tissue)	Chlorophyll a (mg/gm tissue)	Chlorophyll b (mg/gm tissue)
Control *B.pumilus*	HV-39	1.493±0.06 1.695±0.16	0.582±0.02 0.831±0.01	0.923±0.08 0.864±0.15
Control *B.pumilus*	T-17	1.233±0.03 1.937±0.06	0.604±0.03 0.829±004	0.606±0.08 1.112±0.03
Control *B.pumilus*	S-449	0.820±0.03 1.321±0.03	0.395±0.01 0.612±0.03	0.425±0.02 0.709±0.05
Control *B.pumilus*	BSS-2	1.270±0.04 1.364±0.06	0.474±0.03 0.587±0.05	0.797±0.18 0.778±0.01
Control *B.pumilus*	UP-3	0.838±0.05 0.987±0.03	0.385±0.07 0.386±0.02	0.565±0.05 0.601±0.03
Control *B.pumilus*	TV-18	1.069±0.29 1.403±0.02	0.485±0.01 0.549±0.02	0.554±0.05 0.854±0.18

4.13.2. Catechin analysis

Catechins derived from leaves of plants whose rhizosphere was soil drenched with bacteria were analysed in HPLC. Results revealed that there were no major changes in the peaks following bacterization but observed appearance of few new peaks indicating new isomers. (Fig. 9, 10 and 11; Table 33, 34 and 35)

Table 33: HPLC analysis of catechin extracts from leaves of tea plants (cv. BSS-2) grown in untreated and treated (with *B. pumilus*) soil

Peak No.		Ret. Time	Area (mv.S)	Height (mv)	Area (%)	Height (%)
1	Control	2.480	475.3894	24.629	0.089	0.311
2		3.120	17218.8853	997.074	3.228	12.577
3		3.990	3604.7871	260.989	0.676	3.292
4		4.610	5859.0140	212.786	1.098	2.684
5		5.950	41722.2667	541.317	7.822	6.828
6		7.800	20455.8179	305.019	3.835	3.847
7		9.550	51121.9293	712.538	9.584	8.988
8		12.370	128895.6883	980.621	24.164	12.369
9		14.070	37264.4918	977.556	6.986	12.330
10		16.050	34571.3428	974.012	6.481	12.286
11		17.220	52108.0618	971.946	9.769	12.260
12		18.550	140120.2215	969.566	26.268	12.228
1	Treated with *B.pumilus*	2.680	5778.0166	467.597	0.890	5.137
2		3.240	31952.9386	1009.981	4.921	11.096
3		4.000	15194.7796	1009.398	2.340	11.089
4		4.660	15925.3609	676.130	2.453	7.428
5		5.110	3749.7174	222.369	0.577	2.443
6		5.930	37916.0413	738.907	5.839	8.118
7		8.110	32205.1821	327.824	4.960	3.602
8		9.790	26807.4281	640.050	4.128	7.032
9		12.660	128246.5328	1004.046	19.750	11.031
10		14.270	48861.9702	1003.015	7.525	11.019
11		15.200	32948.8814	1002.400	5.074	11.012
12		17.960	269758.6119	1000.690	41.543	10.993

Table 34: HPLC analysis of catechin extracts from leaves of tea plants (cv. HV-39) grown in untreated and treated (with *B. pumilus*) soil

Peak No.		Ret. Time	Area (mv.S)	Height(mv)	Area(%)	Height(%)
1	Control	2.150	32.8467	4.299	0.010	0.065
2		2.620	884.8221	23.468	0.283	0.355
3		3.890	7656.5542	601.786	2.447	9.095
4		4.500	2747.9053	111.081	0.878	1.679
5		5.890	5808.0080	154.650	1.856	2.337
6		7.680	4301.7337	198.085	1.375	2.994
7		8.360	2121.0916	76.457	0.678	1.156
8		9.060	5868.1514	210.666	1.875	3.184
9		10.120	782.7649	31.727	0.250	0.479
10		10.520	956.4171	34.915	0.306	0.528
11		11.790	58392.0233	986.706	18.660	14.912
12		13.790	24063.2510	984.191	7.690	14.874
13		14.690	6993.7403	258.686	2.235	3.910
14		16.120	32019.2394	981.295	10.232	14.830
15		16.920	50890.1126	980.351	16.263	14.816
16		18.480	109406.4298	978.423	34.962	14.786
1	Treated with *B.pumilus*	2.560	97.9829	4.912	0.034	0.071
2		3.060	173.7390	7.621	0.059	0.110
3		3.440	1031.4689	76.106	0.353	1.094
4		3.860	4814.5449	506.681	1.649	7.285
5		4.440	3601.7054	205.404	1.233	2.953
6		5.460	1056.3126	43.626	0.362	0.627
7		5.790	3673.4167	218.680	1.258	1.014
8		6.320	1020.7940	70.522	0.350	0.685
9		6.630	1341.4991	47.621	0.459	0.459
10		7.560	4890.0459	257.035	1.674	3.695
11		8.170	3015.3947	118.047	1.033	1.697
12		8.840	5425.3266	239.111	1.858	3.438
13		9.390	1415.5217	63.003	0.485	0.906
14		10.330	1675.8800	41.837	.574	0.620
15		11.550	62544.8490	992.681	21.417	14.272
16		13.670	29526.6908	991.149	10.111	14.250
17		14.950	5863.7555	152.196	2.008	2.188
18		15.710	32905.7934	976.823	11.268	14.044
19		16.790	58495.6025	988.943	20.030	14.218
20		18.390	69464.6642	953.401	23.785	13.707

Table 35: HPLC analysis of catechin extracts from leaves of tea plants (cv. S-449) grown in untreated and treated (with *B. pumilus*) soil

