Experimental

4.1. Charcoal stump rot disease

The fungus, *Ustulina zonata*[lev.] Sacc. *Ustulina deusta* (Fr.) Petrak. cause charcoal stump rot of tea and other trees in forest areas in tropics. The fungus was originally named *Sphaeria zonata* Leville. Agnihothrudu mentioned that it was different from *Sphaeria deusta* Hoffmann in its reddish brown colour and the presence of zonation. The imperfect form has not been referred to and Saccardo transferred the fungus to *Ustulina*.. This disease occurs on all soils and is probably the most common of root diseases of tea. It is one of the primary root disease of tea through out north east Indian tea plantation. Younger bushes which are attacked die suddenly and there are instances when the older bushes are only partially dead with the withered leaves remaining attached to the bush. In Malaya the disease caused by this fungus is found on old rubber areas in every part of the peninsula. It has been recorded attacking *Hevea* in java, Fiji and Ceylon. In the latter country, it has been known for many years as the commonest root disease on tea.

As the charcoal stump is primary root disease the fungus (*U. zonata*) remain in the root system for several years and appear normal up till the time of death, which takes place suddenly. The foliage wilts and dies but the withered leaves remain attached to the branches for some time before they drop off. Gradually the disease extends along the roots killing the host tissues until it reaches the collar, the water conducting cell which make up the wood become gradually blocked by the mycelium or threads of the fungus which weave in and out of the tissues. Eventually the fungus completely blocked the water conducting tissues at or near the collar, so that passage of water from the roots to the leaves is stopped and the bush suddenly dies. The process may take about 1-4 yrs, according to age and size of the bush.

The disease shown up prominently on young trees, though occasional attacks on trees not more then two to three years of age have been recorded in Temi Tea Estate. On one side of the stem a hollow at the base may be formed due to the rotting tissue disintegrating under the influence of the weather and falling away. When a cut was made below the surface at such a point, it was found that the rotting tissue has spread to a distance of three to four feet above ground level. When an attacked root system was

examined, it was found that the taproot and some of the larger laterals were involved, and as a result water conduction was stopped along these roots.

In some cases, conspicuous black lines have been noticed on the outer surface of the wood and they form thin black plates running through the internal tissues of the woody stem , their edges showing as black lines on the exterior (Plate 5, fig.D).

A longitudinal section taken through the collar of a diseased tree shows these black lines running irregularly in the rotting tissues, often forming circles surrounding dark colored patches of diseased wood. The black lines are composed of aggregated fungus tissue, formed by the massing of hyaline hyphae; this massing always commences in the medullary rays cells. Tracts of connecting cells between the rays later become filled with similar tissue and a continuous line is formed. What appears to be carbonaceous material is deposited in the infested cells after the aggregation therein of the fungus hyphae, and as time passes it is difficult to detect the exact origin of the lines. The cells bordering the black lines are crowded with hyaline hyphae. Such aggregations of fungal hyphae might be considered rhiozomorphs, but as they are strictly confined to the diseased tissues they have not the power of growing freely along the exterior of the root and so cannot effectively function as organ for vegetative spread in area. But they retain their vitality for considerable periods and if portions of diseased wood, containing black lines, become scattered about in the soil, there is a definite chance that roots of healthy trees, which come into contact with such infective material, will contract the disease. Young specimens showed a well defined zoning on the surface. When these plates were closely examined their was an opening into a small globose cavities, the perithecia, from which the black ascospores are ejected. The asci numerous with paraphyses, the mature ascospore measures 28-32μ×7-10μ.

4.1.1. Disease incidence in Sikkim hills

Temi Tea Estate is turning in fully organic, manuring is usually done with organic fertilizers like Cattle manure, Sterilized animal meal etc. are being used at the time of planting. Shading is not necessary because of its high altitude and Pruning is usually done during the winter season between December to February. Charcoal stump rot disease caused by *Ustulina zonata* has been located in various plantation sectors in the said

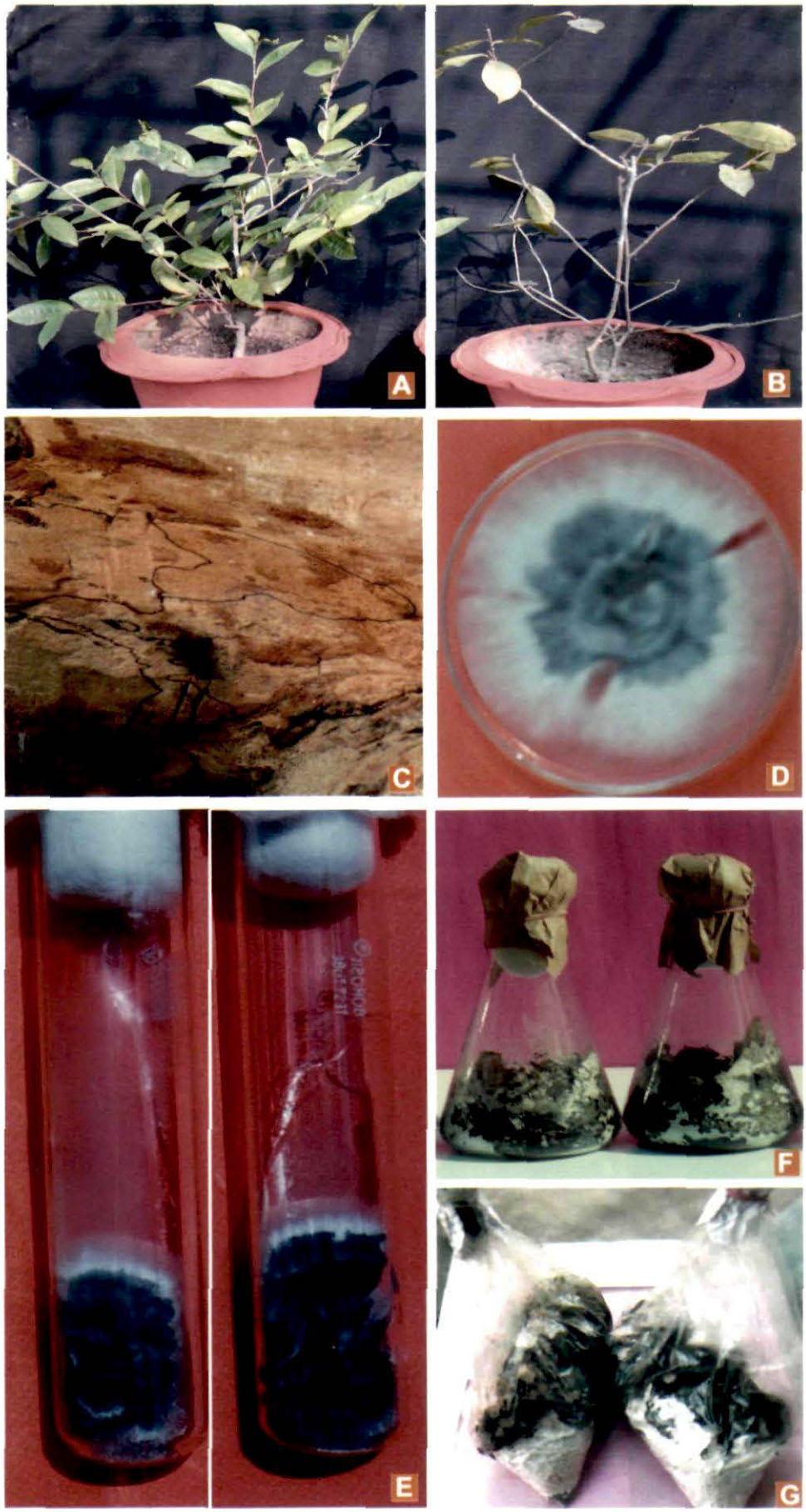


Plate 5(A-G): Healthy tea plants grown in pot (A), infected tea plants, 60 days after inoculation with *U.zonata* (B), tea plants showing symptoms of charcoal stump rot disease (C), Radial growth of *U.zonata* (D). *U. zonata* grown in PDA slants (E), sand maize meal medium in conical flask (F) and polythene bags (G)

garden during the survey period. The disease was identified in the garden following six standard keys.

i. external appearance of bushes in the field.
ii. indication on the collar region.
iii. external appearance of the surface of the roots when the bush is dug out.
iv. indicators when the bark is peeled away from the roots.
v. identifications when cut is made into the root with knife.
vi. leaf diseases.

Whole bushes die suddenly, leaves green to reddish in color, withered leaves remain attached or part of bush dies suddenly, mature bush with part dead and remainder apparently normal and healthy. Dead part completely rotten; collar with encrustation, encrustation initially white, then turned greenish-grey, finally black. Surface undulated, brittle, charcoal like when broken. Roots without cords or strands of mycelium on the surface, roots with cushions of lumps, white or black, thin white film of mycelium on wood surface, wood with dark bands or reticulation with dark black bands only wood normal. All above given symptoms confirms that plants is infected with primary root disease of tea *i.e* charcoal stump rot caused by *Ustulina zonata.* When a disease is in the early stage, all the symptoms described in the keys may not be noticeable. Naturally infected tea roots showing symptoms of charcoal stump rot (Plate 5, fig.D) in Temi Tea Estate, Sikkim was uprooted and brought to Immuno-Phytopathology Laboratory. The pathogen (*U. zonata*) was isolated (Plate 5, fig. C & E) from the naturally infected tea roots and after completion of Koch's postulate the pathogen was used for present investigation.

4.1.2. Varietal resistance test against *Ustulina zonata*

Varietal resistance test of tea against *U. zonata* was carried out with five tea varieties (TeenAli-17, TV-18, TV-23, TV-25, TV-26). Three year old plant roots were inoculated with *U. zonata* and disease assessment was done on the basis of visual observation of symptoms and disease index (ranging from 1-6) was calculated after 20,40 and 60 days following inoculation. Results have been presented in Table-1. Among the tested tea varieties TV-25 and TV-18 were found to be highly susceptible in comparison with other three tea varieties (T-17, TV-23 and TV-26).

Defoliation of leaves following infection with *U. zonata* was evident in TV-25 after 60 days of inoculation (Plate 5, fig.B) in relation to healthy control plant (Plate 5, fig A).

Table 1: Varietal resistance test of tea varieties against *U. zonata*

Tea varieties	Disease index		
	20 days	40 days	60 days
T-17	1.1	1.9	2.0
TV-18	1.0	2.0	3.7
TV-23	0.5	2.0	2.8
TV-25	1.9	3.5	4.8
TV-26	0.5	2.0	2.9

Average of 50 separate inoculated plants of each variety

4.2 Cultural characteristics of the pathogen (*U. zonata*)

Mycelial growth of *U. zonata, in vitro* showed variation depending on different factors like medium, pH, temperature and available carbon and nitrogen sources. The young mycelia of *U. zonata* were white or hyaline initially, which turned into grayish black in clour, gradually become brittle crust. The mycelial growth was generally superficial with fan shaped dull silky white mycelia or small black or white cushion like growth was found depending on the medium. Growth is equal from all sides; reach to its optimum during rainy season (July-August). Optimal growth condition was determined by growing the *U. zonata* in different growth conditions by keeping the culture in different temperature, pH, incubation period and various carbon and nitrogen sources (Figure 1A&B; Tables 2, 3, 4, 5 and 6).

4.2.1 Media

U. zonata was grown in eight different media i.e. Potato dextrose agar (PDA), Potato sucrose agar (PSA), Richard's Agar (RA), Carrot juice agar (CJA) Czapek-Dox agar (CDA), Flentze's soil agar (FSEA), Malt extract peptone dextrose agar (MPDA), Yeast extract- dextrose agar (YDA). Results revealed that the maximum growth was recorded in PDA followed by PSA and RA but minimum growth was recorded in CDA. The fungus shows submerged, translucent mycelia to surfaced thick white growth which gets shriveled and turn dark, with black grey sporulation in oval patches (Table 2).

Table 2: Effect of different media on mycelial growth of *U. zonata*

Medium	Diameter of mycelium (mm)			
	4day	7 day	10day	13 day
Czapek dox agar	2.5±0.5	4.1±0.6	7.4± 1.0	9.5±1.5
Malt extract agar	-	-	-	-
Nutrient agar	1.4±0.5	2.0± 0.5	4.1± 0.7	8.0±1.0
PDA	2.6±0.5	4.1±0.6	8.3±1.0	14.4 ±2.1
Richard's medium	3.1± 0.5	5.2± 0.7	9.5±1.0	15.7±2.5

** Incubation temperature 25±1 °C, average of three replicates.

4.2.2. Temperature

U. zonata was grown in different temperature range. And the result revealed that it grew best at 25 °C -28°C.

4.2.3. Incubation period

U. zonata was grown in PDA medium for a period of 30 days, mycelial growth was recorded after 5,10,15,20,25,30,35 and 40 days of growth and incubated at 25± 1°C. Maximum growth was recorded after 20 days of incubation after which it declined. After 5 days of incubation the growth was negligible (Table 3).

Table 3: Effects of incubation period on mycelial growth of *U. zonata*

Incubation period (Day)	Mean mycelial dry weight (mg) [a]
5	07.60 ±0.42
10	19.5± 0.80
15	37.3 ± 0.25
20	69.6± 0.40
25	77.10± 0.50
30	74.8 ± 0.51
35	66.2±0.40
40	67.2±0.37

a= Average of three replicates, ± = standard error, temp.25 ±1 °C, pH of medium is 5.0

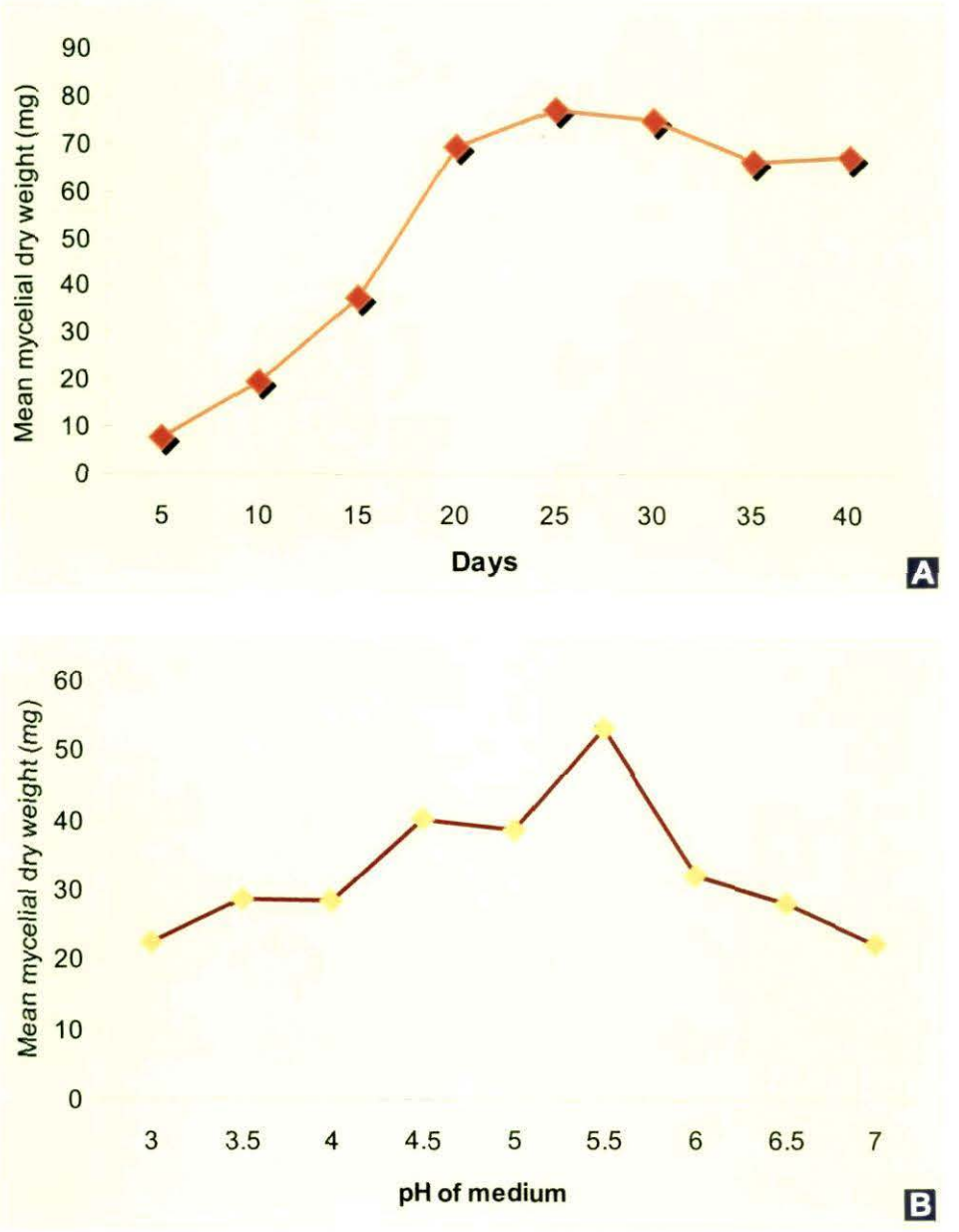


Fig.1(A-B): Effects of incubation period (A) and different pH (B) on mycelial growth of *U.zonata*

4.2.4 pH

pH of the medium plays an important role in the growth of microorganism. In order to determine the effect of pH, buffer system have to be used to stabilize the pH, initially buffer solution with pH values ranging from 3.0 to 8.0 (3.0, 3.5,4.0, 4.5, 5.0,5.5, 6.0, 6.5, 7.0 and 8.0) were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of 0.03M. The pH finally adjusted using N/10 HCl or N/10 NaOH in each case. Potato dextrose medium and phosphate buffer was sterilized separately by autoclaving for 15min at 15 lb p.s.i pressure and equal parts of the buffer solution and medium were mixes before use in Laminar Flow Bench. After mixing flasks were inoculated and incubated for 20 days after which dry wt. was taken as described previously. Results revealed that *U. zonata* grew to a lesser or greater extent over a wide range of pH (3.0-8.0), maximum growth was observed at pH 5.5 and then growth gradually declined. (Table 4).

Table 4: Effects of different pH on mycelial growth of *U. zonata*

pH of medium	**Mean mycelial dry weight (mg)a**
3.0	22.50± 0.55 -
3.5	28.80 ± 0.48
4.0	28. 34 ± 1.20
4.5	40.12 ± 0.24
5.0	38.62 ± 2.14
5.5	53.32 ± 2.52
6.0	32.4 ± 2.41
6.5	28.10 ± 2.30
7.0	22.21 ± 1.47
8.0	-

A= average of three replicates, temperature = 25±1°C, incubation time 25 days.