Peak No.		Ret. Time	Area (mv.S)	Height(mv)	Area(%)	Height(%)
1	Control	2.810	473.3173	12.257	0.144	0.150
2		3.410	868.7875	65.448	0.264	0.803
3		3.870	7682.3199	857.601	2.331	10.523
4		4.420	4259.0464	197.280	1.292	2.421
5		5.420	1044.2576	57.297	0.317	0.703
6		5.710	6447.4943	358.672	1.956	4.401
7		6.260	2041.9531	128.611	0.619	1.578
8		6.620	575.5837	41.603	0.175	0.510
9		6.890	493.4300	40.140	0.150	0.493
10		7.450	5257.1728	271.776	1.595	3.335
11		8.010	4513.3562	174.622	1.369	2.143
12		8.630	4563.9448	188.224	1.385	2.309
13		9.170	2978.0295	142.535	0.903	1.749
14		9.880	2265.8441	54.195	0.687	0.665
15		11.360	67655.9305	990.888	20.525	12.158
16		12.780	4809.0689	109.204	1.459	1.340
17		13.400	12530.5400	474.985	3.801	5.828
18		14.460	4986.1522	202.144	1.513	2.480
19		15.220	8726.9136	269.448	2.648	3.306
20		15.630	26869.0730	988.138	8.151	12.124
21		16.720	46211.7512	987.483	14.020	12.116
22		17.490	10799.8614	551.024	0.276	6.761
23		18.280	103569.5928	986.499	31.420	12.104
1	Treated with *B.pumilus*	3.000	533.9653	19.365	0.172	0.236
2		3.420	951.7176	64.073	0.306	0.779
3		3.860	6133.5504	621.490	1.974	7.559
4		4.440	3776.2338	135.153	1.215	1.644
5		5.730	10168.8955	408.078	3.273	4.963
6		6.730	1036.6882	52.698	0.334	0.641
7		7.500	4255.8639	147.582	1.370	1.795
8		8.100	3996.8123	141.639	1.286	1.723
9		8.760	4912.9617	123.339	1.581	1.500
10		10.040	2694.0107	59.404	0.867	0.722
11		11.520	64728.0004	992.513	20.833	12.071
12		13.090	3469.2315	91.235	1.117	1.110
13		13.630	7343.2052	171.530	2.363	2.086
14		14.590	5020.4748	204.134	1.616	2.483
15		15.680	28249.8214	989.352	9.092	12.033
16		16.740	35009.4416	988.600	11.268	12.023
17		18.150	35306.9967	952.921	11.364	11.590
18		18.440	43797.5820	987.336	14.096	12.008
19		19.630	40439.0283	920.235	13.015	11.192
20		21.260	8875.1528	151.601	2.858	1.842

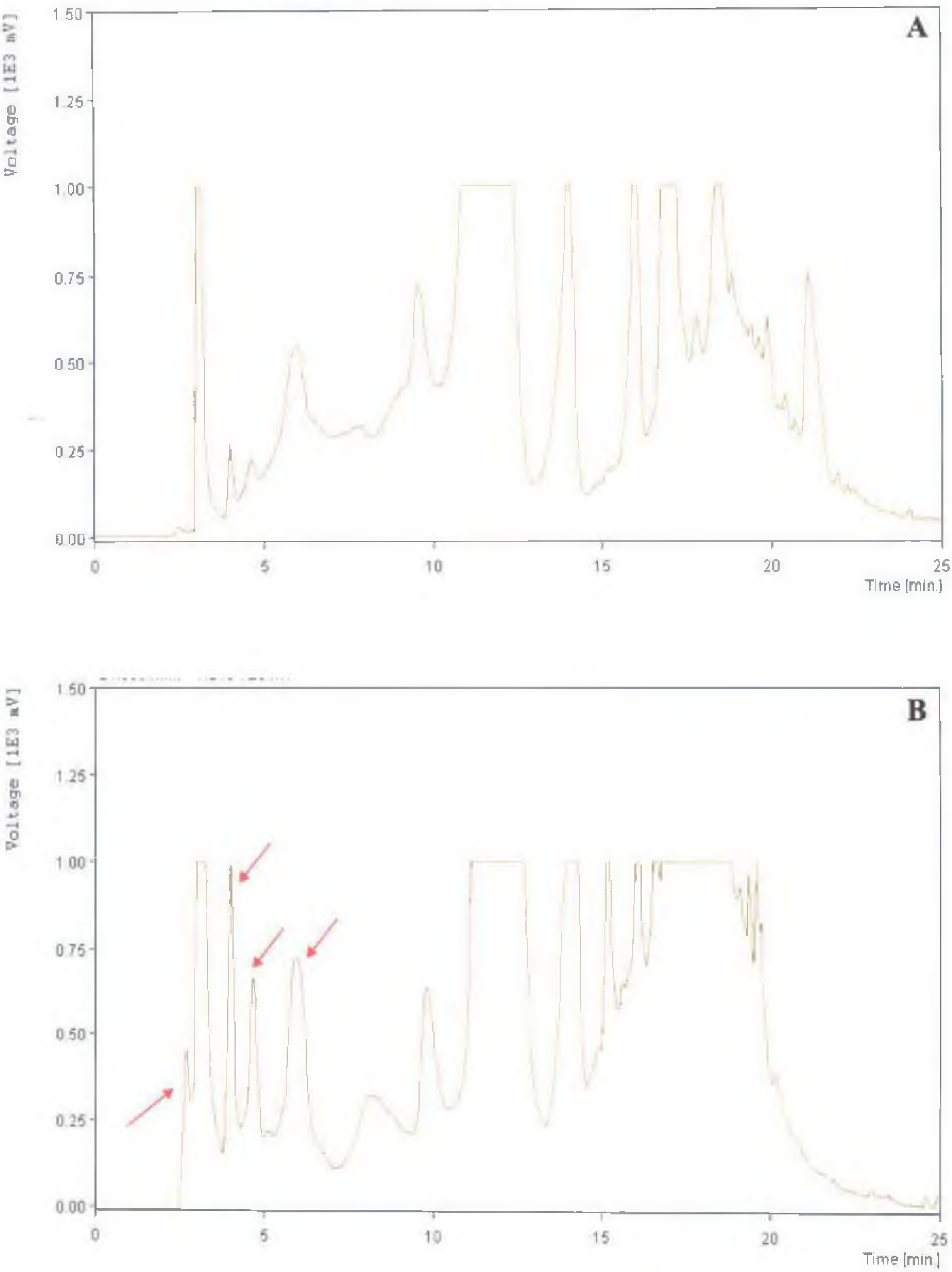


Fig. 9: HPLC analysis of catechin extracts from leaves of tea plants (BSS-2). A: Control; B: Soil treated with *B.pumilus*.

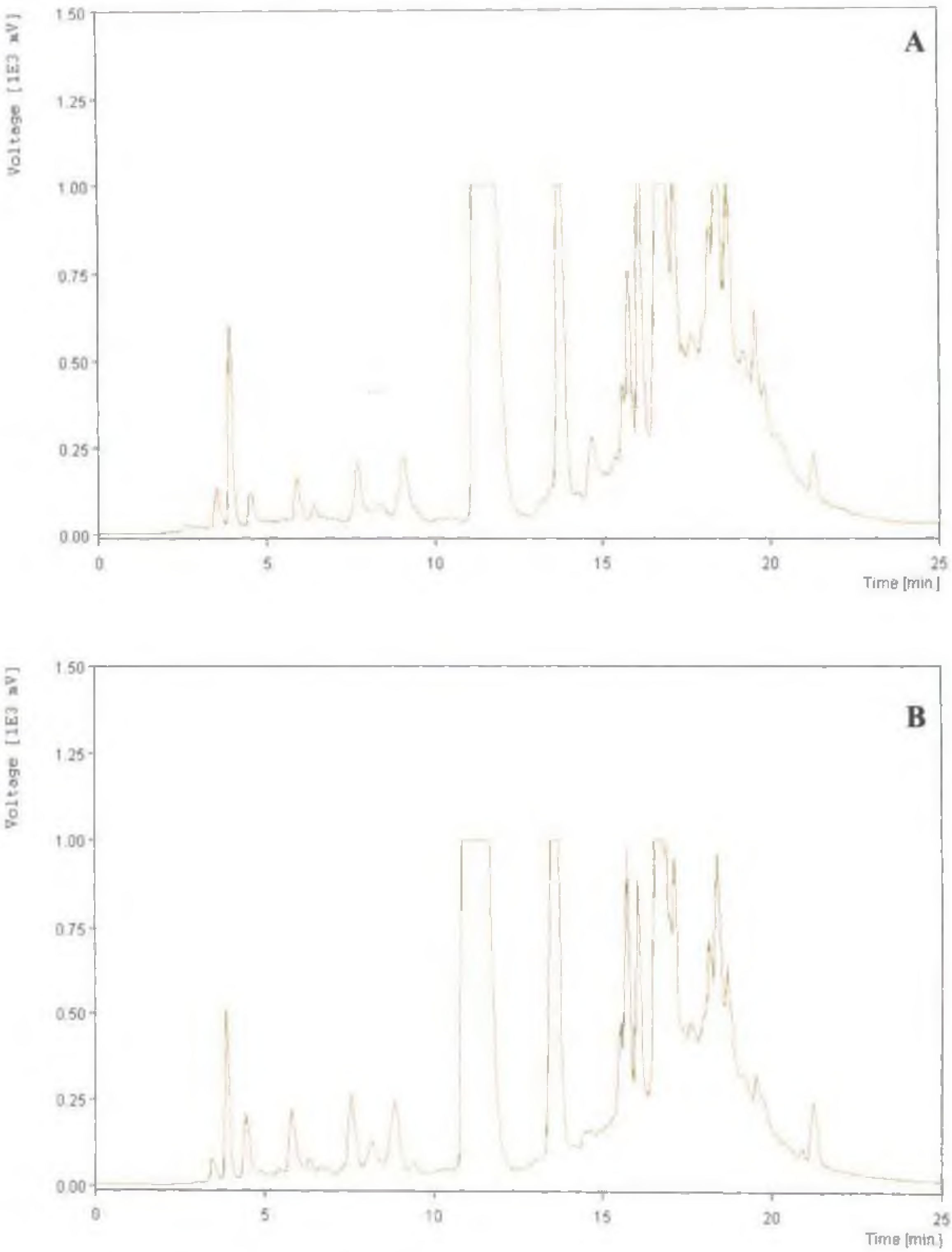


Fig. 10: HPLC analysis of catechin extracts from leaves of tea plants (HV-39). A: Control; B: Soil treated with *B.pumilus.*