4.2.5 Carbon source

Like the pH of the surrounding medium the growth of fungus is greatly influenced by available nutrients. The ability of fungi to grow in different media depends on their capacity to utilize by 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 investigations, eight different carbon sources (dextrose, fructose, lactose, mannitol, maltose, sorbose, starch and sucrose) were tested for their effect on the growth of *U. zonata*. These were added separately to the basal medium. PDA medium without sugar was used as the basal medium which served as control set. Data were recorded after 20 days of incubation. Results (Table 5, Figure 2A) revealed maximum growth using lactose as the carbon source while no growth was observed in sorbose which was similar to control set. Fructose and sucrose also supported comparatively good growth.

4.2.6. Nitrogen source

The availability of nitrogen for growth of the organism depends on the form in which it is supplied. Hence the most suitable medium for any particular microorganism can only be determined by testing a number of sources including both organic and inorganic. The effect of inorganic nitrogen sources (ammonium nitrate, ammonium sulphate, calcium nitrate, potassium nitrate and sodium nitrate) as well as complex organic sources (casein hydrolysate, beef extract, peptone, urea and yeast extract) on the mycelial growth of *U.zonata* was tested. A basal medium without any nitrogen source was considered as control. Results (Table 6, Figure 2B) revealed maximum growth in beef extract followed by yeast extract and then in peptone. Among the inorganic sources calcium nitrate supported maximum growth. Other inorganic sources supported lower growth than organic sources, though no growth was observed in urea and insignificant growth was noted in basal medium without nitrogen.

Table 5: Effect of different carbon sources on mycelial growth of *U. zonata*

Carbon Sources	**Mean mycelial dry wt (mg)***
Fructose	152.66±2.745
Sorbose	02.50± 0.056
Dextrose	65.40± 0.289
Mannitol	50.20± 1.233
Sucrose	49.80±1.326
Starch	46.13± 1.322
Maltose	39.21± 0.945
Lactose	158.23± 1.234
Control	02.70± 0.175

*Average of three replicates, ± = standard error; temperature 25±1°C: incubation period 20 days.

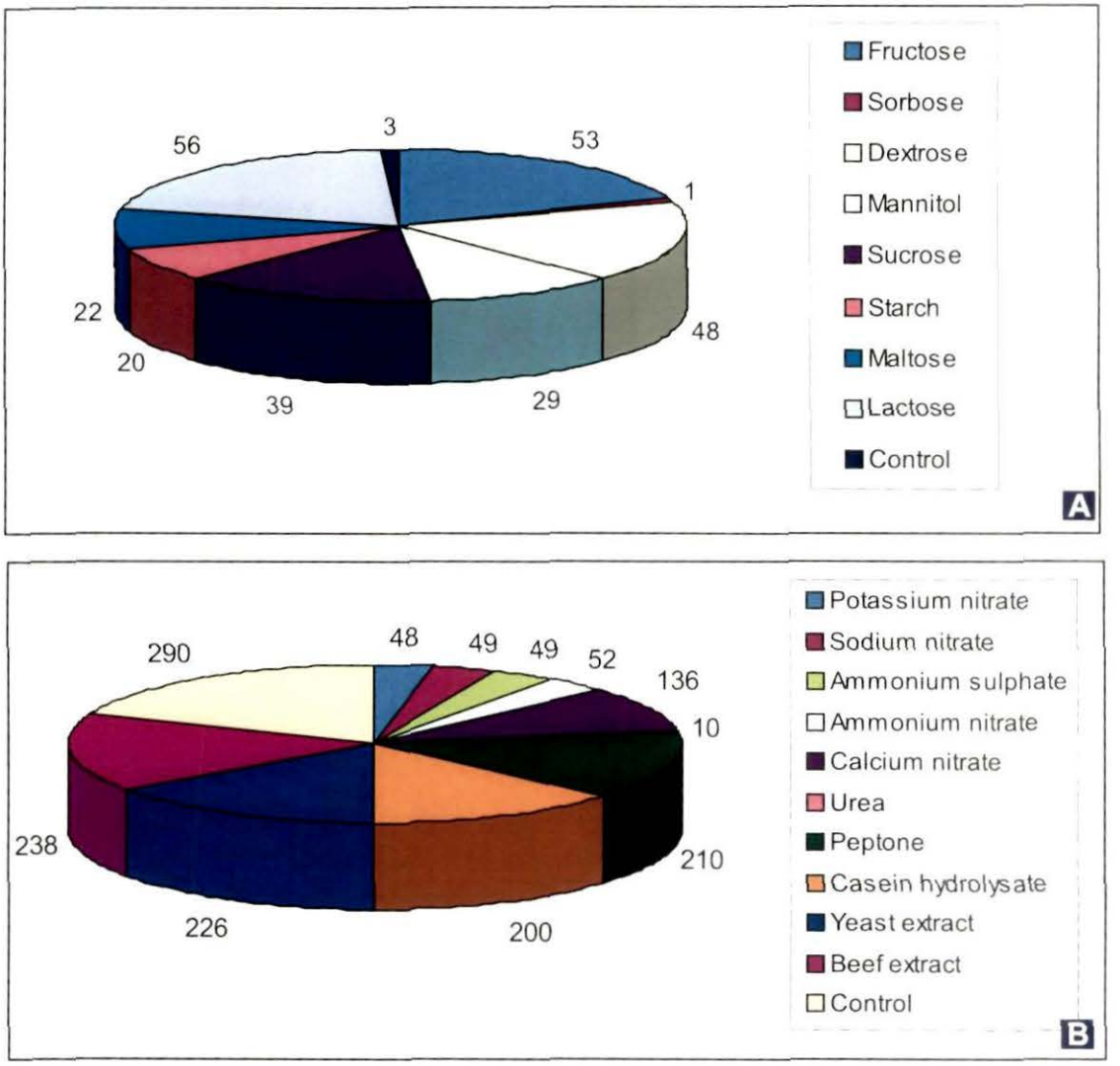


Fig 2 (A-B): Effect of Carbon sources (A) and Nitrogen sources (B) on mycelial growth of *U.zonata*

Table 6: Effects of different nitrogen sources on mycelial growth of *U. zonata*

Nitrogen Sources	Mean dry wt of fungal mass (mg) *
Inorganic	
Potassium nitrate	30.3± 2.97
Sodium nitrate	31.3±1.72
Ammonium sulphate	32.1± 0.26
Ammonium nitrate	45.9± 1.50
Calcium nitrate	119± 4.25
Organic	
Urea	--
Peptone	205.8± 2.63
Casein hydrolysate	189.7± 1.57
Yeast extracts	219.6± 5.19
Beef extract	285.5± 5.19
Control (without nitrogen)	3.4± 0.36

* Average of three replicates, ± standard error,
Temperature 25± 1°C; incubation period 25 days

4.3. Analyses of soil samples of Temi Tea Estate

The soil samples were collected from six different locations from each block of Temi Tea Estate and these were coded with TTS1 , TTS2, TTS3, TTS4, TTS5 and TTS6 and were given for analysis in Soil Testing laboratory, Institute of Plantation Science and Management, North Bengal University soil before the isolation of microorganisms. Moisture content, pH for soil type, soil texture, carbon and nitrogen ratio, K and P available etc were determined for all six soil samples. Results have been presented in Table 7.

Table 7: Analyses of soil samples of Temi Tea Estate, Sikkim

Sample code	Moisture %	pH	Organic carbon	nitrogen	Soil type	Soil texture			Available	
						% of silt	% of clay	% of sand	K_2O (ppm)	P_2O_5 (ppm)
TTS1	16.37	4.38	1.05	0.11	Clay	10.00	51.00	39.00	120.96	38.09
TTS2	19.37	4.11	1.15	0.12	Sandy Clay	5.00	45.00	50.00	154.57	40.26
TTS3	14.58	3.90	1.10	0.11	Sandy Clay	2.00	43.00	55.00	88.70	31.56
TTS4	26.35	4.43	1.27	0,13	Sandy Clay	4.00	40.00	50.00	123.65	26.12
TTS5	18.67	4.69	1.35	0.14	Sandy Clay	10.00	34.00	56.00	177.41	50.06
TTS6	20.25	4.76	1.09	0.11	Sandy Clay	13.00	42.00	52.00	168.00	54.41

4.4. Isolation of microorganisms from tea rhizosphere and their identification

4.4.1. Isolation of microrganisms

The soil samples collected from the rhizosphere of the healthy tea plants of different age growing in different sites of the Temi Tea Garden, South Sikkim were brought to the laboratory and 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 rhizosphere. Microbial population determined in soils, ranged between $5x10^5$- $15x\ 10^5$cfu in case of fungi and $10x10^6$cfu-$30x10^6$cfu in case of bacteria. Results show that the maximum population was observed in the rhizosphere of 35 year old bushes and minimum in 10 year old bushes is summarized in Table (8 & 9). Individual fungal and bacterial isolates were transferred in PDA and NA slants (Plate 6, figs A-C). The maximum microbial population was obtained in the hot humid months, which decreased during winter.

Table 8: Fungal population and number of isolates from tea rhizosphere

Soil samples (cfu g^{-1}soil)	Age/years isolates	Microbial population	No. of fungal isolates
Block 1	10	5×10^5	4
Block 2	20	5×10^5	4
Bolck 3	30	10×10^5	5
Block 4	35	15×10^5	5

Table 9: Bacterial population and number of isolates from tea rhizosphere

Soil sample	Age/year	Microbial population (Cfug-1 soil)	No. of bacterial isolates
Block 1	10	10×10^6	4
Block 2	30	$12x\ 10^6$	4
Bolck 3	20	16×10^6	5
Block 4	35	30×10^6	6

4.4.2. Identification of fungal isolates

The isolated fungi were allowed to grow in Petri dishes containing sterile PDA medium for 7-15 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 suspension 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, Colletotrichum* and *Macrophomina* (Table 10).

Plate 6(A-C): Microorganisms isolated from tea rhizosphere of Temi Tea Garden. Fungal isolates (A&B) and bacterial isolates (C)

Table 10: Characterization and Identification of fungal isolates of tea rhizosphere.

Isolates code	Mycelial character	Conidiophores	conidia	name of organisms
Ttf1	slow growing	large globose vesicles, bearing inflated, club metulae and narrow phialides	Globose to Ellipsoidal smoo- Th, walled 2.5 3.5 mm in dia., uninuc leate.	*Aspergillus candidum*
Ttf2	Blue green color	1.5- 3.0mm tall, arising From specialized wide- ned hyphal cells which becomes the branch- ing foot cells.	Smooth-walled, 3.0-4.5x2.5-3.5µm and Uninuclate.	*Apergillus clava- tum.*
Ttf3	Yellow green to greenish brown.	Hyaline, 0.4- 1.0 mm Long, rough- walled, present.	Globose to sub-globose, roughed to echi- Nucleate, 3.5-4.5 µm in dia. 1-4 nucleate.	*Aspergillus flavus.*
Ttf4, Ttf19, Ttf20	Typically black, Powdery.	Arising from long, broad Foot cells, 1.5-3.0mm Tall	Mostly globose, Irregularly rough- ened . 4.0-5.0 µm dia., uninucleate	*Aspergillus niger*
Ttf5	Black	Simple, straight, 1-3 septate, 5µm long, 3- 6 µm wide, golden brow obclavate.	pigmented conidia for long branching c Chains, ovoid, obyri- Form with a short ap- Ical beak, smooth wall 3-8 septa, base Broadly round, each Portion of lower part With 1-2 longitudinal Septa, 18-65x 7-118 µm	*Alternaria alternata*
Ttf6	Black	simple, become genicul sympodial elongation,	conidia have borne singly, muriform, beak 5-10 transverse septa, and 120-295 x12=20 µm	*Alternaria solani*
Ttf7	brown	Metulae present.	globose to subglobose	*Aspergillus nidulae*
Ttf8		Pigmented conidiophore With clavate vesicles ar From clearly differentiat thick walled foot cells.	strictly columnar conidial heads, con- idia globose to sub- globose, echinulate, 2.5-3.0 µm.	*Aspergillus fumigatus*

Ttf9	Black	Conidiophore erect, pigr nted, geniculated from s mpodial elongations, 3-1 septate.	Conidia olive brown Curved ellipsoidal, 3- septate,rounded at the apex slightly acuminate at the base, the mid-dle septum below the centre and the third cell strongly curved, 20-30x 9-15 µm.	*Curvularia lunata*
Ttf10		Pigmented ,erect conidio hore.	conidia predominantly 4-septate, the central cell Distinctly geniculated, ta ering gradually towards each end, 18-37x8-14 µm.	*Curvularia geniculatum*
Ttf11	white	Simple, lateral phialides short, sparsely branched.	microconidia never in chain, mostly non-sepa-tate. Ellipsoidal to cylin -drical, 5-12x2.3-3.3 µm Macroconidia fusiform, hyaline, poin-ted at both ends basal cells pedicellate, 27-46x 3.0-4 µm	*Fusarium oxysporum*
Ttf12	Fast growing, green to bluish brown.	Short ,branched conidiophores 8-16x 2-4 µm.	Microconidia abundant , chlamydospores borne Singly, sometimes in pairs, in terminal. Latera ,hyaline, smooth walle-d 6-10 µm	*Fusarium solani*
Ttf13	Greenish blue	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	*Penicillum oxalicum*
Ttf14		Conidiophores are hyaline, short obpyriform to cylindrical producing aseptate, hyaline, oval. enteroblastic conidia		*Macrophomina phaseolina*
Ttf15	Freely branched Filaments,cottony white mycelium la on become black	Sporangiophores pale to dark brown, straight, mostly 1.5mm tall, 20-25 µm wide, each sporangiophores	sporangium, biconical to oval, ridged, mostly 4 nucleate 7-12x6-8.5 µm.	*Rhizopus stolonifer*

		bear globose to subglobose multispored columellate sporangium.		
Ttf16		Conidiophores are hyaline to brown, septate, branched at the base, smooth, formed from the upper cells of the frutification.	Conidia hyaline, unicel-Lular, falcate or lur cylindrical, more or less Guttu;ate, muticate or v apex prolonged into a cellular appendage, p from phialides.	*Colletotrichum sp.*
Ttf17	Light yellow	Smooth –walled, 500-700 µm long, vesicles elongate with metulae and phialides	Globose, echinucleate ,2-3 µm in diameter: uninuclate	*Aspergillus versicolor*
Ttf18	Velvety, orange-brown	100-250 µm long, smooth-walled, hyaline, metulae present	globose to slightly ellipsoidal, 1.8-1.4 µm in diameter: uninuclate	*Aspergillus terreus*

4.5. Rhizosphere microorganisms of tea and their *in vitro* interaction with root pathogens

All the isolated fungus and bacteria were grown in solid and liquid media were tested for their antagonistic activity against certain root pathogens – *Ustulina zonata, Fomes lamaoensis, Sclerotium rolfsii,* by dual paring tests. Their interactions were categorized into four type-A: Homogenous; free intermingling between pairing microorganisms, B: Overgrowth; pathogen overgrown by the test organisms, C: Inhibition; a clear zone of inhibition and D: Cessation of growth at time of contact. Different types of reactions developed in the pairing experiments are enlisted in Table 11 & 12.

Table 11: Effects of interactions between the fungal isolates and tea root pathogens

Source of soil	Total no. isolates	Isolate No.	Type of reactions in test against		
			S. rolfsii	*F.lamaoensis*	*U. zonata*
Block-1	04	Ttf1	B	C	-
		Ttf2	B	B	B
		Ttf3	C	B	B
		Ttf4	C	C	C
Block-2	05	Ttf5	B	B	B
		Ttf6	B	C	B
		Ttf7	B	B	C
		Ttf8	B	C	C
		Ttf9	B	B	B
Block-3	03	Ttf10	B	C	B
		Ttf11	B	B	B
		Ttf12	B	C	B
Block-4	04	Ttf13	B	B	B
		Ttf14	B	B	B
		Ttf15	C	B	C
		Ttb16	C	B	B
Block-5	04	Ttf17	C	C	B
		Ttf18	C	C	B
		Ttf19	B	B	C
		Ttf20	B	B	B

A: Homogenous, B: Overgrowth, C: Inhibition, D: Cessation.

Table 12: Effects of interactions between the bacterial isolates and tea root pathogens

Source of soil	Total no. isolates	Isolate No.	Type of reactions in test against		
			S. rolfsii	*F.lamaoensis*	*U. zonata*
Block-1	04	Ttb1	B	B	B
		Ttb2	B	B	B
		Ttb3	B	B	B
		Ttb4	C	C	B
Block-2	05	Ttb5	B	B	B
		Ttb6	B	C	B
		Ttb7	B	B	B
		Ttb8	B	B	B
		Ttb9	C	C	-
Block-3	03	Ttb10	B	C	C
		Ttb11	B	B	B
		Tta12	C	B	B
Block-4	04	Ttb13	B	B	B
		Ttb14	C	C	C
		Ttb15	C	B	B
		Ttb16	B	C	B
Block-5	04	Ttb17	B	C	B
		Ttb18	B	C	C
		Ttb19	B	B	B
		Ttb20	C	B	C

A: Homogenous, B: Overgrowth,C: Inhibition, D: Cessation.

Among all tested microorganism, it was observed that five of the isolates viz. Ttf4 , Ttf8, Ttf17, Ttb9,Ttb10, Ttb14 and Ttb20 showed antagonistic activity against the soil borne pathogens. The fungal isolate Ttf4 Showed highest inhibitory effect on against all the tested pathogens (Table 11). The antagonistic test was also done in liquid culture. In case of bacterial isolates, Ttb14 showed maximum inhibitory effect on all the test pathogen (Table 12). Among these fungal and bacterial antagonists only one fungal (Ttf4) and bacterial isolate (Ttb14) were selected for further investigation. Based on the morphological as well as antagonistic study the fungal isolate Ttf 4 was identified as *Aspergillus niger.*

Among bacterial isolates Ttb14 which showed maximum inhibitory effect against the tested tea root pathogens (*U. zonata*, *F. lamaoensis* and *S. rolfsii*) was further studied

under microscope after suitable staining and characterized based on morphological and biochemical studies following Bergeys manual of systematic Bacteriology as mentioned in Table 13 as well as scanning electron microscopic observations (Plate 7, fig.C) identified it as *Bacillus pumilus* which was also confirmed by CABI- Bioscience, UK.