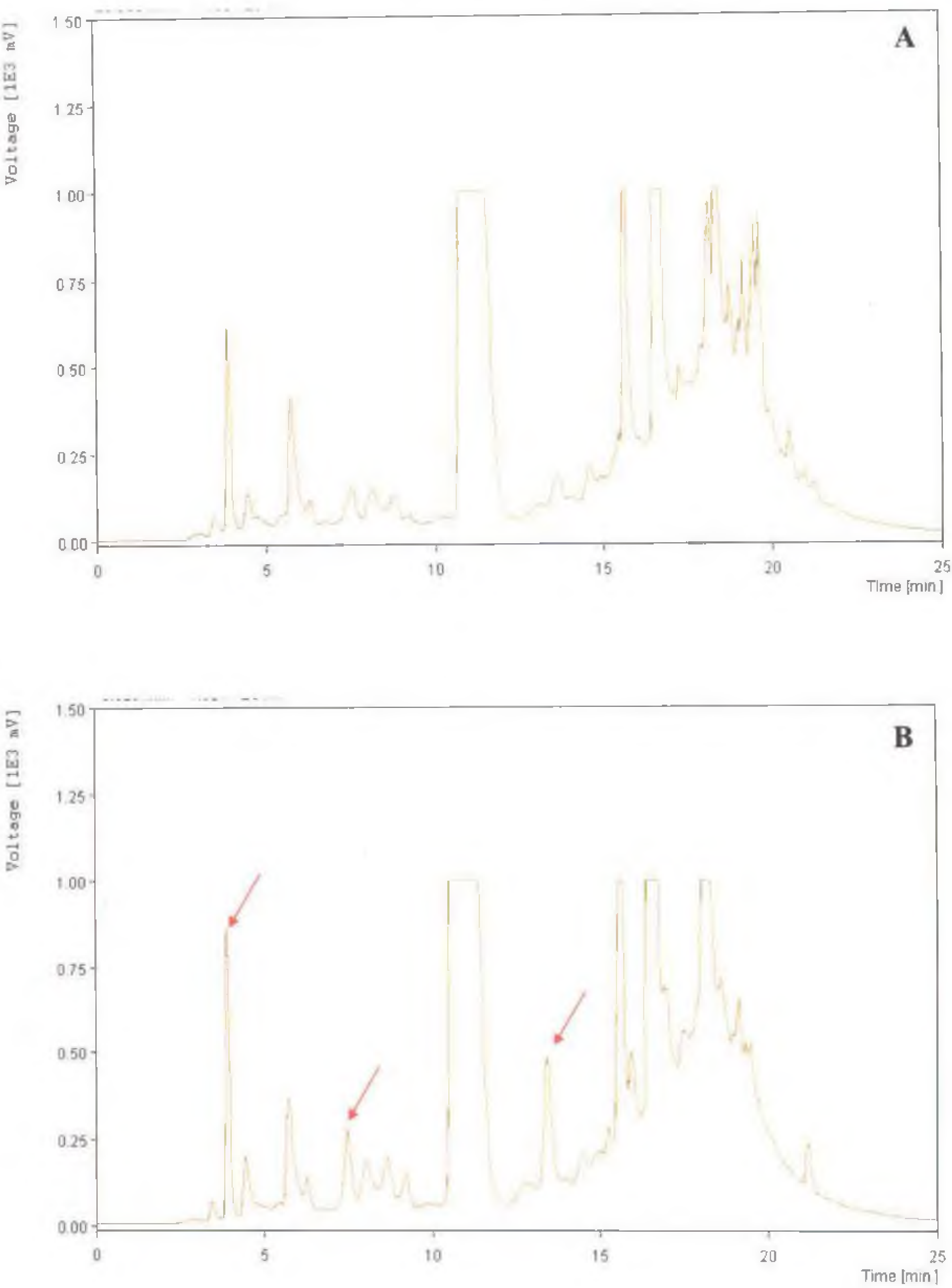


Fig. 11: HPLC analysis of catechin extracts from leaves of tea plants (S-449). A: Control; B: Soil treated with *B.pumilus*.

4.13.3. Phenols and Defense enzymes

Since phenols and defense enzymes are known to be involved in defense of plants against pathogen, analysis of these were done not only after bacterial inoculation, but also with pathogen inoculation.

4.13.3.1. Phenol content

Both the Total and O-dihydroxy phenol contents of the tea leaves were increased significantly after application of *B.pumilus* as compared to untreated control in different varieties of tea. Results revealed that maximum accumulation occurred when there was joint inoculation by PGPR and pathogen. (Table 36)

Table 36: Phenol contents in the tea leaves following treatments in the rhizosphere

Varieties	Treatment	Phenol content (mg/g tissue)	
		Total	O-dihydroxy
T-17	Control	30.2±3.2	8.2±1.4
	P.hypobrunnea	33.4±2.1	8.6±0.8
	B.pumilus	42.5±2.2	11.3±1.1
	P.hypobrunnea+ *B.pumilus*	44.6±3.6	11.5±1.0
HV-39	Control	28.2±1.6	7.5±1.4
	P.hypobrunnea	34.1±2.9	8.9±1.3
	B.pumilus	36.4±3.9	10.1±0.6
	P.hypobrunnea+ *B.pumilus*	43.4±3.8	13.2±1.5
UP-3	Control	34.4±0.4	6.5±1.3
	P.hypobrunnea	39.1±0.6	7.8±0.8
	B.pumilus	46.0±2.5	8.3±1.0
	P.hypobrunnea+ *B.pumilus*	49.5±3.9	14.2±1.6
TV-18	Control	30.4±1.4	7.9±4.1
	P.hypobrunnea	34.7±3.9	9.1±2.9
	B.pumilus	41.5±2.8	9.8±1.8
	P.hypobrunnea+ *B.pumilus*	45.3±1.1	11.3±2.1
S-449	Control	24.1±0.8	7.6±1.0
	P.hypobrunnea	38.1±0.6	9.2±1.2
	B.pumilus	41.2±0.4	10.4±2.1
	P.hypobrunnea+ *B.pumilus*	45.6±0.9	11.8±1.7
BSS-3	Control	22.5±1.2	7.8±1.8
	P.hypobrunnea	33.7±1.3	9.2±1.4
	B.pumilus	45.0±1.1	14.2±1.6
	P.hypobrunnea+ *B.pumilus*	48.1±2.2	14.8±2.3

Sampling done after a period of two month of treatments; Average of three replicates; ± S.E.

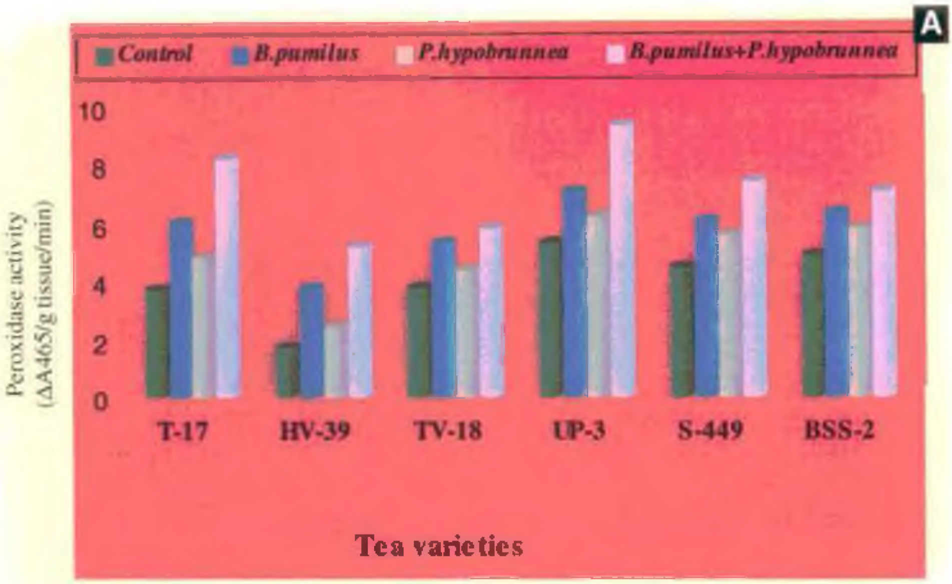
4.13.3.2.Enzymes activities.