Table 13: Morphological and biochemical test of isolate Ttb14

Characterization/morphological	**Isolate Ttb14**
Colony Shape	circular
Cell shape	Rod
Size (μm)	2-3x0.7-0.8
Capsule/slime layer	-
Motility	+
Gram reaction	+
Endospore	+
Margin	+
Surface	wavy
Pigmentation	white
Density	opaque
Biochemical	
V.P reaction	-
Catalase production	+
Oxidase production	+
Urea digestion	-
Esculine hydrolysis	+
Casein hydrolysis	-
Starch hydrolysis	-
Indole test	+
Nitrate reduction	-
Phenol red Tartarate	-
Citrate utilization	+

+ positive reaction, - negative reaction

4.6. Screening of phosphorus solubilizing fungi and bacteria from tea rhizosphere

Phosphorus solubilizers produce clearing zones around the microbial colonies in media. Insoluble mineral phosphates such as tricalcium phosphate or hydroxyapatite are contained in the media. The principal mechanism for mineral phosphate solubilization is

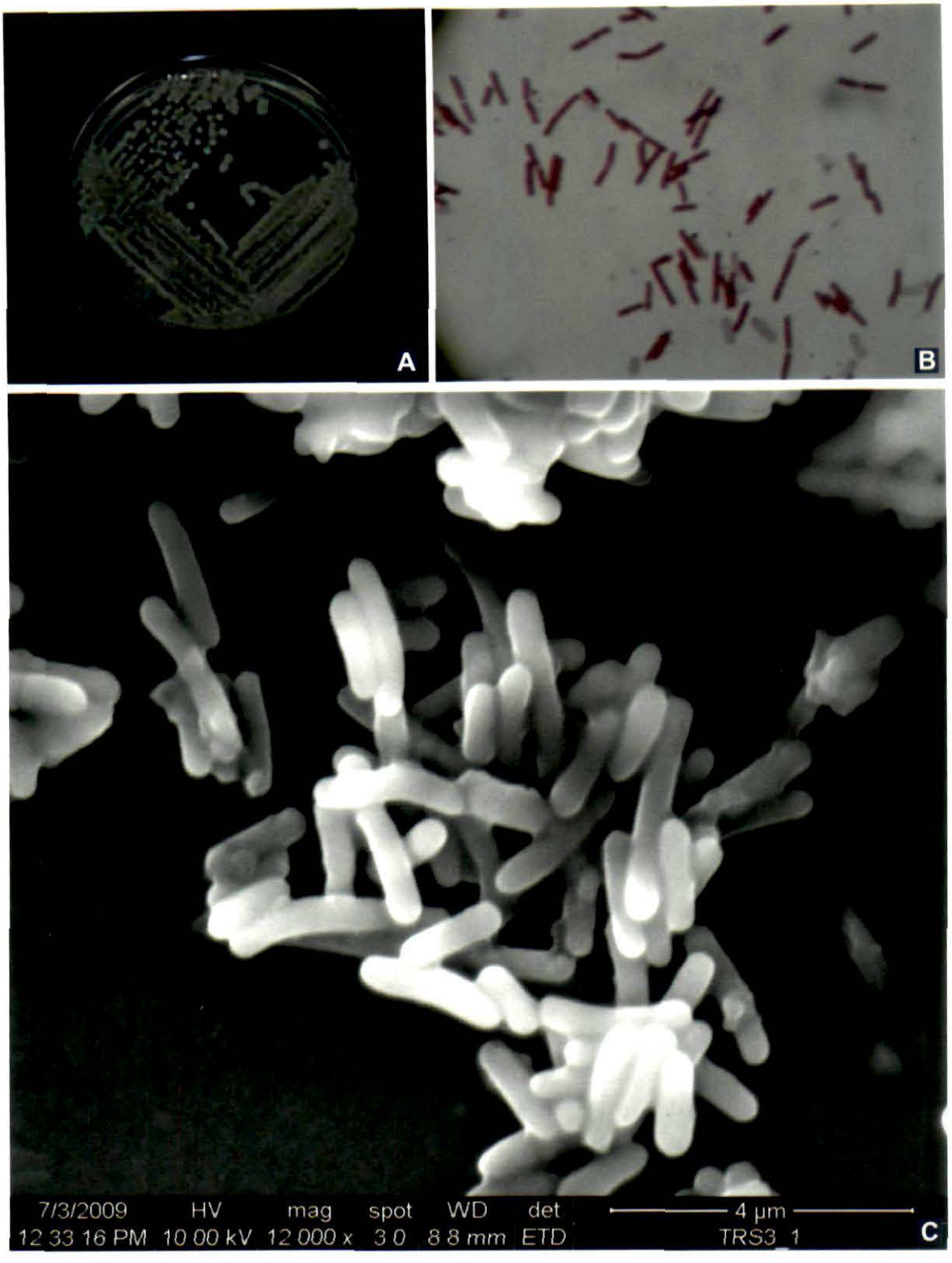


Plate 7(A-C): Phosphate solubilizing bacterium; *Bacillus pumilus*. Streaking NA media (A); Microscopic view after gram staining (B); Scanning electron microscopic view (C)

the production of organic acids, and acid phosphatases play a major role in the mineralization of organic phosphorus in soil. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Production of organic acids results in acidification of the microbial cell and its surroundings. The fungal and bacterial isolates were screened for phosphate solubilizing activity in PVK medium. Formation of halo zones around the colony indicated positive results (Plate 8,figs A-G) . Among fungal isolates 5 and among bacterial isolates 4 showed phosphate solubilizing activity.

4.6.1. Phosphorus solubilization efficiency on PVK plates

Preliminary screening for phosphate solubilization was done by a plate assay method using Pikovskaya (PVK) agar medium supplemented with tricalcium phosphate (TCP). The pH of the media was adjusted to 7.0 before autoclaving. Sterilized PVK media was poured into sterilized Petri plates after solidification of the media, a pinpoint inoculation of fungal and bacterial isolates was made onto the plates under aseptic conditions. They were incubated at 28±2°C for 7 days with continuous observation for colony diameter. The halo zone formations around the growing colony showing phosphate solubilization. Solubilization index was evaluated according to the ratio of the total diameter (colony + halo zone) and the colony diameter.

Phosphate solubilizing activities of both fungal isolates and bacterial isolates as evident in PVK medium have been presented in Plate 8(figs A-G). In case of *Aspergillus niger* (Ttf4) reached to maximum on the day fourth then remain constant at the end of the week whereas *Aspergillus nidulae* (Ttf7) solubilization started within 24 h and reached maximum value on day three and remain constant throughout the week. Among the isolates maximum phosphate solubilizing activity was shown by isolates *Aspergillus niger* (Ttf 4) and *Aspergillus versicolor* (Ttf 17) and the zone of inhibition formed by both fungal isolates and bacterial isolates have been presented in Tables 14 and 15.

4.6.2. Evaluation of phosphorus solubilization by fungal isolates in PVK broth amended with tricalcium phosphate (TCP) and rock phosphate (RP)

Five PSF isolates *Aspergillus niger* (Ttf4); *Aspergillus nidulae* (Ttf7); *Aspergillus fumigatus* (Ttf8), *Aspergillus versicolor* (Ttf17) and *Aspergillus terreus* (Ttf18) were

further evaluated in PVK liquid media amended with tricalcium phosphate (TCP) and rock phosphate (RP) to assess their phosphorus solubilization capacity. Results have been presented in Table 16; Figure 3.The pH of the cultural broth samples dropped significantly as compared to the control where it remained constant around pH 7.0. *Aspergillus niger* (Tff 4) caused decrease in pH from 7, at the beginning to 3.7 which was attributed to the varying diffusion rates of different organic acids secreted by the tested organisms. *Aspergillus terreus* (Ttf18) showed better efficiency of TCP solubilization after seven days of incubation (Table 16)

Table 14: Showing clear zone formation by the selected fungal isolates

Name of isolates	Clear zone (cm)*					
	24 h	48 h	72 h	96h	120h	144h
Aspergillus niger (Ttf4)	-	0.2	0.5	0.6	0.6	0.6
Aspergillus nidulae (Ttf7)	0.2	0.4	0.5	0.5	0.5	0.5
Aspergillus fumigatus (Ttf8)	-	0.3	0.4	0.4	0.5	0.5
Aspergillus versicolor (Ttf17)	-	0.3	0.4	0.5	0.6	0.6
Aspergillus terreus (Ttf18)	0.2	0.2	0.3	0.5	0.5	0.5

*Average of three replicates.

Table 15: Showing clear zone formation by the selected bacterial isolates

Bacterial isolates (Code)	Clear zone (cm)					
	24 h	48 h	72h	96h	120h	144h
Ttb9	-	0.2	0.4	0.5	0.5	0.5
Ttb14	0.2	0.4	0.5	0.6	0.6	0.6
Ttb17	0.2	0.3	0.4	0.5	0.5	0.5
Ttb20	0.2	0.3	0.5	0.5	0.5	0.5

*Average of three replicates.

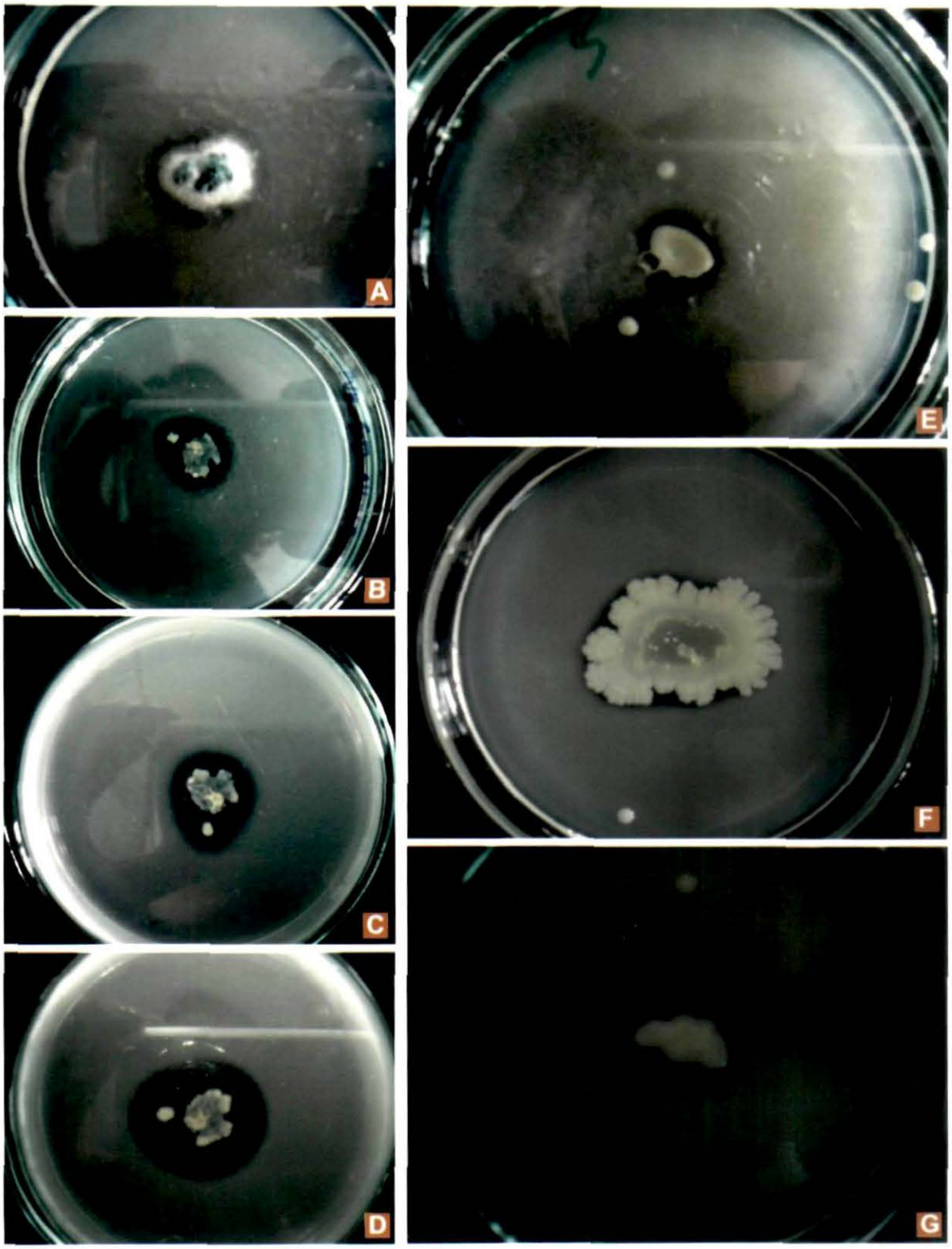


Plate 8(A-G): *In vitro* characterization of microorganisms for phosphate solubilization in PVK medium. Fungal isolates (A-D); Bacterial isolates (E-G)

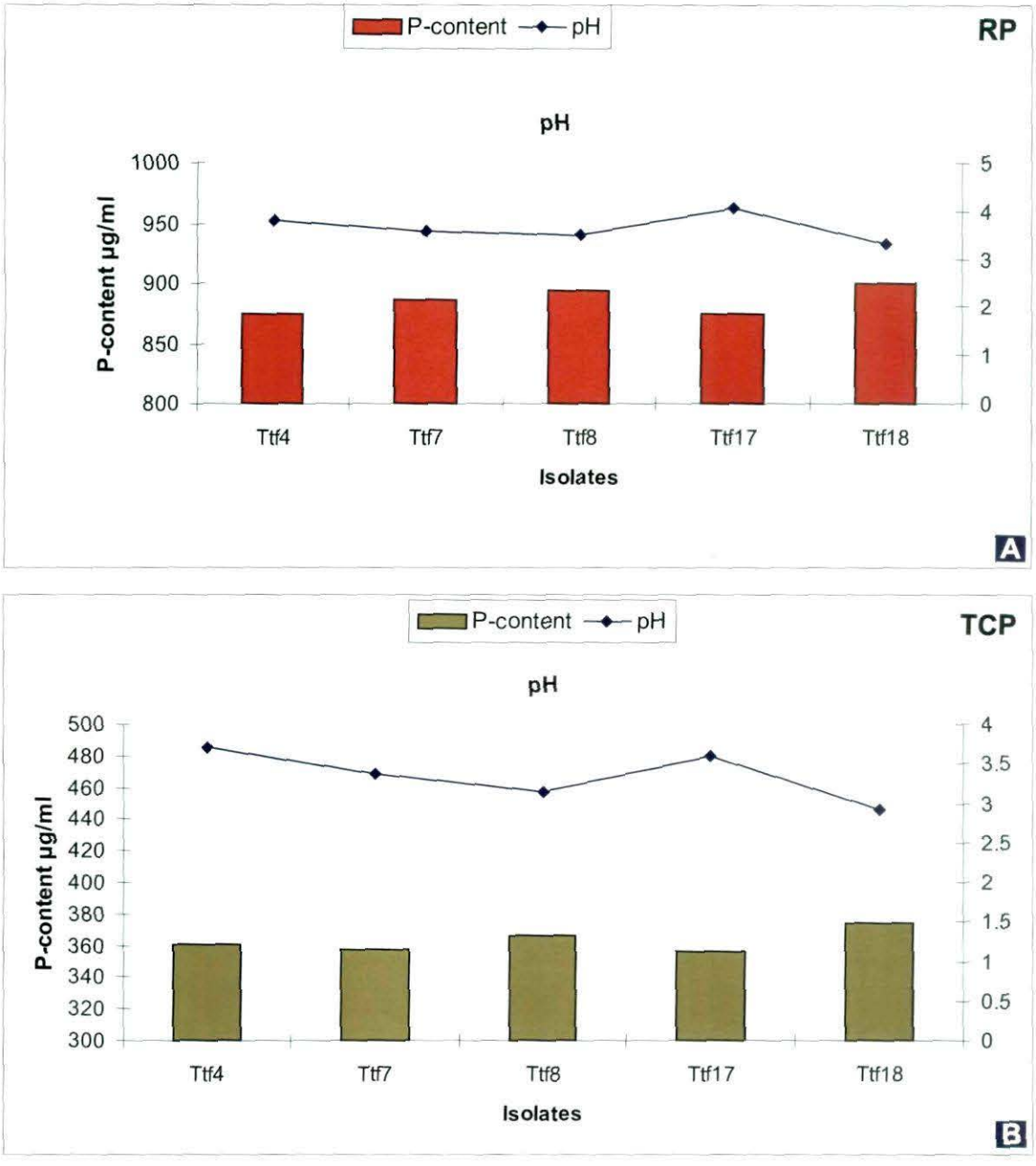


Fig 3 (A-B): Solubilization of rock phosphate (RP) (A) and tricalcium phosphate (TCP) (B) with phosphate solubilizing fungal isolates

Table 16: Evaluation of phosphorus solubilization by fungal isolates in liquid media amended with tricalcium phosphate (TCP) and rock phosphate (RP)

PSF isolates	TCP (mg/l)	pH	RP (mg/l)	pH
Aspergillus niger (Ttf4)	875	3.8	361	3.7
Aspergillus nidulae (Ttf7)	886	3.6	357	3.37
Aspergillus fumigatus (Ttf8)	894	3.5	366	3.15
Aspergillus versicolor (Ttf17)	874	4.05	356	3.6
Aspergillus terreus (Ttf18)	900	3.32	374	2.92

PSF = Phosphate solubilizing fungi; TCP = tricalcium phosphate (P=997mg/l); RP= rock phosphate (P=500mg/l)

The increase of P concentration in the later stages might be due to the action of the fungi on the substrate for demands of nutrients, thus releasing more P from insoluble sources. Increasing the released P during the later stages was also attributed to cell lysis and P precipitation brought about by organic metabolites. The tested isolates reached their maximum biomass level after seven days of incubation. Such result indicated the ability of the fungal strains to solubilize P and change it to available form. Culture media with no TCP produced poor growth.

4.7. Searching for Arbuscular Mycorrhizal Fungi from tea plants grown in Sikkim hills and plains

Soil and root samples collected from tea plants growing at various locations of Temi Tea Estate, Sikkim as well as from Tea Garden of Matigara Tea Estate were used for determining association of Vesicular Arbuscular Mycorrhiza, Extracted spores from each soil samples were divided into groups of the same characteristics for identification under dissecting microscope. Spore attachment, size and color of spores were noted.The identification in this study was done following the monograph and the manual of Gerdemann & Trappe (1974), Trappe and Schenck (1982) and Hall (1984). The semi permanent slides were prepared using PVL (polyvinyl alcohol + lactic acid + Glycerol) which effected certain spore characteristics, such as wall characteristics and spore color, diameter, attachment and hyphal wall thickness etc. Detailed photographic illustrations of AM spores and their associations with tea root system obtained from Temi Tea Estate, Sikkim hills have been presented in Plate 9 (figs A-M) and AM spores and root association obtained from Matigara Tea Estate, plains of Siliguri have been presented in

Plate 10 (figs A-K). Morphological characteristics of some AM spores are described below and population of AM fungi in the rhizosphere of tea collected from different blocks of Temi Tea Gardens have been presented in Table 17.

Glomus fasciculatum **Gerdemann and Trappe-**

Colour pale yellow to bright brown with globose to subglobose in shape. Spores are produced directly with one or more subtending hyphae attached to it. Spore wall is continuous. Spore wall consisting of three layers (L1, L2, and L3). Spore size ranges from 70-120µm in diameter. (Plate 9, fig. D)

Glomus aggregatum **Koske, Gemma and Olexia-**

Spores globose to oval in shape. Size ranges from 40-120µm in diameter, color- pale yellow. Formed singly or in sporocarps. Spore wall consist of 1-2 layers. Sporocarps are formed in loose clusters, from a single stalk, diameter ranges from 200-1800 x 200-1400 µm in size. (Plate 10,fig. H)

Glomus mosseae **Nicolson and Gerdemann-**

Brown to orange-brown in colour, shape, globose to sub-globose with an average diameter of 200µm. Presence of three hyaline layers with subtending hyphae attached. Hyphae are double layered. (Plate 9 fig. B)

Glomus drummondii **Blaszk. & C. Renker-**

Spores occur singly in the soil; develops from the tip of extraradical hyphae of mycorrhizal roots. Spores are golden yellow, globose to subglobose, average diameter is 70µm in diameter; single subtending hypha attached with the spore. Spore wall consists of three distinct layers. (Plate 9,fig. E)

Glomus clarum **Nicol. & Smith**

Spores single in the soil; hyaline to pale yellow, globose to subglobose; 150 µm diam; sometimes ovoid; 90-100 x 140-180 µm; with one subtending hypha. hyaline to pale yellow straight to curved;wall of subtending hypha hyaline to pale yellow ,thick at the spore base; composed of three layers (Plate 10; fig.G)

Gigaspora gigantea **(Nicol. & Gerd.) Gerd. & Trappe**

Spores single in the soil; formed terminally or laterally on a bulbous sporogenous cell; greenish yellow (globose to subglobose; 300µm diam; sometimes ovoid; 250-270 x 265-370 µm. Subcellular structure of spores consists of a spore wall with two layers and one germinal wall. (Plate 10 , fig.C)

Gigaspora margarita **W.N. Becker & I.R. Hall**

Spores produced singly in the soil, blastically at the tip of a bulbous sporogenous cell. Spores yellowish white to sunflower yellow; globose to subglobose; 357 µm diam; sometimes ovoid; 300-340 x 360-380 µm. Sporogenous cell orange to brownish yellow (clavate; 52.5-60.0 x 75.0-100.0 µm. Structure of sporogenous cell composed of two layers. Layer 1 hyaline, 1.7 µm thick approximately. Continuous with spore wall layer 1. Layer 2 orange to brownish yellow, 5.6 µm thick, continuous with spore wall layer 2. (Plate 10, fig. D)

Acaulospora bireticulata **F.M. Rothwell & Trappe-**

Spores are globose and brownish in colour, diameter ranges from 280-410µm. Surface ornamentation is prominent. Spore wall consists of three layers. Spores are borne laterally from the neck of a sporiferous saccule. (Plate 10, fig. E)

Acaulospora capsicula **Blaszk.**

Spores are bright red or orange red in colour, globose to subglobose with three distinct layers. (Plate 10, fig.F)

Scutellospora pellucida **(Nicol. & N.C. Schenck) C. Walker & F.E. Sanders**

Spores single in the soil; formed terminally on a bulbous subtending hypha; hyaline to yolk yellow; globose to subglobose; 195µm diam; sometimes ovoid; 130-155 x 160-235 µm.

Scutellospora rubra **(Nicol. & N.C. Schenck)**

Spores color: dark orange-brown to red-brown, most tending toward the latter at maturity. Immature spores are white to cream with a rose tint under a dissecting microscope. Shape: globose to subglobose. Size 180 µm in average.

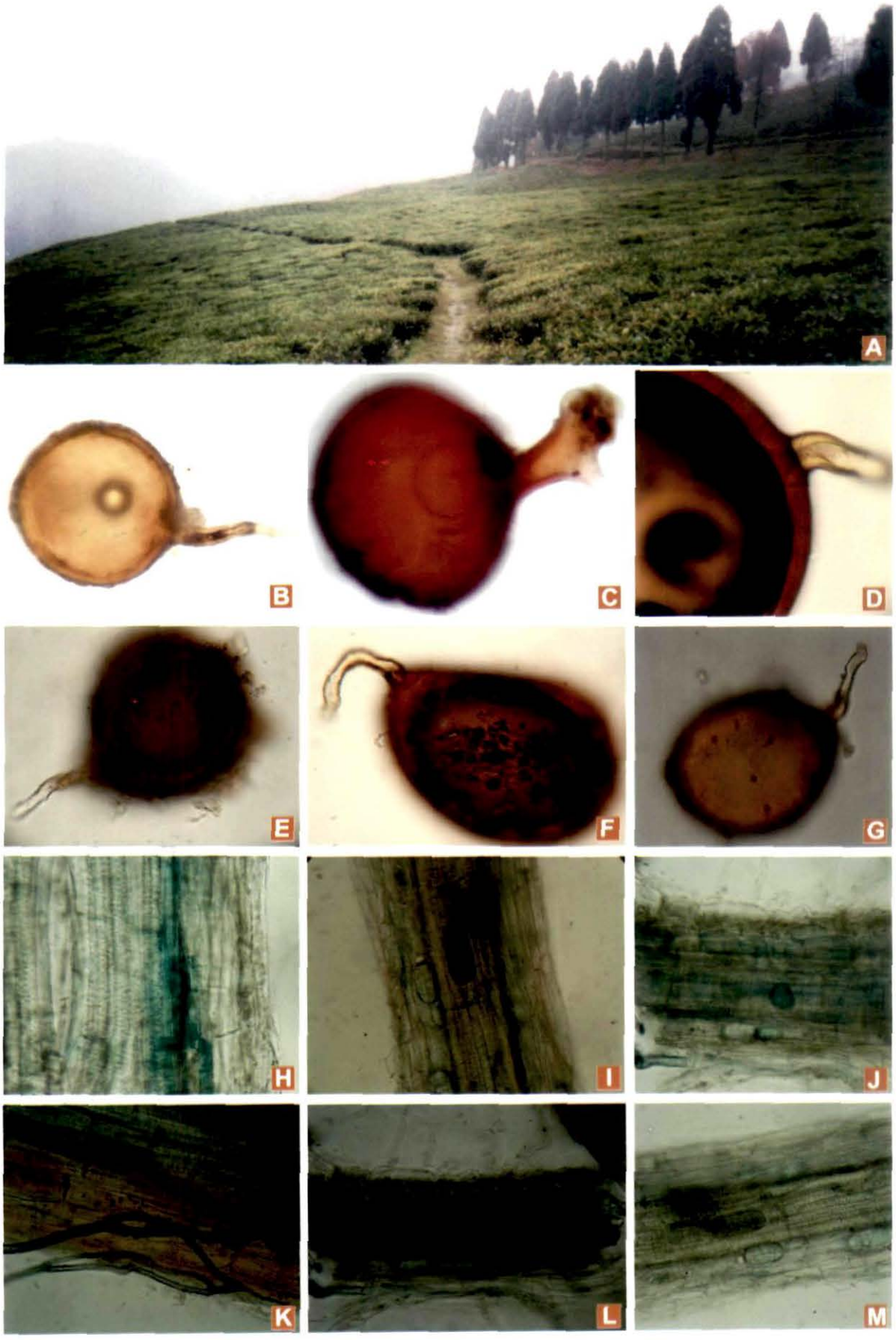


Plate 9(A-M): Plantation site of Temi Tea Garden (A). Spore of *Glomus mosseae* (B); *G.badium* (C), *G.fasciculatum* (D), *G.drummondii* (E) *G.clarum* (F) and*G.mosseae* (G) Histopathological study of tea roots showing intraradical hyphae, vesicles and hyphal structure (H-M)

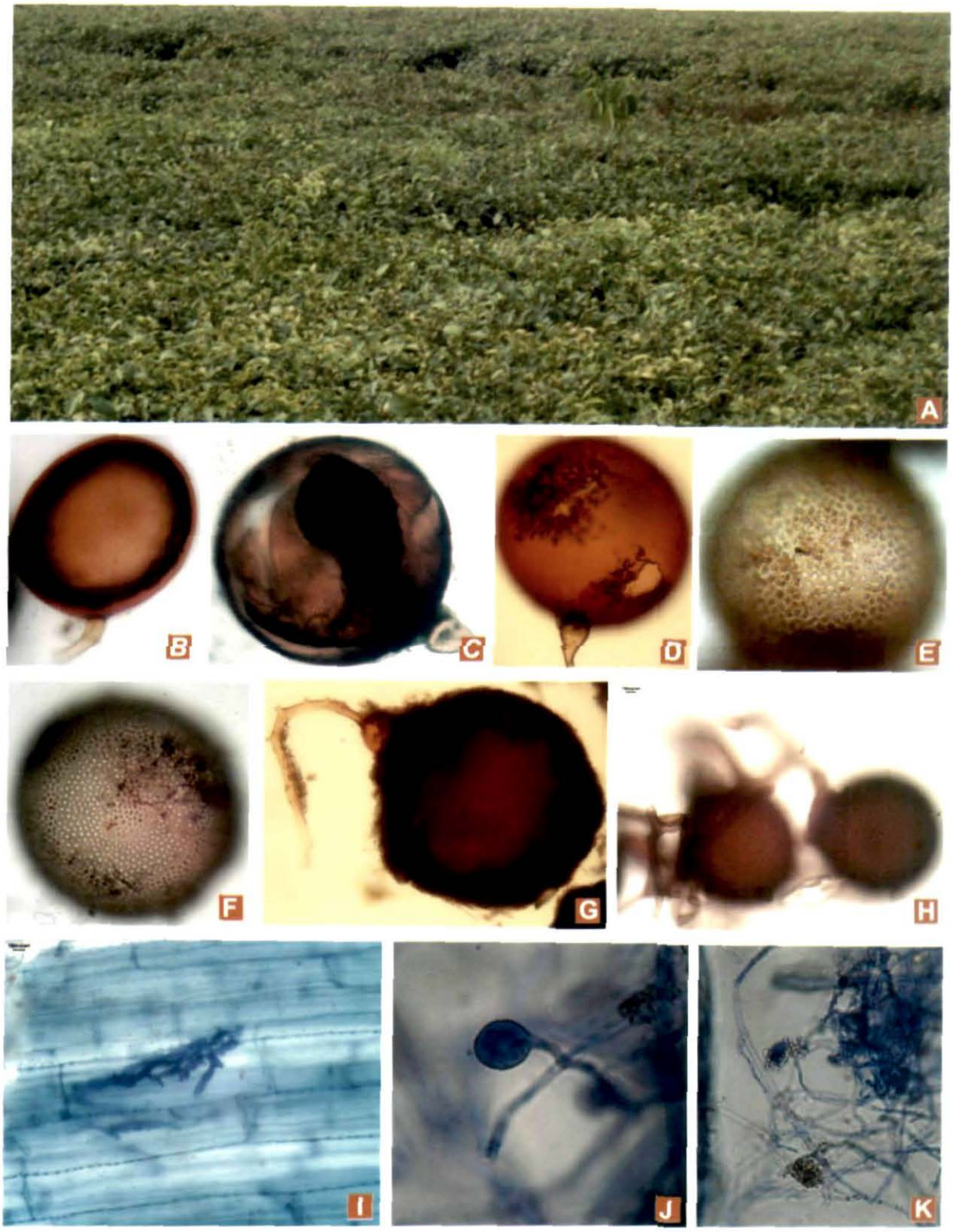


Plate 10(A-K): Plantation site of tea gardens in plains (A). Spore of *Glomus mosseae* (B), *Gigaspora gigantea* (C), *G. margarita* (D), *Acaulospora biteticulata* (E) *A.spinosa* (F) *Gigaspora margarita* with bulging hyphal attachment (G), Glomus aggregating(H), Histopathological study of tea roots showing intraradical hyphae, vesicles and hyphal structure (I-K)

Table 17: Population of AM fungi in the rhizosphere of tea collected from different blocks of Temi Tea Garden

Genus & species	% of AM spores from different blocks			
	Block 1	Block 2	Block 3	Block 4
Glomus aggregatum	44	23	48	82
G. mosseae	47	22	18	86
G. fasciculatum	45	30	38	82
G. drummondii	-	-	-	12
G. clarum	05	08	08	10
G. microaggregatum	13	12	10	18
G. versiforme	-	-	-	06
Gigaspora decipiens	60	57	48	03
G. gigantea	75	78	82	14
G. margarita	65	72	70	34
G. rosea	25	20	-	08
Acaulospora bireticulata	15	18	16	24
A. spinosa	08	12	10	22
A. denticulata	10	12	10	10
A. capsicula	02	-	01	04
Scutellospora rubra	04	02	04	05
S. pellucida	03	02	04	02

Table 18: Comaparison of soil characters of tea rhizosphere and colonization of AM fungi in Temi Tea Estate, Sikkim and Matigara Tea Estate, Siliguri

Parameters	**Temi Tea Garden (Sikkim)**	**Matigara Tea Garden (Plains)**
Soil type	Sandy-Clay	Sandy-clay
Sand (%)	55	60
Silt (%)	7	02
Clay (%)	43	38
pH	4.43	5.01
Moisture (%)	22.08	13.68
P_2O_5 (ppm)	40.26	34.82
K_2O (ppm)	123.65	91.46
Organic Carbon (%)	1.27	1.39
Nitrogen (%)	0.11	0.08
Spore No. / gm of soil	115	80
% Colonization	85	88

Microscopic observations of AM fungi associated with tea roots in hills and plains have been illustrated in Plates 9 & 10. Maximum root colonization of tea roots with AM fungi of both in hills and plains revealed the presence of *Glomus mosseae* and *Glomus fasciculatum.* Pure line of these two AM fungi were made and scanning electron microscopic observations have been presented in Plate 11 (figs.A-C).

4.8. Biochemical changes in tea plants following inoculation with *U. zonata*

4.8.1. Estimation of Phenol content

As polyphenols are the major constituents of tea plants it was decided to compare the levels of total phenol and ortho-dihydroxy phenol in tea roots following inoculation with *U. zonata.* Five tea varieties were selected for this experiment.

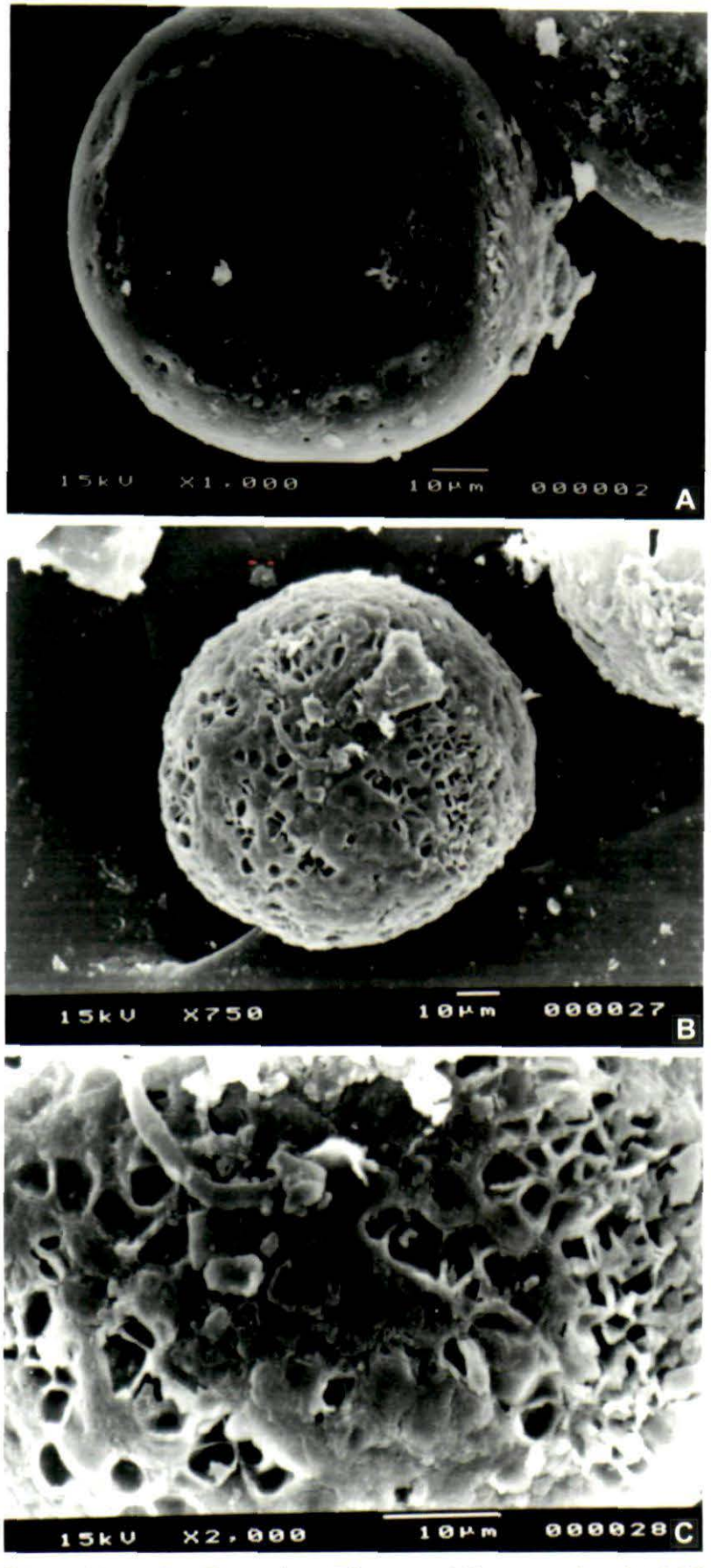


Plate 11(A-C): Scanning Electron Micrograph of AMF spores obtained from tea rhizosphere. *Glomus fasciculatum* (A) *G. mosseae* (B), highly magnified image of spore wall structure of *G. mosseae* (C)

4.8.1.1.Total phenol

Total phenols from healthy and *U. zonata* inoculated tea roots of different varieties were extracted after 10 days of inoculation and estimated. Results (Table 19) revealed that total phenol decreased following inoculation with *U. zonata* in the susceptible varieties. However there is an increase in the phenol content of resistant varieties following inoculation. Among all the varieties tested TV -26 showed maximum increase in total phenol following inoculation with the pathogen.

Table 19: Total phenol content in healthy and *U. zonata* inoculated tea varieties

Tea varieties	Total phenol (mg/g tissue)*	
	Healthy	**Inoculated**
TV-18	4.5±0.25	7.1±0.05
TV-23	5.3±0.15	8.8±0.10
TV-25	4.6±0.40	5.0±1.21
TV-26	6.2±1.25	9.7±0.35
T-17	5.7±0.05	6.8±1.22

* Average of three replicates.± = S.E

4.8.1.2.Ortho- dihydroxy phenols

Ortho-didydroxy phenols were also extracted from healthy and *U. zonata* inoculated tea roots of different varieties after 10 days of inoculation with the pathogen and estimated. Results (Table 20) revealed that ortho-dihydroxy content decreased in susceptible varieties increase in resistant varieties following inoculation with *U. zonata*. Responses of TV-25 variety against the pathogen were towards increasing the levels of orthodihydroxy phenol.