Application of *B. pumilus* to the tea plants resulted increase in activities of all the four tested enzymes in the leaves of the bacteria treated plants compared with untreated plants. However, further enhancement was observed when *P.hypobrunnea* was also inoculated. (Table 37; Fig. 12 and 13)

Table 37: Enzyme activities in leaves of different tea varieties grown in soil following treatments in the rhizosphere

Varieties	Treament	Enzyme activities			
		POX[a]	PAL[b]	CHT[c]	GLU[d]
T-17	Control	3.7±0.2	66±0.5	12.5±0.9	360±2.0
	P.hypobrunnea	4.8±0.1	95±0.8	17.5±0.4	443±5.1
	B.pumilus	6.0±0.4	97±0.7	22.2±1.6	465±3.8
	P.hypobrunnea+ B.pumilus	8.2±0.2	141±0.3	26.4±1.0	532±1.2
HV-39	Control	1.7±0.1	96±0.4	12.5±1.1	336±3.9
	P.hypobrunnea	2.4±0.2	101±0.4	13.2±0.4	356±2.0
	B.pumilus	3.8±0.2	110±0.6	18.0±1.8	476±5.2
	P.hypobrunnea+ B.pumilus	5.2±0.3	173±0.7	30.0±2.2	542±2.4
TV-18	Control	3.8±0.1	65±0.2	16.4±1.4	340±1.8
	P.hypobrunnea	4.4±0.7	73±0.6	17.3±1.2	492±0.8
	B.pumilus	5.3±0.3	75±0.8	20.1±1.7	560±2.4
	P.hypobrunnea+ B.pumilus	5.8±0.6	132±0.4	29.2±1.1	610±2.1
UP-3	Control	5.3±0.2	60±0.4	14.3±2.1	340±1.8
	P.hypobrunnea	6.2±0.2	69±0.8	17.7±1.1	489±1.2
	B.pumilus	7.1±0.5	82±0.3	20.4±1.0	520±3.7
	P.hypobrunnea+ B.pumilus	9.3±0.3	137±0.5	29.2±2.3	556±2.9
S-449	Control	4.5±0.3	84±0.5	18.3±1.7	410±2.9
	P.hypobrunnea	5.6±0.7	97±0.8	26.5±1.8	470±2.3
	B.pumilus	6.1±0.4	130±0.3	32.0±1.4	510±5.2
	P.hypobrunnea+ B.pumilus	7.4±0.5	139±0.9	35.2±2.1	560±4.2
BSS-2	Control	4.9±1.0	64±0.8	13.8±1.3	450±3.9
	P.hypobrunnea	5.8±1.1	68±3.4	17.4±3.1	490±4.2
	B.pumilus	6.4±0.3	82±0.4	25.0±1.8	560±4.1
	P.hypobrunnea+ B.pumilus	7.1±0.7	96±0.6	29.6±1.7	580±4.4

[a] POX activity assayed as Δ A465 min^{-1} g $tissue^{-1}$; [b] PAL activity assayed as μg cinnamic acid produced by enzyme from 1 g tissue min^{-1}; [c] CHT activity assayed as μg N-Acetyl glucosamine released by enzyme from 1 g tissue min^{-1} and [d] β 1,3- GLU activity assayed as μg glucose released by enzyme from 1 g tissue min^{-1}



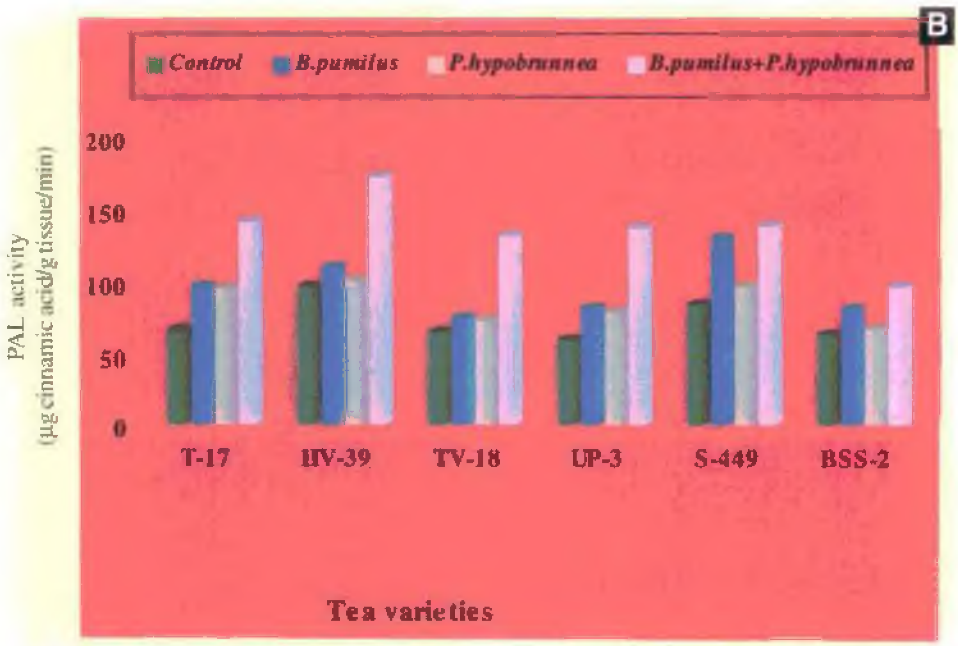


Fig. 12: Peroxidase (A) and PAL activity (B) in tea leaves of different varieties following treatment in the rhizosphere.

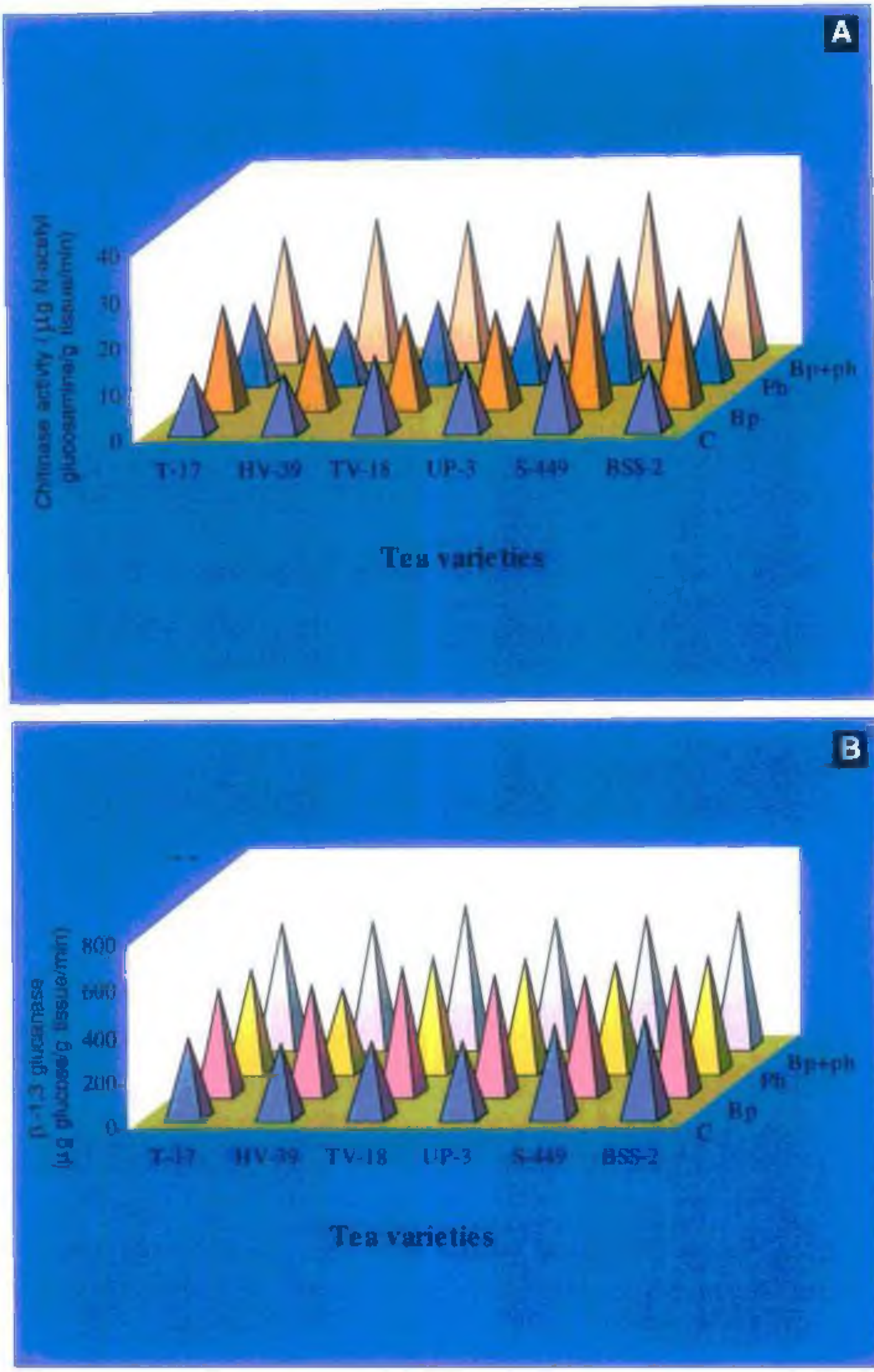


Fig. 13: Chitinase (A) and β-1,3 glucanase activity (B) in tea leaves of different varieties following treatment in the rhizosphere. C-Control; Bp-*B. pumilus;* Ph-*P. hypobrunnea.*

4.14. Immunodetection of *P. hypobrunnea* and *B. pumilus* in soil

Polyclonal antibodies (PAbs) were raised in rabbits against mycelial antigen of *P. hypobrunnea* as well as from bacterial antigen of *B.pumilus* as described previously. Prior to injection, the quantity of protein was measured and these were also analyzed by SDS-PAGE. Good bands were evident in all cases indicating the yield of bacterial protein and fungal proteins (Plate XIX). Antisera were collected for 6 bleeds and IgG purified from these. For each antigen source normal serum were collected before immunization. These were used for further experimental purposes.

4.14.1. Immunodiffusion

The effectiveness of antigen preparations from bacterial cell and fungal mycelia in raising PAbs was checked by homologous cross reaction following agar gel double diffusion tests. Control sets involving normal sera and antigen of bacteria and fungus were all negatives. Strong precipitin reaction was occurred when PAbs reacted with its own antigen and good titre was obtained in 2^{nd} and 5^{th} bleedings with maximum reaction occurring in 3^{rd} and 4^{th} bleed. For subsequent immunoassays, 3^{rd} /4^{th} bleed PAbs were used. Only weak precipitin bans were observed in 1^{st} and 6^{th} bleedings. (Plate XIX)

4.14.2. ELISA

The observed reduction in disease intensity could be either due to reduction of pathogen population in soil or by induction of resistance; or by a combination of both. Pathogen population can be specifically determined in soil by immuno-detection techniques using PAbs raised against the specific pathogens. PAbs were used for detection of pathogens both by ELISA and Dot-blot.

4.14.2.1. For pathogen

Fungal root rot pathogen, *P. hypobrunnea* in the soil was detected by ELISA using specific PAbs of *P. hypobrunnea*. Antigens were prepared from treated and untreated soil and tested by ELISA, using PAbs of specific pathogens. The results showed that population of *P.hypobrunnea* in soil were reduced when PGPR was also applied to soil. It was also shown that when soil was inoculated with bacteria and pathogen lower A405 values were obtained as compared to antigens from soil treated only with *P.hypobrunnea* (Table 38)