Table 20: Level of ortho-dihydroxy phenol content in healthy and *U. zonata* inoculated tea varieties

Tea varieties	ortho-dihydroxy phenol (mg/g tissue)*	
	Healthy	**Inoculated**
TV-18	1.7±0.20	2.5±2.35
TV-22	3.1±2.10	3.5±0.65
TV- 23	2.1±0.35	2.8±2.34
TV- 25	3.1±1.15	3.8±0.56
TV- 26	2.2±1.00	3.6±2.15
T-17	1.7±0.25	2.9±1.32

*= average of three replicates. ± = S.E

4.8.2 Determination of enzyme activities

4.8.2.1.Phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase (PAL) is the first enzyme of phenyl propanoid metabolism in higher plants and it has been suggested to play a significant role in regulating the accumulation of phenolics, and phytoalexins as well as lignins, three key factors responsible for disease resistance. In present study, PAL activity was assayed in tea roots following inoculation with *U. zonata*. PAL activity was assayed in each case after 7, 14 and 20 days after inoculation. Results have been presented in Table 21. It showed that PAL activity increased after 14 days of inoculation markedly in all the varieties except in TV-18.

Table 21: Changes in PAL activity in tea roots following inoculation with *U. zonata*

Tea variety	PAL activity in tea roots (µg cinnamic acid g^{-1} m^{-1})*	
	Healthy	Infected
TV-18	123.4± 2.34	089.5± 2.10
TV-23	081.0±1.02	158.6± 1.00
TV-25	076.4±0.03	173.8± 0.00
TV-26	087.6±1.25	142.6±2.01
T-17	189.0±0.21	292.7±0.34

* Average of three replicates. ± = S.E

4.8.2.2.Peroxidase

Peroxidase activity was assayed as increase in absorbance when o- dianisidine was oxidized by the oxygen released from H_2O_2 which was oxidized by the enzyme. Peroxidase was extracted from healthy and *U. zonata* inoculated tea roots and their activity was also assayed after 7 and 14 days of inoculation. Peroxidase activity increased in all the varieties tested following inoculation(Table 22).

Table 22: Changes in peroxidase activity in tea roots following inoculation with *U.zonata*

Tea variety	PO activity in tea roots (Δ OD/g tissue /min)* Healthy	Infected
TV-18	1.46±0.03	1.57±0.00
TV-23	0.64± 2.56	1.80±1.25
TV-25	1.11±3.65	1.22± 2.11
TV-26	1.29±2.01	1.77±0.34
T-17	1.03±1.90	1.62± 0.26

*Average of three replicates. ± = S.E

4.8.2.3.Polyphenol oxidase (PPO)

Tea varieties were selected and enzyme activity following inoculation with the pathogen was assessed. PPO activity in tea roots increased markedly after 4 days of inoculation with *U. zonata* in all the varieties tested (Table 23)

Table 23: Changes in polyphenol oxidase activity in tea roots following inoculation with *U. zonata.*

Tea variety	PPO activity in tea roots (Δ OD/g tissue /min)* Healthy	Infected
TV-18	0.14±0.34	0.19±1.25
TV-23	0.11±2.01	0.21±0.34
TV-25	0.16± 2.78	0.23± 1.80
TV-26	0.25±0.11	0.29±0.03
T-17	0.04± 2.10	0.16±0.02

* Average of three replicates. ± = S.E

4.8.3. Detection of antifungal compound

Drop diffusates collected from tea roots contained some antifungal compounds. Further experiments were carried out to detect the antifungal phenolics from relatively large samples of freshly harvested tea roots following artificial inoculation with *U. zonata* using facilitated diffusion technique. Antifungal compounds were extracted separately from healthy and *U. zonata* inoculated tea roots. Ethyl acetate fractions of both healthy and *U. zonata* inoculated tea root extracts were loaded on TLC plates, developed in chloroform: methanol (9:1,v/v) and sprayed with Folin- Ciocalteau's reagent. Color reaction was noted at Rf 0.56. Crude extract (ethyl acetate fraction dissolved in methanol) prepared from healthy and *U. zonata* inoculated roots were bioassayed following radial growth inhibition assay. Results have been presented in Table 24.

Table 24: Effect of antifungal compounds extracted from healthy and inoculated tea root extracts on radial growth of *U. zonata*.

Variety	Diameter of mycelial growth (mm)*	
	Healthy	**Infected**
	15.1	4.6
	13,2	5.3
	19.6	10.4
	18.4	12.8

Distilled water control 30mm
* Average of three replicates

4.9. Cultural characteristics of PSB (*B.pumilus*) and PSF (*A. niger*) isolates

4.9.1. PSB isolate (*B. pumilus*)

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

4.9.1.1 pH

B.pumilus grew best at pH 6.0 and did not grow well below pH 4.0 and above 8.0.

4.9.1.2. Temperature

The growth of *B.pumilus* was observed at different temperatures ranging from 20 to 50 °C. NB medium was inoculated with the bacteria and flask was incubated at 20 .25.30, 35,40,45,50°C. Bacteria grew well within these ranges of temperature but grew best at 35 °C.

4.9.1.3. 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 NB is the best medium for growth of *B.pumilus.* (Table 25)

Table 25: Growth of *B.pumilus* in different media

Media	***B.pumilus* (cfu/ml)**
PDA	$3.31x10^{7}$
NB	$6.02x10^{13}$
KB	$1.90x10^{11}$
LB	$5.88x10^{8}$
NSA	$1.81x10^{6}$
GYP	$2.60x\ 10^{9}$

* Average of three replicates; incubation period 4 days.

4.9.1.4. Incubation period

B.pumilus was grown in NB for a period of 10 days with growth being recorded after 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days of growth at temperature of 35 °C. Maximum growth was recorded after 4 days and then growth gradually decreased.

4.9.1.5. Antibiotic sensitivity

Antibiotic sensitivity test was performed to know the strength of rhizobacteria against particular antibiotic. Antibiotics was mixed at rate of 15µg/ml in Nutrient Agar medium; Results showed that *B. pumilus* is highly sensitive to amoxicillin, streptomycin, gentamycin. (Table 26)

Table 26: Antibiotic sensitivity of the rhizobacterial isolate (*B. pumilus*)

Antibiotics	*B.pumilus*
Amoxicillin	HS
Penicillin	PH
Chloramphenical	HS
Streptomycin	HS
Gentamycin	HS
Norfloxacin	HS
Tetracycline	HS
Kanamycin	MS

PS- partially sensitive; HS- highly sensitive; MS- moderately sensitive

4.9.2. PSF isolate (*A. niger*)

Before further work was carried out with the isolated phosphate solubilizing fungi, their cultural characteristics were determined in order to obtain information about their optimal growth conditions. Effects of different pH, temperature and media on growth of the fungi were determined.

4.9.2.1. pH

Phosphate solubilizing fungi grew best at pH 4.5 and almost did not grow at pH 2.0 and 10.0

4.9.2.2.Temperature

The growth of these PSF was observed at different temperatures ranging from 20 to 50°C. PDB medium was inoculated with the PSF and the flasks were incubated at 20.30,40,50 °C. The fungi grew well within 30°C temperature.

4.9.2.3. Media

Five different media were selected for testing the growth of the fungi. These were Potato Dextrose agar, (PDA), Potato dextrose broth (PDB), Carrot juice Agar, Czapek dox Agar, Potato sucrose Agar. The fungi grew well in almost all the media but it showed maximum growth on PDB and PDA.

4.10. *In vitro* studies of selected PSF and PSB isolates against test pathogen

4.10.1. Pshosphate solubilizing fungus (PSF)

The inhibitory effects of the isolates Ttf 4 viz. *Aspergillus niger* were further confirmed by dual culture methods in solid and liquid media.

4.10.1.1 Solid medium

In solid medium, inhibition of the growth of different fungal pathogens by the antagonistic fungi and the zone of inhibition were recorded. The results revealed that *Aspergillus sp.* inhibited the growth of all test pathogens *viz. Ustulina zonata, F.lamaoensis, S. rolfsii, P. lamonensis* and results were summarized in (Table 27).

Table 27: *In vitro* antagonistic effect of phosphate solubilizing fungus (*Aspergillus niger*- Ttf4)

Test pathogens	Diameter of inhibition zone (cm)*
U. zonata	1.6± 0,03
F. lamaoensis	1.0± 0.01
S. rolfsii	2.0±0.05

± = standared error; * Average of 3 replicates.

4.10.1.2 Liquid medium

Aspergillus niger was tested for their activity against the test fungi in liquid medium. After 7 days of growth, mycelia were harvested; dried and mycelial dry weight was taken. Results (Table-28) revealed similar observation as in solid medium..

Table 28: *In vitro* test of phosphate solubilizing fungus (*Aspergillus niger*- Ttf4) with different tea root pathogens

Interacting microorganism	Mycelial dry weight (mg)	% of reduction in growth
U. zonata	189.3± 1.30	74.4%
U. zonata+ *A. niger*	043.0± 2.21	
F. lamaoensis	265.5±2.43	74.76%
F. lamaoensis+*A. niger*	058.0±1.56	
S. rolfsii	306.6± 4.30	82.25%
S. rolfsii+*A. niger*	054.4 ± 2.42	

± = standared error; * Average of 3 replicates.

4.10..2 Phosphate solubilizing bacterium (PSB)

Antagonistic activity of selected bacterial isolate Ttb14 viz. *Bacillus pumilus* was confirmed by dual culture method both in solid and liquid media.

4.10.2.1. Solid medium

In case of solid medium tests, diameter of fungal growth alone or with plant growth promoting bacteria and zone of inhibition were noted. The results (Table 29) revealed that the bacteria inhibited *Fomes lamaoensis* most, followed by *Ustulina zonata* and *Sclerotium rolfsii*.

Table 29. Effects of *B. pumilus* against fungal pathogens in solid medium

Test organisms	Diameter of inhibition zone (cm)	Mycelial dry wt.(g)
Ustulina zonata	1.4± 0.03	0.125
Fomes lamaoensis	1.6± 0.08	0.101
Sclerotium rolfsii	0.6± 0.05	0.387

± = Standard error
Average of three replicates
Temperature 30± 2°C; after 7 days of incubation

4.10.2.2 Liquid medium

The bacterium (*B. pumilus*) was also tested for their activity against the test fungi (tea root pathogens) in liquid medium. After 7 days of growth, mycelia was harvested, dried and mycelial dry weight was noted. Lowest mycelial dry weight was recorded in *F. lamaoensis* and percentage of reduction was less in *Sclerotium rolfsii.* In dual culture, it was found that the *B. pumilus* inhibited all the test pathogens (Table 30)

Table 30: *In vitro* pairing test of *B.pumilus* with different pathogens in liquid medium

Interacting microorganisms	Mycelial dry weight (mg)	% reduction over control
U. zonata	190.3± 0.30	
U. zonata + *B. pumilus*	52.4 ± 0.21	72.2%
F. lamaoensis	260.0± 0.25	
F. lamaoensis+*B. pumilus*	43.5±1.20	75.5%
S. rolfsii	312.0± 0.15	
S. rolfsii+*B. pumilus*	125.0 ±1.23	59.1%

Average of three replicates. ± = Standard error

4.11. Effects PSF application on growth of tea plants

The growth promotion of different varieties of tea seedling and two year old tea plants was observed in terms of increase in height of seedlings, number of shoots and number of leaves. It was observed that treatment with the *A. niger* increased the rate of growth of the seedling in relation to untreated control. Percentage increase in height of plants as well as number of leaves after two and four months of application of fungus to the soil were calculated (Table 31). The results showed that the PSF efficiently promoted growth in tea plants irrespective of their variety.

Table31: Effect of Phosphate Solubilizing Fungus (*A. niger*) on the growth of tea seedling.

Tea variety	Treatment	2 months after treatment		4 months after treatment	
		% increase in height	% increase in no. of leaves	% increase in height	% increase in no. of leaves
TV-18	Control	6.6 ±1.20	15 ± 1.71	16.0± 1.21	25 ± 1.93
	A. niger	35.4 ±1.32	30±6.01	55.0± 2.46	49.0± 1.00
T -17	Control	7.0± 1.16	10.0± 1.50	15±1.92	20± 5.20
	A. niger	36.0± 0.80	36.0± 1.20	50± 0.92	48± 1.74
TV-25	Control	10 ±2.0	18±1.20	18.2±0.89	30.0±1.90
	A. niger	29±1.20	42.0±1.25	56.2±1.20	53.0± 1.20
TV-26	Control	10.5 ±1.30	18.0±1.92	15.6 ±1.20	34.0±2.05
	A. niger	29.4±0.47	45.0 ± 1.82	49.8 ±1.28	53.0 ± 1.65
TV -23	Control	8.4±2.12	17.0±1.34	17.3±1.92	38.0 ±1.23
	A. niger	23±1.20	39.0±2.10	48.5±1.28	49.0±1.00

Ten plants per treatments; * difference of all tests with control significant at P= 0.05 rest significant at P= 0.01 as tested by Students t test.

4.12. Effects of PSF application on Charcoal stump rot disease development of tea

Since the PSF isolate showed antagonistic activity *in vitro*, experiments were further conducted to determine whether these could also alter disease reaction in tea plants. Three tea varieties (TV-26, T-17, TV-23) were grown and following application of PSF

mass multiplied in FYM, each plant was inoculated with *U. zonata*. Disease development was assessed 20, 35 and 55days after inoculation with the pathogen. Results (Table 32) revealed that plants prior treated with PSF treatment reduced the severity of the disease.

Table 32: Effects of PSF (*A. niger*) application on the development of charcoal stump rot disease of tea

Varieties	Treatments	Stump rot index Days after inoculation		
		20	35	55
TV-26	*U. zonata*	1.20	2.00	4.55
	U. z +A. niger	0.79	1.26	2.36
T-17	*U. zonata*	1.23	2.12	4.65
	U. z +A. niger	0.60	1.02	1.89
TV-23	*U. zonata*	1.00	2.45	3.43
	U. z +A. niger	0.56	0.90	1.56

Rot index: 0= no symptom; 1= small roots turn brownish and start rotting; 2= leaves start withering and 20-30% of roots turn brown; 3= leaves withered and 50% of roots affected; 4= shoot tips also start withering; 60-70% affected; 6= whole plants die, with upper withered leaves still remaining attached; roots fully rotted

4.13. Biochemical changes in PSF treated tea plants following inoculation with *U. zonata*

Application of the PSF (*A. niger*) to soil prior to inoculation with the pathogens resulted in a decrease in disease intensity in all cases. Disease alteration is associated with changes in biochemical constituents in the host. Major biochemical components of tea leaves such as proteins and chlorophylls were analyzed. Protein content was lesser in pathogen inoculated plants then in uninoculated plants but protein content was higher in *A. niger* treated plants. Phenol contents were even more significantly increased when challenged with pathogen. Chlorophyll content was higher in treated plants than in pathogen inoculated plants (Table 33).

Table 33: Effect on protein, phenol and chlorophyll content of *A. niger* inoculated tea plants

Biochemical Components (mg g^{-1} tissue)	Treatments	
	Control	PSF
Protein	30	46.2
Phenol		
Total	12.8	34.21
Ortho	2.6	3.56
Chlorophyll		
Chl a	0.596	0.686
Chl b	0.289	0.426
Total chl	0.885	1.112

Difference with control significant at P=0.05, rest significant at P= 0.01 as tested by student 't' test.

Both the total and O-dihydroxy phenol contents of the tea leave were significantly higher following treatment with PSF as compared to non treated control plants in different varieties of tea (Table 34).

Table 34: Phenol contents in tea leaves of different varieties following fungal (*A. niger*) and pathogen application in the rhizosphere.

Varieties	**Treatment**	**Phenol content (mg/g tissue)**	
		Total	**O-dihydroxy**
T-17	Control	30.2± 3.2	8.2±1.4
	U. zonata	34.4 ± 2.2	8.1± 0.9
	U. zonata + *A. niger*	38.5± 2.5	9.5±0.9
TV-26	Control	28.2±1.2	7.5±1.4
	U. zonata	30.8±2.9	8.1±1.4
	U. zonata + *A. niger*	35.9±2.5	9.6± 0.9

Higher activities of defense enzymes like peroxidase were observed in *U. zonata* treated and *A. niger,* treated plants. Other defense enzymes like PAL, β, 1, 3-glucanase and chitinase also showed enhanced activity (Table 35).

Table 35: Enzyme activities in PSF treated tea varieties following inoculation with pathogen (*U. zonata*)

Varieties	Treatments	Enzyme activities			
		PO	CHI	PAL	GLU
T-17	Control	3.4± 0.2	10.2±1.0	64± 0.4	436±1.4
	A. niger	5.3±0.2	17.2 ±2.1	96± 0.7	544±2.3
	A. niger + *U. zonata*	8.0±0.2	135±0.32	103.4±1.6	620± 1.5
T-23	Control	3.3±0.1	12.2± 0.2	58 ± 0.21	389± 2.1
	A. niger	4.0±0.2	16.2± 2.2	99± 0.13	497± 1.0
	A. niger+ *U. zonata*	9.0± 1.1	24.3± 1.2	112± 0.12	523± 2.1
TV-26	Control	3.7± 0.0	11.5 ± 1.4	66 ± 0.23	376±1.1
	A. niger	4.6± 1.4	15.7± 0.3	98± 0.01	450±2.0
	A. niger + *U. zonata*	5.6±0.2	26.5±0.3	109.5±1.6	525±2.2

POX activity assayed as Δ A 465 min^{-1} g $tissue^{-1}$; PAL activity assayed as µg N-Acetyl glucosamine released by enzyme from 1 g tissue min^{-1} and ß 1,3- GLU activity assayed as µg glucose released by enzyme from 1g tissue min^{-1}.

4.14. Effect of PGPR (*Bacillus pumilus*) application on the growth of tea plants

When bacterial inoculums was applied in the rhizosphere of tea plants or seedlings, increase in the growth was observed in terms of increase in height of seedlings, number of shoots and number of leaves. It was observed that treatment with the bacteria increased the rate of growth of the plants in relation to untreated control (Plate 12). Increase in growth was recorded from two month onwards. Bacterial inoculation led to as much as 125% increase in growth, as against 15-25% in control. (Tables 36 & 37). Percentage increase in height of the plants, no. of leaves and leaf area index have been presented in Figure 4.