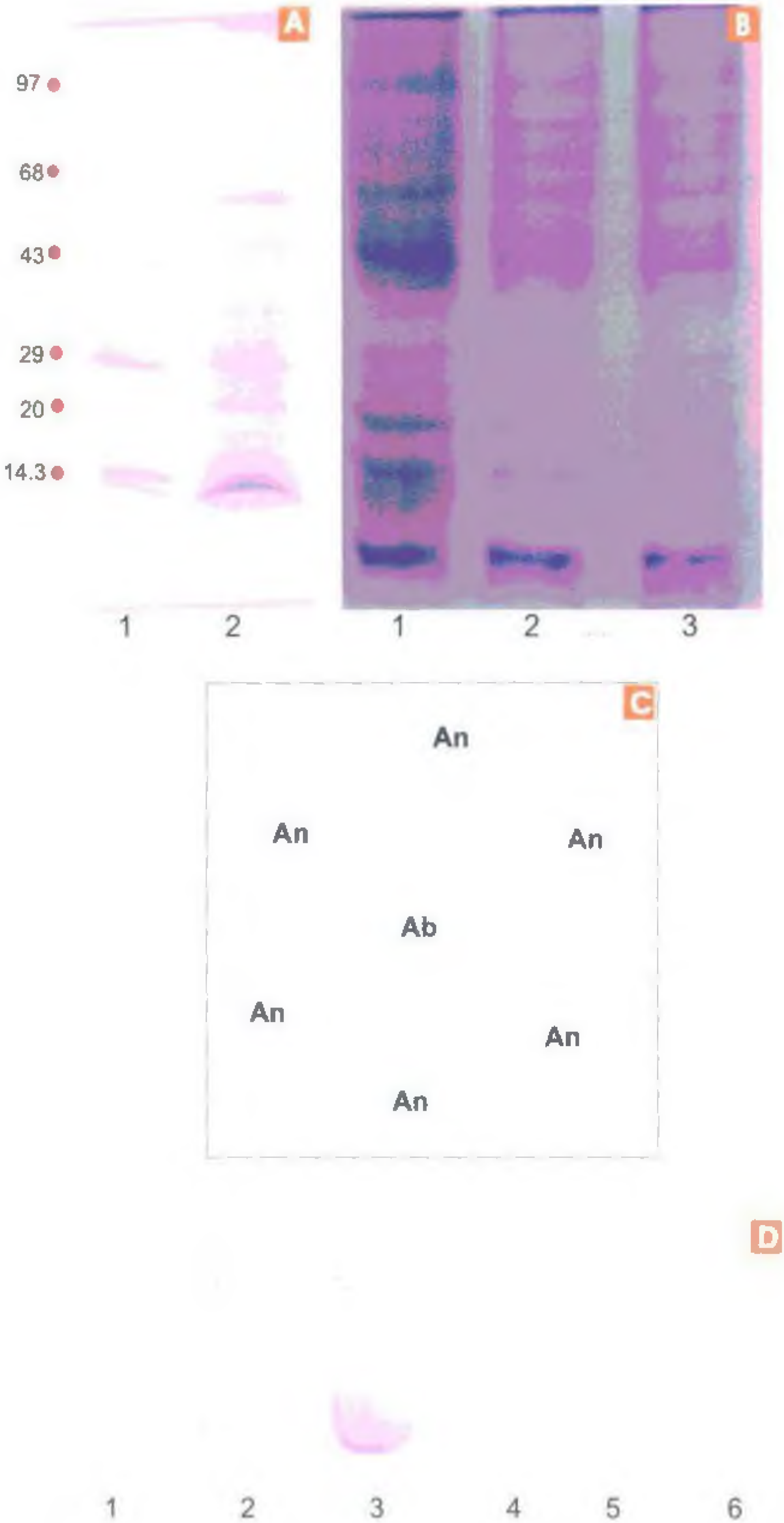


Plate XIX: A & D - SDS PAGE analysis of proteins from *B.pumilus* (Lane 1- marker; 2-protein) and *P.hypobrunnea* (Lanes 1,2 and 3); C-Immunodiffusion of antigens from *B.pumilus* reacted with homologus PAb; D-Dot blot assay of antigens from *B.pumilus* reacted with homologus PAb (Lanes 1-6: PAbs from 1-6 bleedings).

Table 38: ELISA and Dot-blot values of soil antigens from different treatments after reaction with PAbs of *P.hypobrunnea*

Soil antigen*	ELISA A 405 values	Dot-blot Colour intensity**
Uninfested soil	0.320±0.06	-
Treatment		
P.hypobrunnea	0.986±0.04	++
B.pumilus	0.005±0.00	-
P.hypobrunnea+ B.pumilus	0.490±0.07	+

Average of 3 replicates; PAb dilution: 1: 500; * Sample collected 30 days after inoculation with pathogen; ** Fast red colour intensity: Pinkish red: ++++; Bright pink: +++; Pink: ++; Light pink: +; No colour: -;

4.14.2.2. For bacteria

The sustainability of applied bacteria in the rhizosphere was determined by ELISA. Different soil antigens were tested against PAbs of *B. pumilus.* The results revealed that bacteria could successfully survive in the tea rhizosphere even after 3 months of inoculation. Maximum A405 values were obtained from the soils collected soon after application of bacteria. The values reduced to some extent with time, though even after 3 months, these were still detectable at fairly high concentrations. It was also noted that bacteria survived equally well in rhizosphere when applied individually or in combination. (Table 39)

Table 39: Detection of survival of *B. pumilus* in soil after direct application

Antigen source: Rhizosphere Soil from	Absorbance at 405 nm			
	Uninoculated	Time after bacterial application		
		Immediate	15 days	3 months
TV-18	0.350±0.06	1.031±0.03	1.023±0.07	1.006±0.06
TV-26	0.442±0.08	1.074±0.09	1.052±00.9	1.011±0.02
S-449	0.245±0.16	1.233±0.06	1.198±0.05	1. 095±0.05
T-78	0.340±0.03	1.165±0.08	1.112±0.17	0.998±0.11
HV-39	0.520±0.09	1.310±0.07	1.123±0.20	1.078±0.08

Average of 3 replicates; PAb dilution: 1: 500; ± S.E.

4.14.3. Dot-blot

Dot immunobloting technique is rapid and sensitive method for detection of survival of bacteria the in soil. The presence of bacteria in the soil was detected by this technique using the antigen from rhizosphere soil inoculated with bacteria and PAb raised against the bacterium. Results revealed that when antigen dots reacted with PAb of *B.pumilus*, colour intensity was highest when soil antigens were prepared soon after bacterial application. With time, the intensity decreased, though not significantly.