Table 36 : Effect of PGPR (*Bacillus pumilus*) application on the growth of tea seedlings

Tea varieties	Treatment	2 months after treatment			
		Height of seedling (cm)		No.of leaves of seedling	
		Initial	final	initial	final
T17	Control	10.2	14.6	7	10
	B. pumilus	12.8	21.5	8	16
TV-20	Control	14.2	15.5	7	12
	B. pumilus	14.0	24.1	8	15
TV-25	Control	14.7	17.0	7	9
	B. pumilus	15.2	19.6	8	14

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

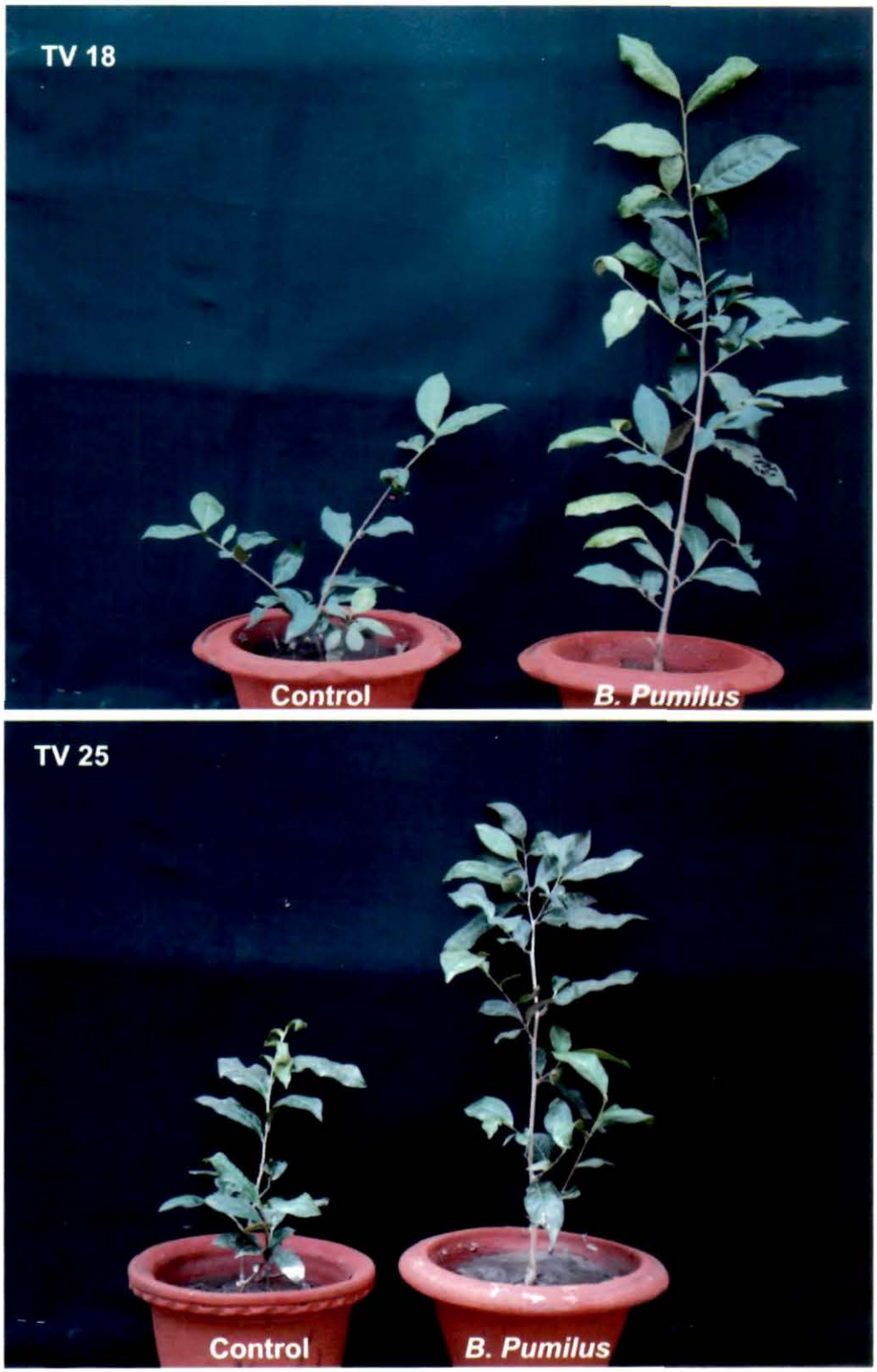
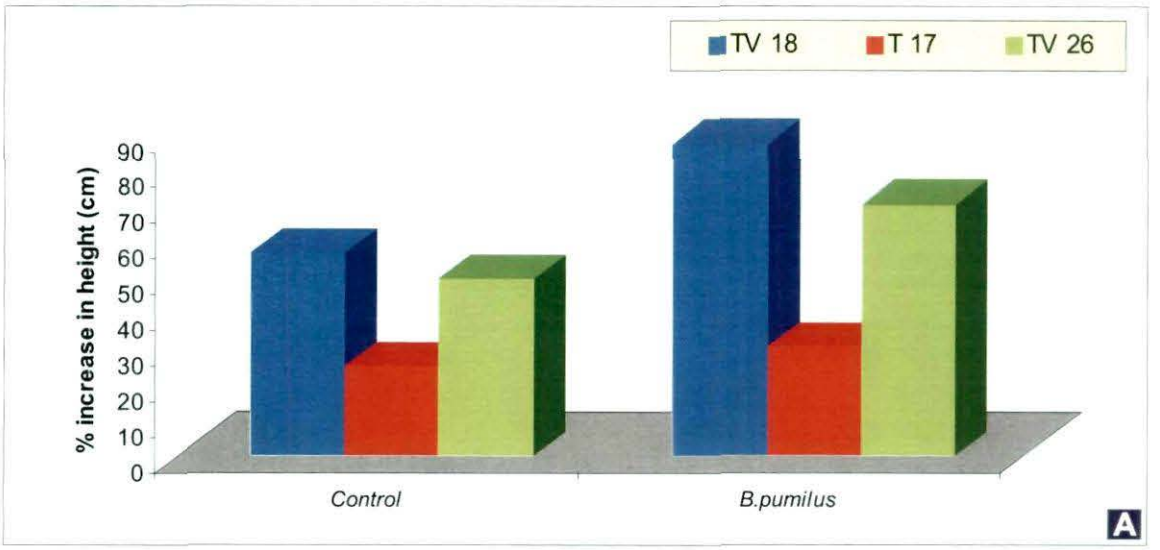
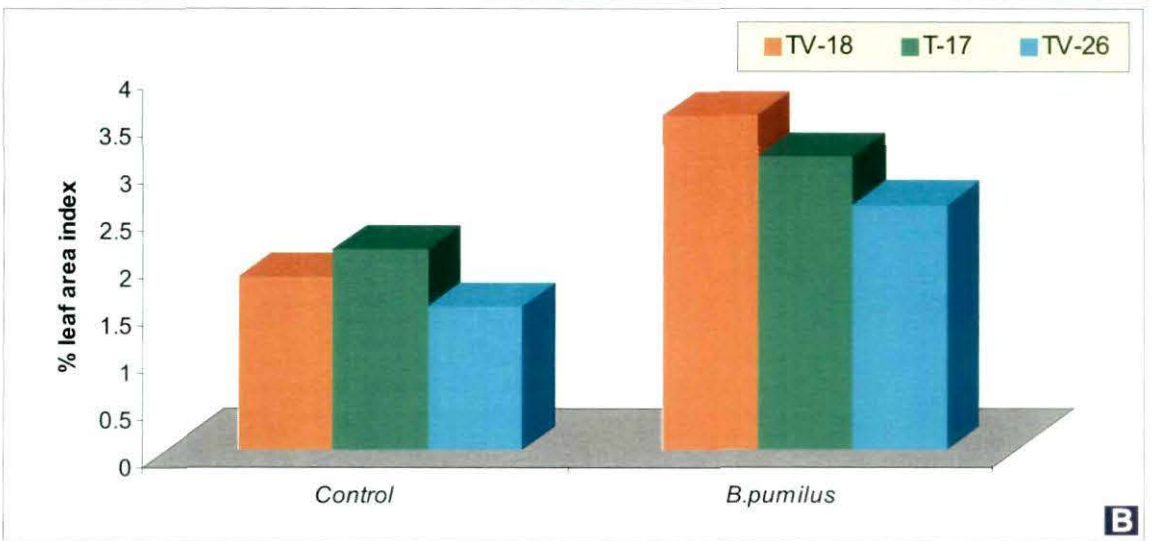


Plate12: Effect of *Bacillus pumilus* on growth of two year old potted tea plants





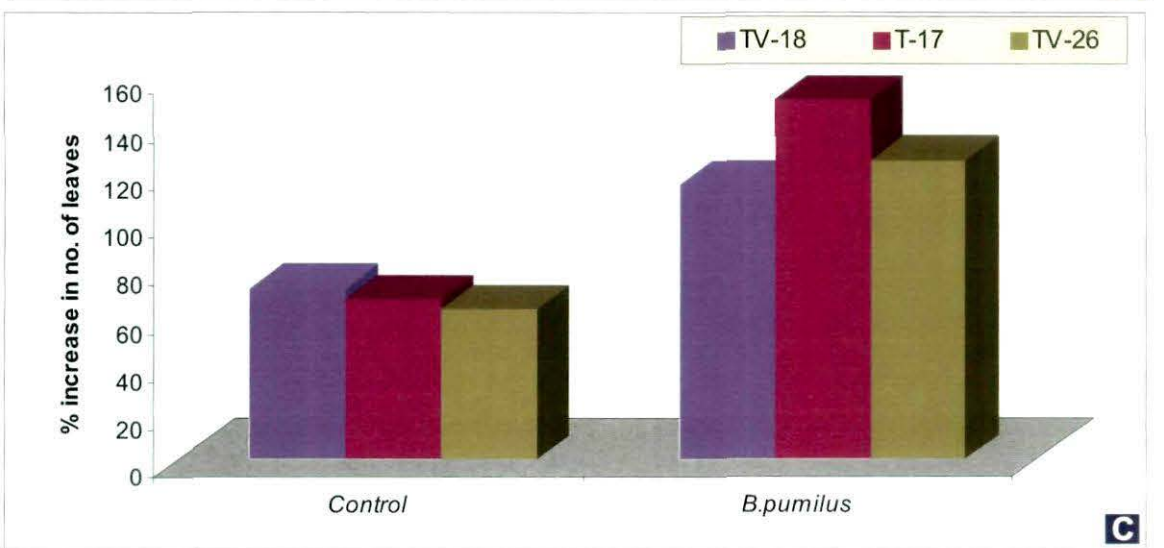


Fig.4 (A-C): Effect on growth of tea plants following application of *B.pumilus*

Table 37: Effect of *B. pumilus* application on growth of two year old plants

Tea Varieties	Treatments	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	22.3	26.4	10	15	40	48
	B.pumilus	26.0	41.0	10	22	43	79
TV- 18	Control	34.6	47.6	14	16	50	80
	B.pumilus	34.0	70.0	10	19	43	95
TV- 26	Control	32.3	42.6	9	10	40	51
	B.pumilus	36.0	82.0	10	15	32	68

Each treatment is average of three replicates.

4.15. Effects of *B. pumilus* application on disease development in tea

In vitro interaction study of PGPR – *B. pumilus* with tea root pathogens (*U. zonata* and *F. lamaoensis*) in dual culture exhibited significant inhibition towards the growth of the tested fungal pathogens (Plate 13). *In vivo* experiments were conducted to determine the effectiveness of this bacterium (*B. pumilus*) for controlling charcoal stump rot diseases. It was observed that *B. pumilus* was successful in reducing intensity of disease (Table 38).

Table 38: Effects of *B. pumilus* on the development of charcoal stump rot disease of tea

Varieties	Treatment	Root rot index Days after inoculation		
		15	30	45
T-17	*U. zonata*	1.90	3.00	5.55
	U. zonata + *B. pumilus*	0.12	1.24	2.25
TV-26	*U. zonata*	1.45	2.64	4.60
	U. zonata + *B. pumilus*	0.52	1.02	2.25
T 25	*U. zonata*	1.34	2.40	4.57
	U. zonata + *B. pumilus*	0.52	0.86	2.24

rot index: 0= no symptom; 1= small roots turn brownish and start rotting; 2= leaves start withering and 20-30% of roots turn brown; 3= leaves withered and 50% of roots affected; 4= shoot tips also start withering; 60-70% affected; 6= whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

4.16. Biochemical changes in tea following 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. In order to determine the effect on photosynthetic apparatus changes in chlorophyll content were also analysed. Besides, catechins the flavonoid flavour component of tea leaves are extremely important and changes in these were also analysed 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, ß-1.3 glucanase were also determined.

4.16.1. 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 (Table 39; Figure 5) revealed that maximum accumulation occurred when there was joint inoculation by PGPR and pathogen. When the plants (T-17 and TV-26) were pretreated with *B. pumilus* and subsequently inoculated with *U. zonata* , the level of total phenol and orthodihydroxyphenol increased in the PGPR treated plants followed by challenge inoculation with the fungal pathogen (Figure 6)

Table 39: Effect of *B. pumilus* on the biochemical components of tea

Tea varieties	Treatment	Protein Content (mg g^{-1} tissue)	Total phenol (mg g^{-1} tissue)	O-dihydroxy phenol (mg g^{-1} tissue)
TV 18	Control	30.1±2.57	27.2±2.65	7.2±0.76
	B. pumilus	40.2±1.80	42.1±2.05	10.4±0.85
T-17	Control	24.5±2.57	22.2±2.75	6.5±0.58
	B. pumilus	39.9±2.44	37.2±2.67	13.5±1.23
TV-26	Control	39.3±3.20	23.5±3.84	7.8±0.92
	B. pumilus	48.5±2.93	38.5±2.63	12.0±1.04

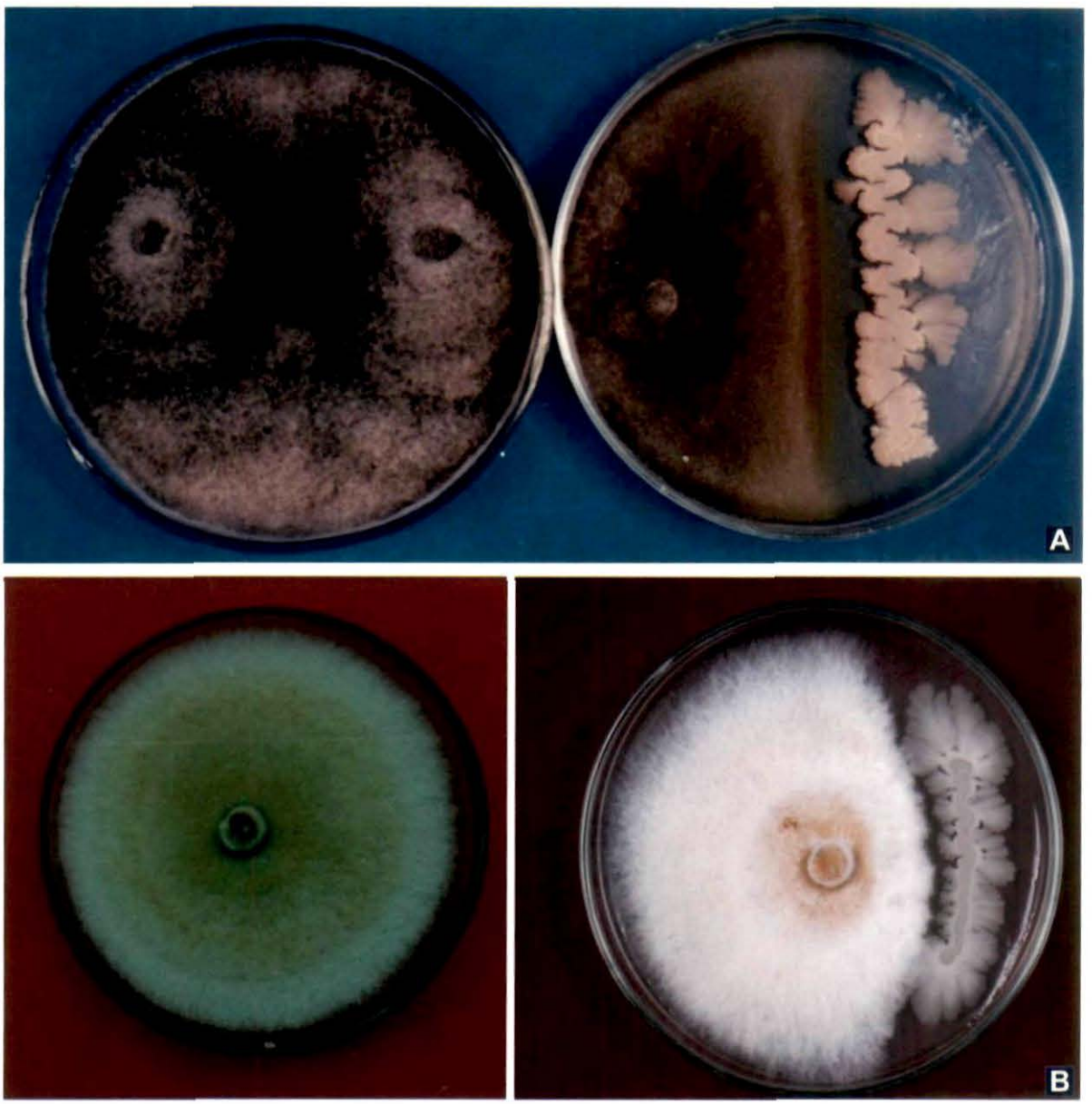
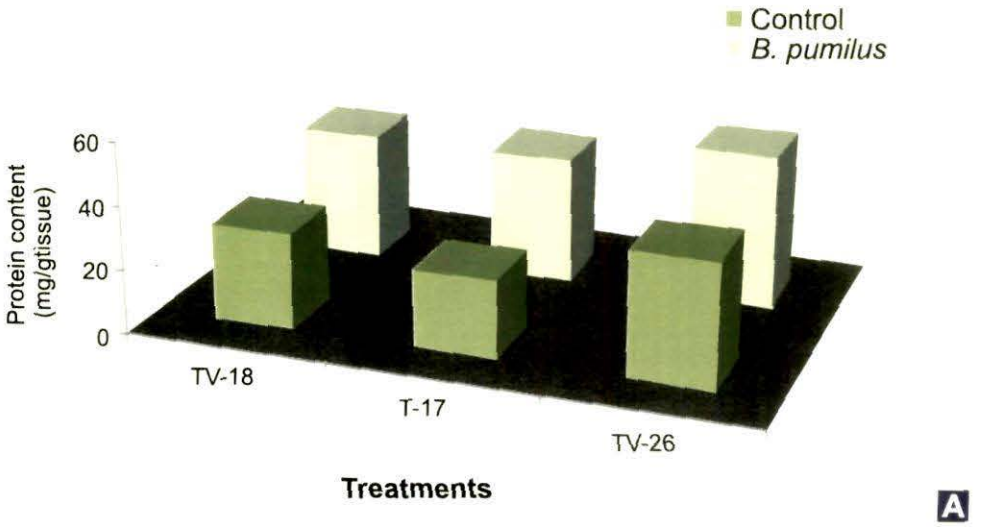
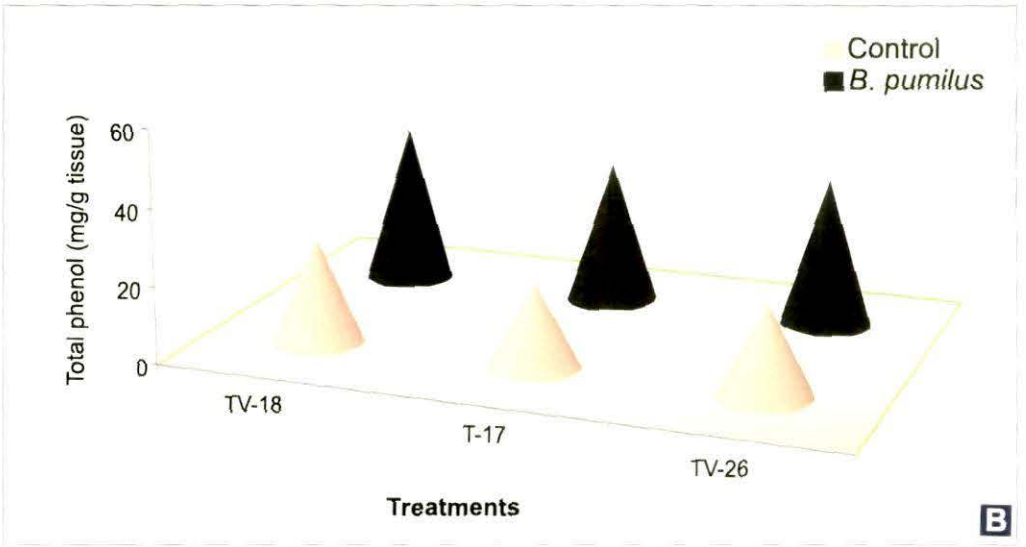


Plate13(A&B): *In vitro* antagonism of *B.pumilus* against *U.zonata* (A) and *F.lamaoensis* (B)





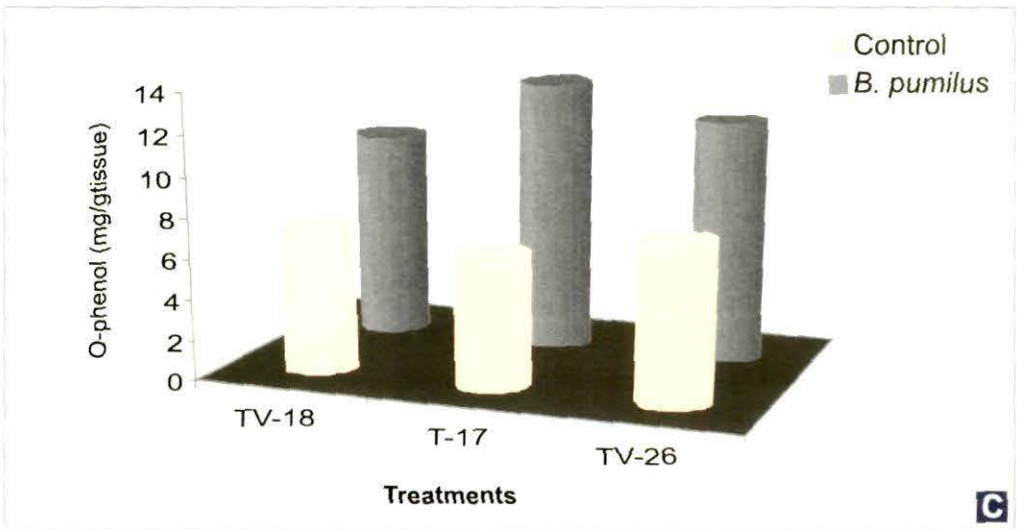


Fig.5 (A-C): Biochemical changes in tea varieties following treatment with *Bacillus pumilus*

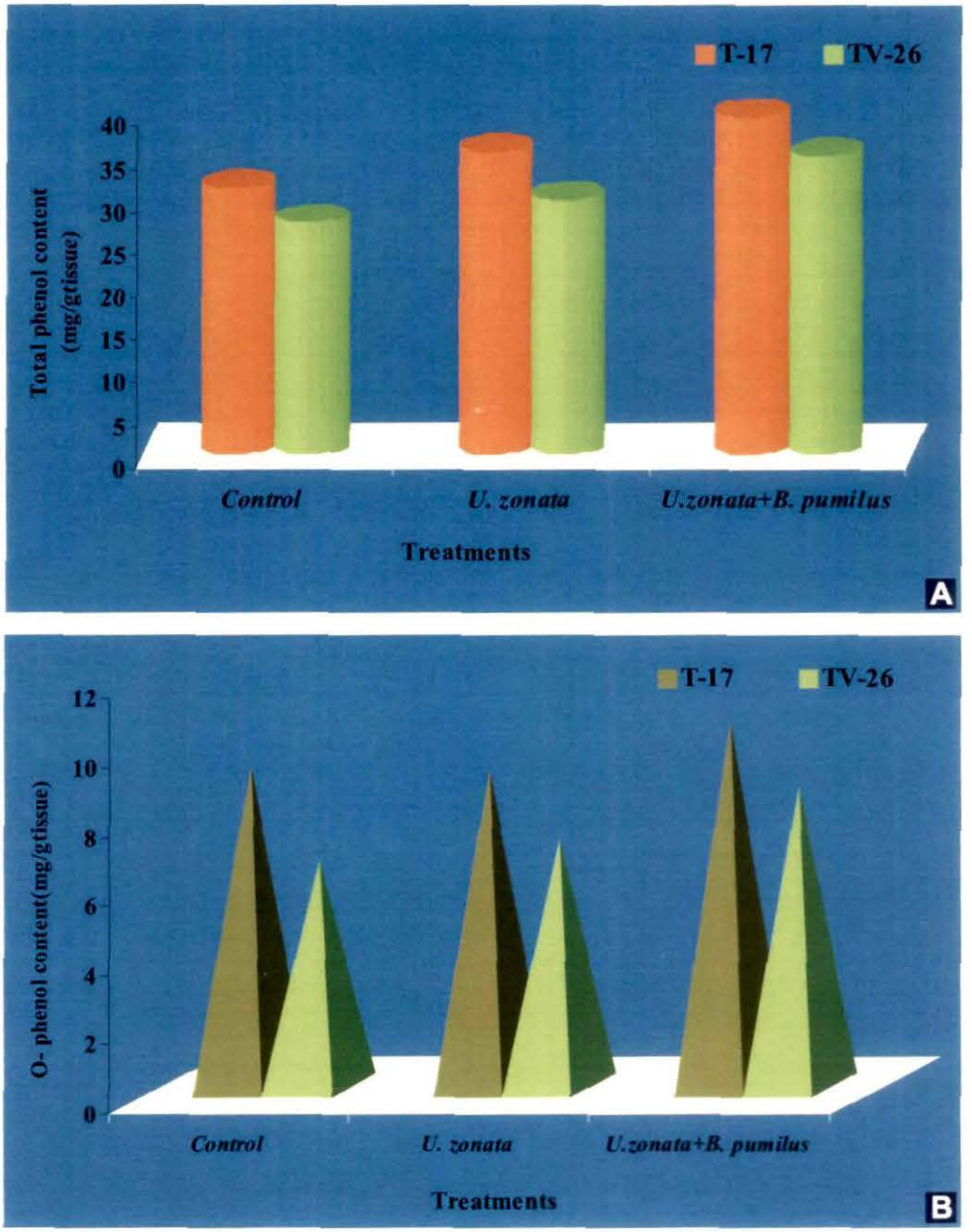


Fig.6 (A&B): Changes in total phenol and orthodihydroxyphenol content following treatment with *B.pumilus* and inoculated with *U.zonata*

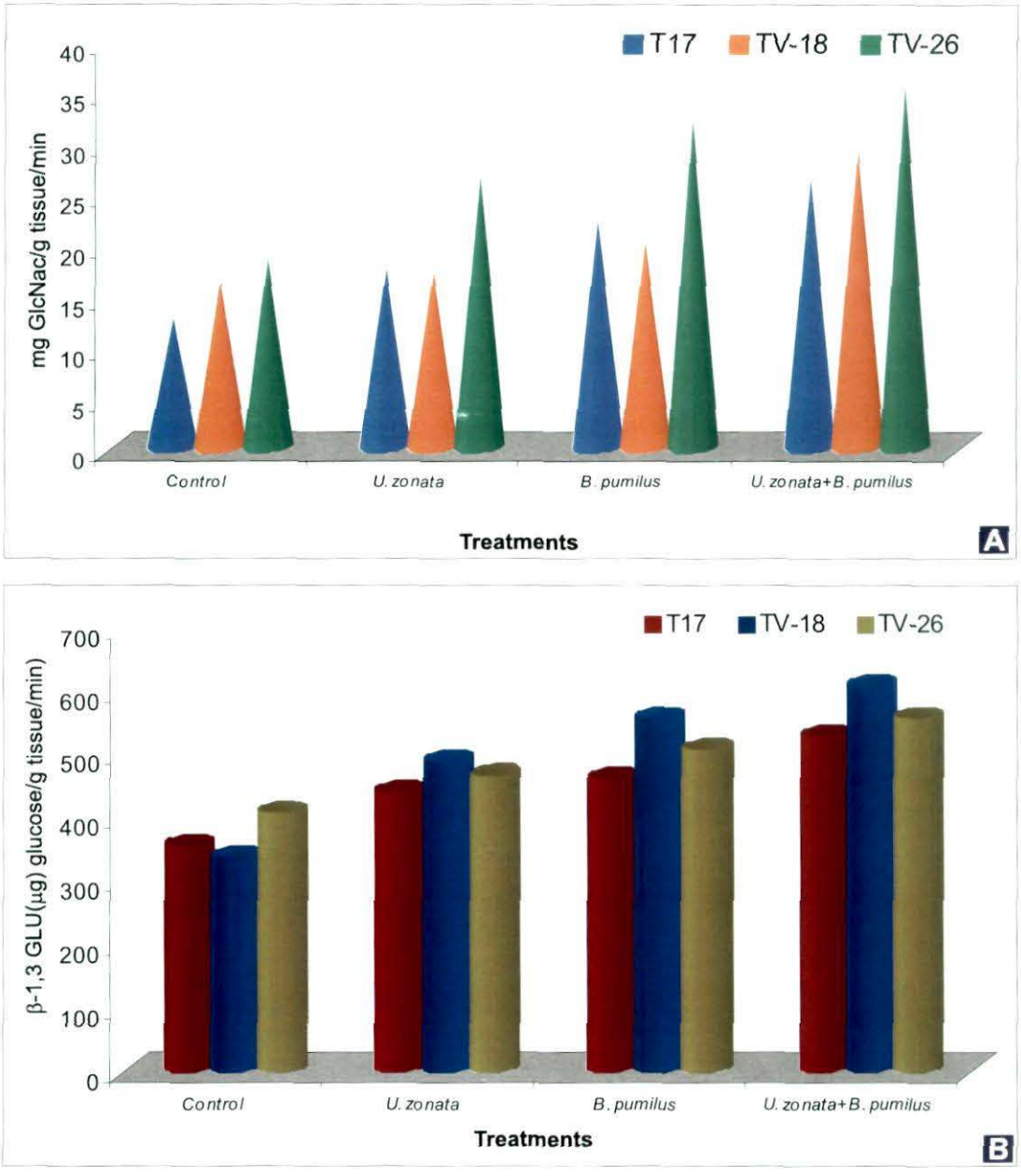


Fig.7(A&B): Glucanase and Chitinase activities in tea plants following treatment with *B.pumilus* and inoculated with *U.zonata*

4.16.2. Chlorophyll

The quantitive analysis (Table 40) revealed that all treatments led to an increase in chlorophyll content both Total as well as chlorophyll a and chlorophyll b.

Table 40: Chlorophyll content of tea leaves following bacterial application in the rhizosphere

Varieties	Treatment	Total chl content (mg/gm tissue	chl A	chl B
TV-26	Control	1.069	0.485	0.554
	B. pumilus	1.083	0.623	0.460
T-17	Control	1.233	0.604	0.99
	B. pumilus	1.233	0.608	0.96
TV-18	Control	1.069	0.485	0.554
	B. pumilus	1.403	0.549	0.854

4.16.3. Enzymes activities

Application of *B. pumilus* to the plants resulted increase in activities of all the four enzymes in the leaves of treated plants compared with untreated plants. However, further enhancement was observed when *U. zonata* was also inoculated (Table 43). Results have been presented in Figures 7 & 8 .

Table 43: Enzyme activities in leaves of different tea varieties grown in soil following treatments in the rhizosphere.

Varieties	treatment	Enzyme activities			
		POX	PAL	CHI	GLU
T17	Control	3.7±0.2	66±0.5	12.5±0.9	360±2.0
	U. zonata	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
	B.p+U.z	8.2±0.2	141±0.3	26.4±1.0	532±1.2
TV-18	Control	3.8±0.1	65±0.2	16.4±1.4	340±1.8
	U. zonata	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
	B.p+U.z	5.8±0.6	132±0.4	29.2±1.1	610±2.1
TV-26	Control	4.5±0.3	84±0.5	18.3±1.7	410±2.9
	U. zonata	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
	B.p+U.z	7.4±0.5	139±0.9	35.2±2.1	560±4.2

POX activity assayed as Δ A 465 min^{-1} g $tissue^{-1}$; PAL activity assayed as µg N-Acetyl glucosamine released by enzyme from 1 g tissue min^{-1} and ß 1,3- GLU activity assayed as µg glucose released by enzyme from 1g tissue min^{-1}.

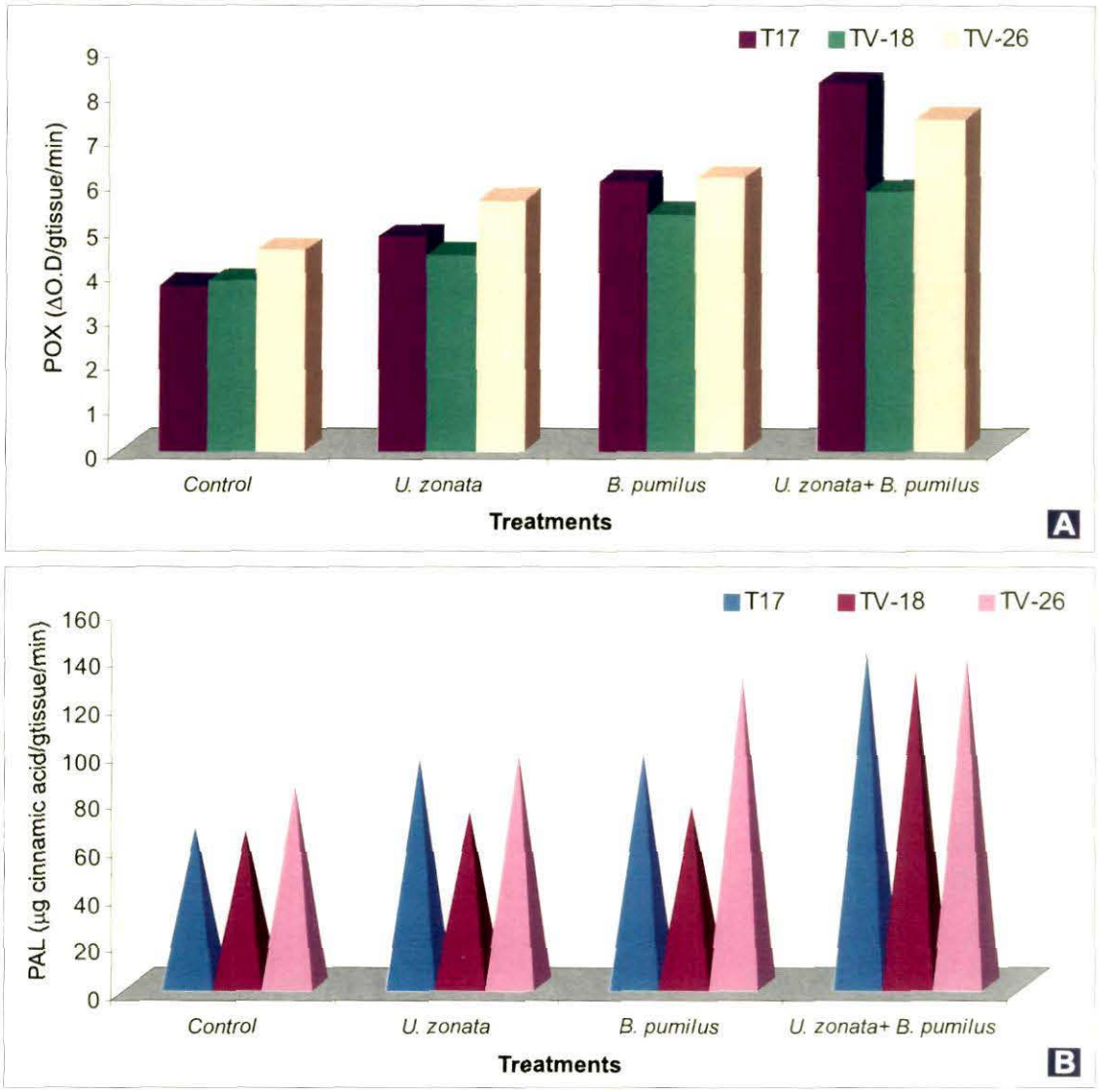


Fig.8 (A&B): Peroxidase and phenylalanine ammonia lyese activities in tea plants following treatment with *B.pumilus* and inoculated with *U.zonata*

4.17. *In vitro* determination of mechanism of action of *B. pumilus*

The growth promotion of plants may be achieved by the ability of bacteria to facilitate phosphorous uptake or produce phytohormones (IAA), HCN, Volatile and Chitinase that trigger responses in a growing plants.

4.17.1. Phosphate solubilization

Formation of clear zone around the colony grown in Pikovskaya's medium is an indication of phosphate solubilization by rhizobacteria. In Pikovskaya's medium *B.pumilus* produced clear zone of diameter 2.6 cm after 4 days of incubation which indicated that the isolates could solubilise the insoluble phosphate unto simpler substances.