Dot-blot was also used for detection of pathogen in the soil. When the antigen prepared from the rhizosphere soil amended with pathogen alone and from soil treated with PGPR followed by pathogen inoculation was probed with PAbs of *P. hypobrunnea*, violet coloured dots were visible in pathogen alone treated samples indicating the survival of the *P. hypobrunnea* in the rhizosphere of the plant whereas the samples from both PGPR and pathogen treated showed very faint colour indicating the inability of pathogen to survive in presence of PGPR.

4.15. Studies on talc- based formulation of PGPR

B.pumilus as soil drench, foliar spray and seed bacterization promoted growth significantly; it was further decided to prepare formulation of this bacterium in a suitable carrier and determine the efficiency of the formulation. Formulation was prepared using carboxy methyl cellulose with talcum powder as carrier material and tested under green house conditions for their effect on growth promotion of tea. Two g of talc- based formulation was applied per pot in the rhizosphere of 3 tea varieties UP-3, TV-18 and BSS-2, and growth of seedlings were observed and compared with control. The percentage of increase in plant height and no. of leaves were significantly enhanced on application of the talcum formulation in comparison to control. (Table 40; Plate XX)

4.15.1. Detection of PGPRs in bioformulation by ELISA

The viability of formulation was tested during storage period of 60 days and 90 days by ELISA. The results showed that bacterial population declined gradually during storage but when applied in the field the bacteria could successfully establish themselves in the rhizosphere. (Table 41)

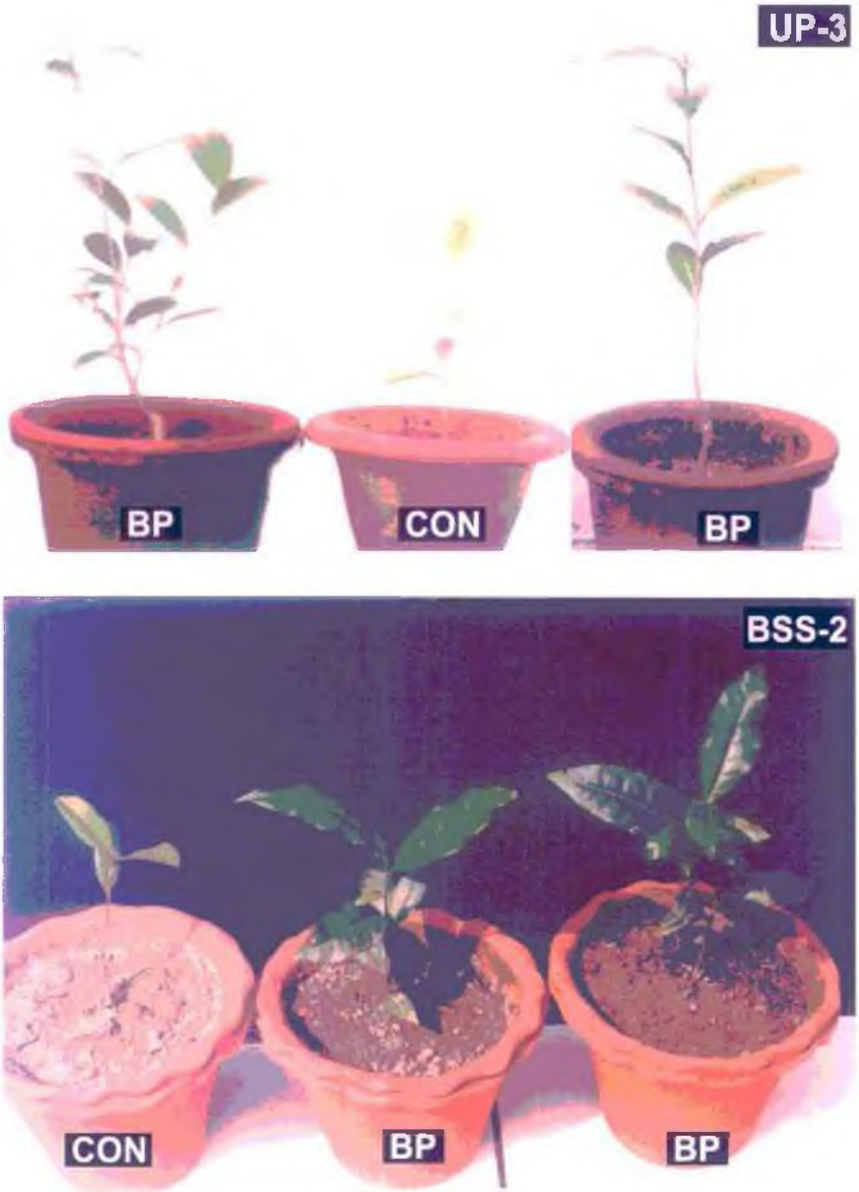


Plate XX: Growth promotion in tea seedling by application of PGPR based talcum formulation; BP = *Bacillus pumilus;* CON = Control.

Table 40: Growth promotion in Tea plants by Talcum based formulation

Varieties	Treament	2 months		4 months	
		Increase in Height(cm)	Increase in No. of leaves	Increase in Height(cm)	Increase in No. of leaves
UP-3	Control	0.2±0.02	0±0.00	0.7±0.03	1.0±0.00
	B.pumilus	3.0±0.06	3.0±0.29	4.5±0.33	5.0±0.58
TV-18	Control	0.5±0.00	1.0±0.00	1.5±0.11	2.0±0.02
	B.pumilus	3.0±0.46	3.0±1.16	6.0±1.33	5.0±1.167
BSS-2	Control	0.5±0.00	1.0±0.00	1.5±0.23	1.0±0.00
	B.pumilus	3.0±0.11	4±0.58	4.5±1.7	7.0±0.58

Average of 10 replicate plants per treatment; ± S.E.

Table 41: Immunodetection of *B.pumilus* in bioformulation after application in rhizosphere

Antigen source	Absorbance at 405 nm*	
	2 months	3 months
Talcum	1.435±0.02	0.876±0.03
UP-26	1.023±0.02	1.011±0.04
UP-3	1.005±0.08	0.941±0.05
CP-1	1.098±0.01	0.974±0.06
HV-39	1.120±0.03	1.026±0.09

Average of 3 replicates; PAb dilution: 1: 500; ± S.E.; Antigens reacted with PAb of *B.pumilus*