4.17.2 IAA production

IAA production by the rhizobacteria was assessed for their ability to produce IAA by growing in Tripticase soya agar supplemented with tryptophane (0.1mM) *B.pumilus* recorded the IAA production of 42mg/L.

4.17.3. Siderophore production

To assess the siderophore production by the antagonistic rhizabacteria, the bacteria was inoculated into Chrome Azurol Sugar agar plates and were incubated for 10-15 days. The appearance of yellow halo region was observed around *B.pumilus* which indicated a bacterium is able to chelate Fe3+ from Chrome Azurol Sugar. Bacteria are able to change the color of CAS- medium from blue to brownish yellow, indicating that they could produce siderophore The diameter of halo region was 1.6 after 12 days of incubation..

4.17.4. HCN production

To determine the ability of *B.pumilus* to produce HCN, the bacterium was 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. *B.pumilus* was found to be non-cyanogenic in nature. This suggests that compound other than HCN may be associated in the inhibition of pathogen in dual culture.

4.17.5. Chitinase production

To determine chitinase production by the antagonistic rhizobacteria spot inoculation was made in the 5% colloidal chitin amended minimal medium and incubated at 28° C for 6-7 days. It was observed that no extracellualr chitinase was secreted by *B.pumilus*.

4.17.6. Volatile production

Volatile compound production by the antagonistic bacteria 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 material and methods. Results showed that the antagonists produced volatile compounds.(Table 44)

Table 44: Effect of volatile compounds on growth inhibition of test pathogen

Pathogens	*B.pumilus*	
	RMG(cm)	GI (%)
U. zonata	4.2	52
F. lamaoensis	3.0	66
S. rolfsii	5.0	44
F. oxysporium	5.1	44
Control	9.0	-

RMG-radial mycelial growth; GI- Growth inhibition; Average of three replicates;

The results obtained from above mentioned experiments suggest plant growth promotion activity by *B. pumilus* which has been summarized in Table 45.

Table 45: PGPR activity of *B. pumilus*

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

Average of three replicates; + positive reaction; - negative reaction

4.18. Bioassay of active principle from *B. pumilus* against test fungi

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 pathogen. Cell free culture filtrate of the bacterial strains were prepared and sterilized either by autoclaving or by passing through sterilized G-5 filter. These were then mixed with the medium in different proportions and the test pathogen (*U. zonata*) was inoculated into it. Results revealed that both autoclaved and cold sterilized cell free culture filtrates significantly restricted the growth of pathogen in vitro. As the ratio of culture filtrates in broth increased, a marked decrease in mycelial growth of the pathogen was recorded (Table 46).

Table 46: Effect of autoclaved culture filtrate on the mycelial groth of *U. zonata*

Treatment	mycelial dry wt (mg)
PDB	280.1
ACF	0(100)
ACF+10%PDB	040 (85.8)
ACF+30%PDB	090 (65.4)
ACF+50%PDB	123.5 (55.2)

PDB- potato dextrose broth; figures in parenthesis indicate percentage (%) of inhibition over control.

4.19. Application of Bioformulations on growth of tea plants

Since the selected bacterial isolate (*B. pumilus*) showed plant growth promoting activity, as well as PSF isolate (*A. niger*) also exhibited growth in tea plants following application with FYM, it was decided to use commercial products of AM fungi – Josh (Plate 14) and PSF product – Kalisena (Plate 15), in order to determine whether they have such growth promoting activity. Josh capsules were applied in tea plant root system of three different varieties (T-17, TV-18 and TV-26) and subsequently their colonization with root systems were examined (Plate 16).

Accordingly, Josh and Kalisena alone and in combination with PGPR were applied to three varieties of tea plants. The growth of the seedlings were noted in each case and increase in growth rate was computed. It was observed that in relation to control, tea plants subjected to all treatments showed increased growth rate (Table 47)

Plate 14 (A&B): Josh commercially available Mycorrizae root inoculants (A); Gelatin capsules containing AMF spores (B)

Plate15 (A-F): Kalisena- commercial formulation (A-D); Phosphate solubilizing fungus - *Aspergillus niger* (E&F).

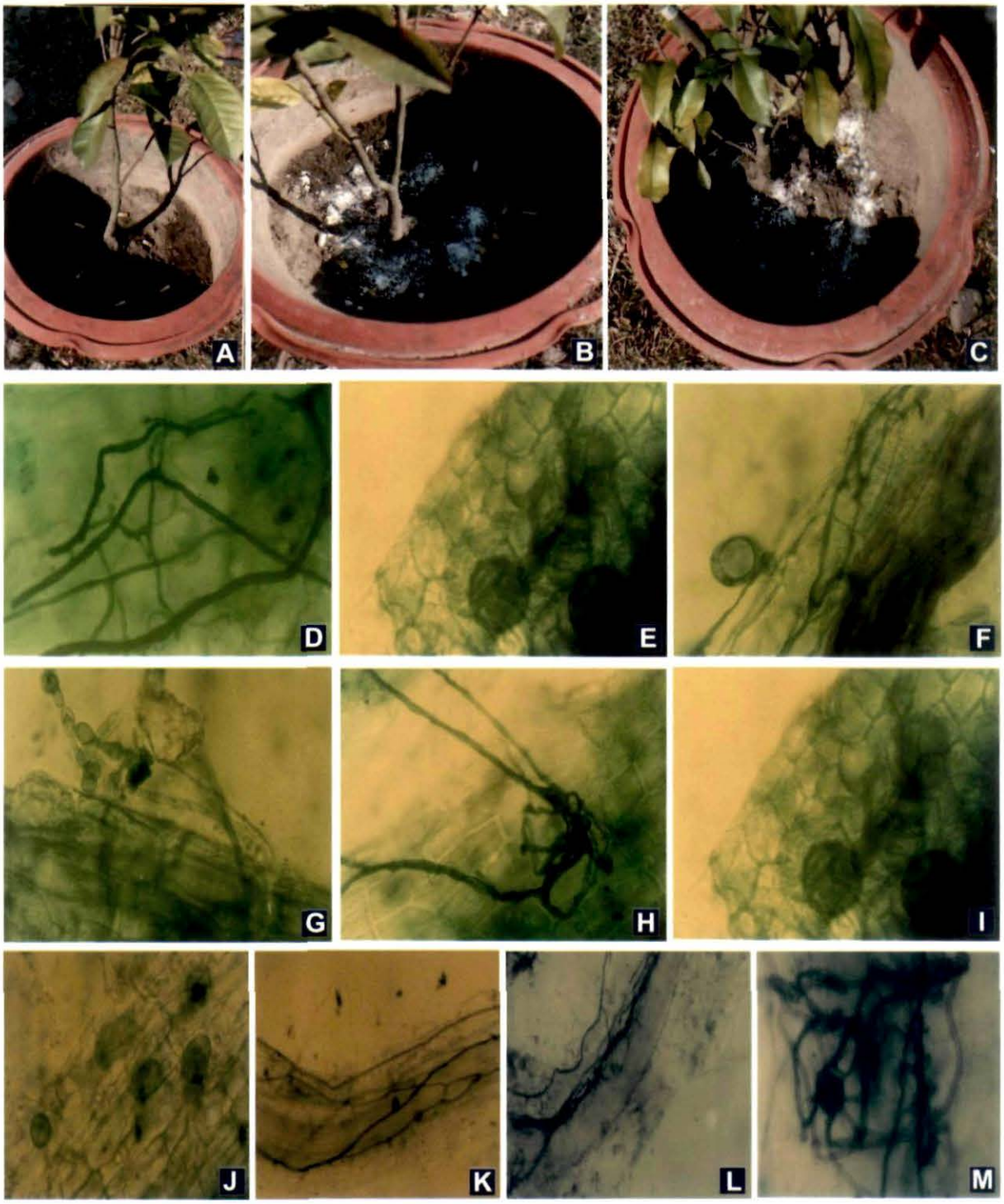


Plate 16 (A-M): Artificial inoculation of tea varieties with Josh capsule (A-C) and mycorrhizal associations in tea roots (D-M); T 17(A,D,E,F); TV 18 (B, G, H, I) and TV-26 (C, J, K, L, M)

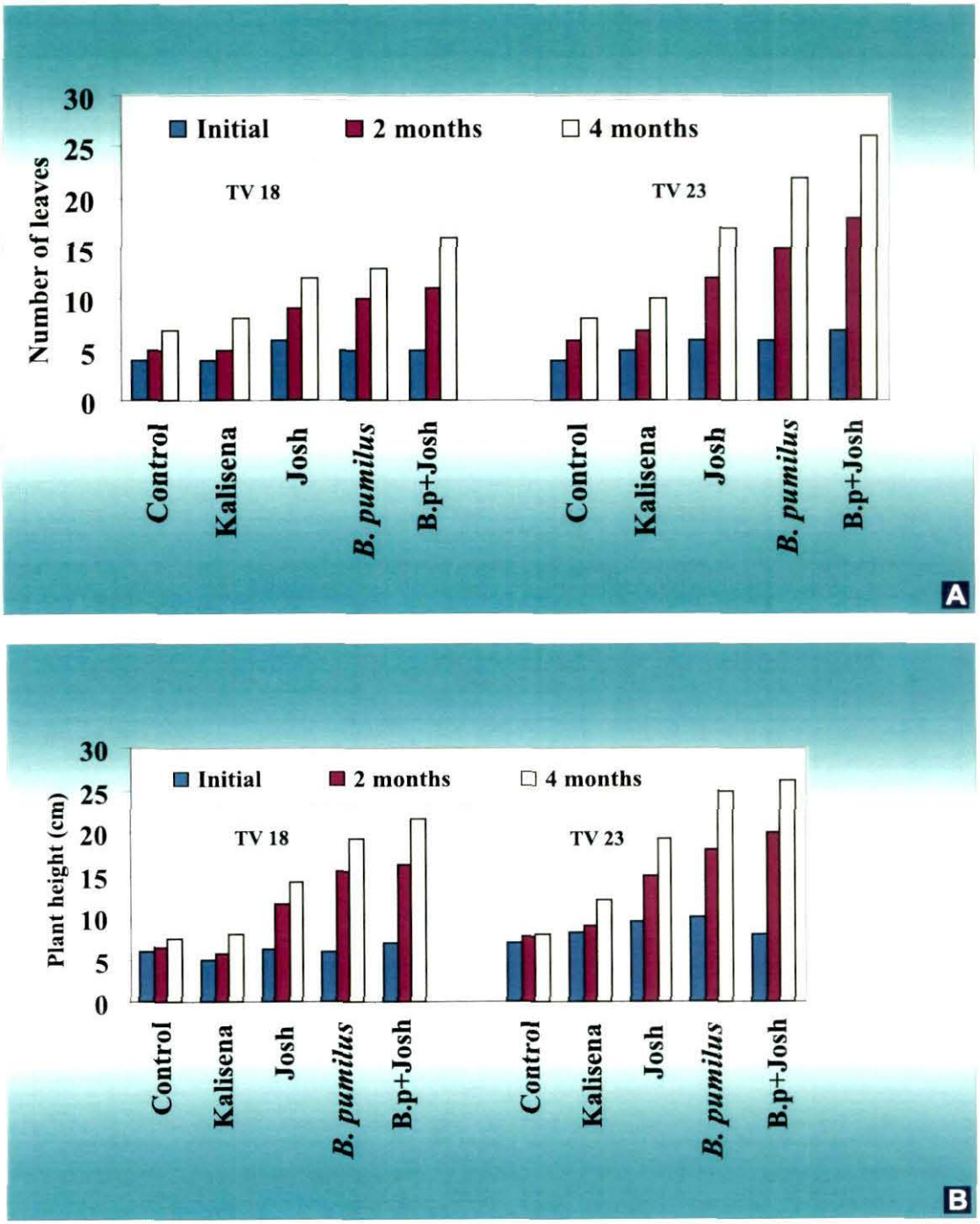


Fig.9 (A&B): Effect on growth of tea plants following application of Kalisena, Josh and *B.pumilus*

Table 47: Effects of Bioformulation treatments on growth of tea seedling.

Treatment	% increment in shoot length over initial		
	TV-18	**T- 17**	**TV- 26**
Control	109.1	120.0	95.7
Kalisena	286.5	267.5	209.0
Josh	300.2	246.2	185.3
PGPR+JOSH+Kalisena	470.3	310.1	245.0

Ten plants per treatment, in triplicate and values are an average of 30 plants. Results were recorded 2 months following the treatments.

It has been postulated that some phosphate solubilizing bacteria behave as mycorrhizal helper bacteria. It is likely that the phosphate solubilized by the bacteria could be more efficiently taken up by the plant through a mycorrhizal pipeline between roots and surrounding soil that allows nutrient translocation from soil to plant. Considerable evidence supports the specific role of phosphate solubilization in the enhancement of plant growth by phosphate solubilizing microorganisms.

Effects of inoculations with Josh, Kalisena and *B. pumilus* singly and jointly on growth of tea plants were evaluated in terms of number of leaves, number of branches and height of the plant. Results have been presented in Table 48 and Figure 9

Table 48. Plant growth promotion in tea varieties following treatments with bioformulation

Treatments	TV-26			TV-18		
	Percentage increase in *			percentage increase in *		
	Height	**No.of branches**	**No. of leaves**	**Height**	**No.of branches**	**No.of leaves**
Untreated	20.0	50.0	62.5	52.6	50.0	70.0
Kalisena	72.6	80.0	160.0	61.5	75.0	68.7
Josh	83.3	100.0	166.6	60.0	80.0	86.6
PSF+ PGPR	105.0	300.0	200.0	110.5	150.0	200.0
Josh+ Kalisena + PGPR	88.8	166.6	141.6	77.3	140.0	155.0

* Percent increase calculated after a period of 60 days, taking into consideration initial and final readings.

Soil sample from rhizosphere of the two varieties were collected after 60 days treatment and phosphorous content in soil was analyzed for the amount of phosphorous depletion on mobilization after treatment with Josh, Kalisena and PGPR. Phosphorous content in soil has been lowered in the treated block due to excess phosphorous mobilization or utilization by those plants treated with 'Josh', 'Kalisena SD' and combination with PGPR (Table 49)

Table 49: Total phosphorous in rhizosphere soil of tea plants subjected to various treatments

Variety	Treatments	Concentration (mg/lit)
TV-18	Control	0.302
	Josh	0.256
	Kalisena	0.190
	PSF+ PGPR	0.185
	Josh+Kalisena+PGPR	0.180
TV-26	Control	0.354
	Josh	0.232
	Kalisena	0.218
	PSF+ PGPR	0.191
	Josh+Kalisena+PGPR	0.195

When the plants were inoculated either with *G. mosseae* and *B. pumilus* separately or jointly the growth of the plants increased markedly (Plates 17 & 18). In experimental garden when tea seedlings were inoculated with *G. mosseae* and subsequently cell suspension of *B. pumilus* was applied to these plants, excellent growth was noticed (Plate 19). Percentage increase in leaf number was also evident (Figure 10). Phosphorous solubilization following these treatment was also calculated (Table 50)

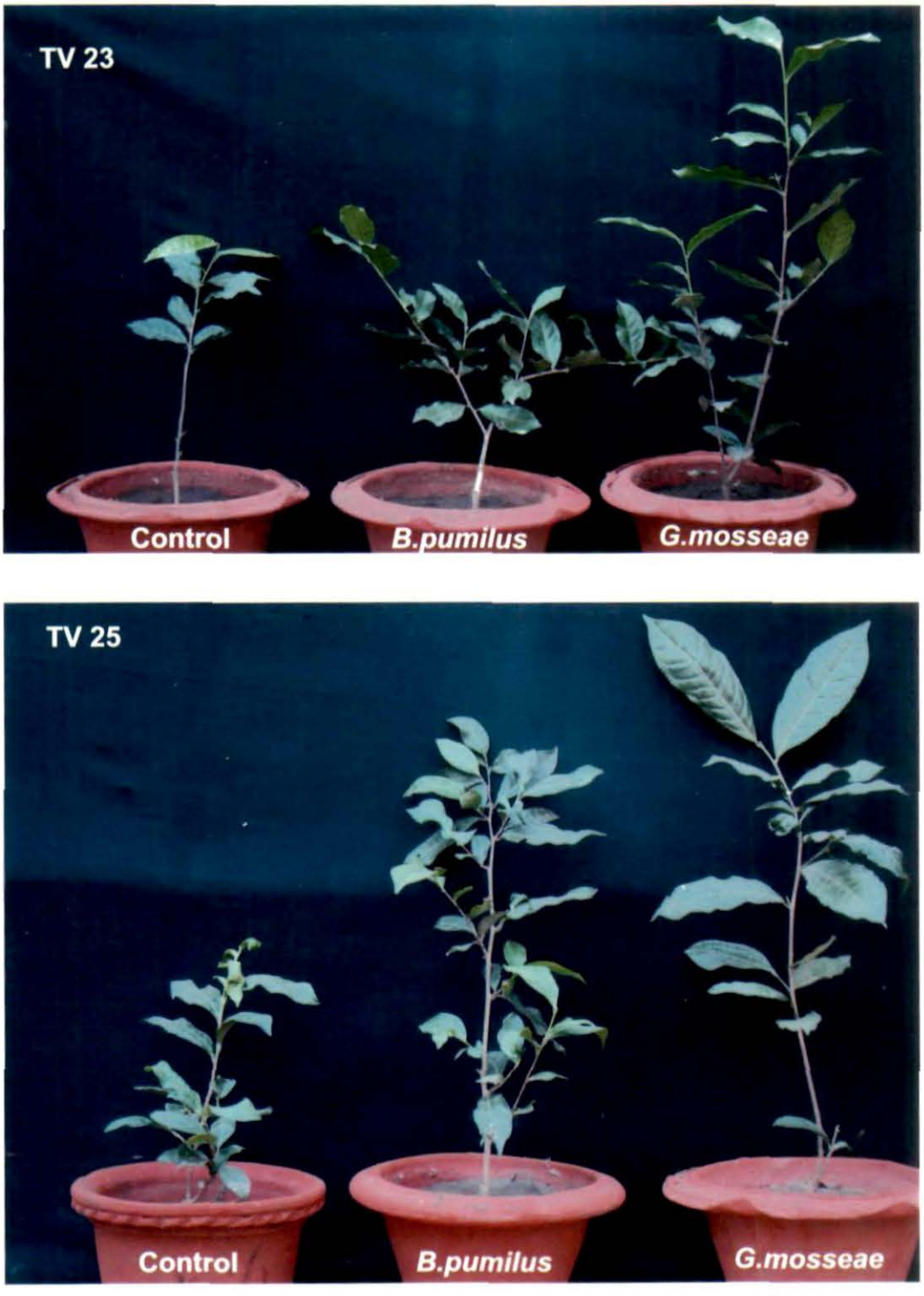


Plate 17: Effect of *Bacillus pumilus and G.mosseae* on growth of two year old potted tea plants

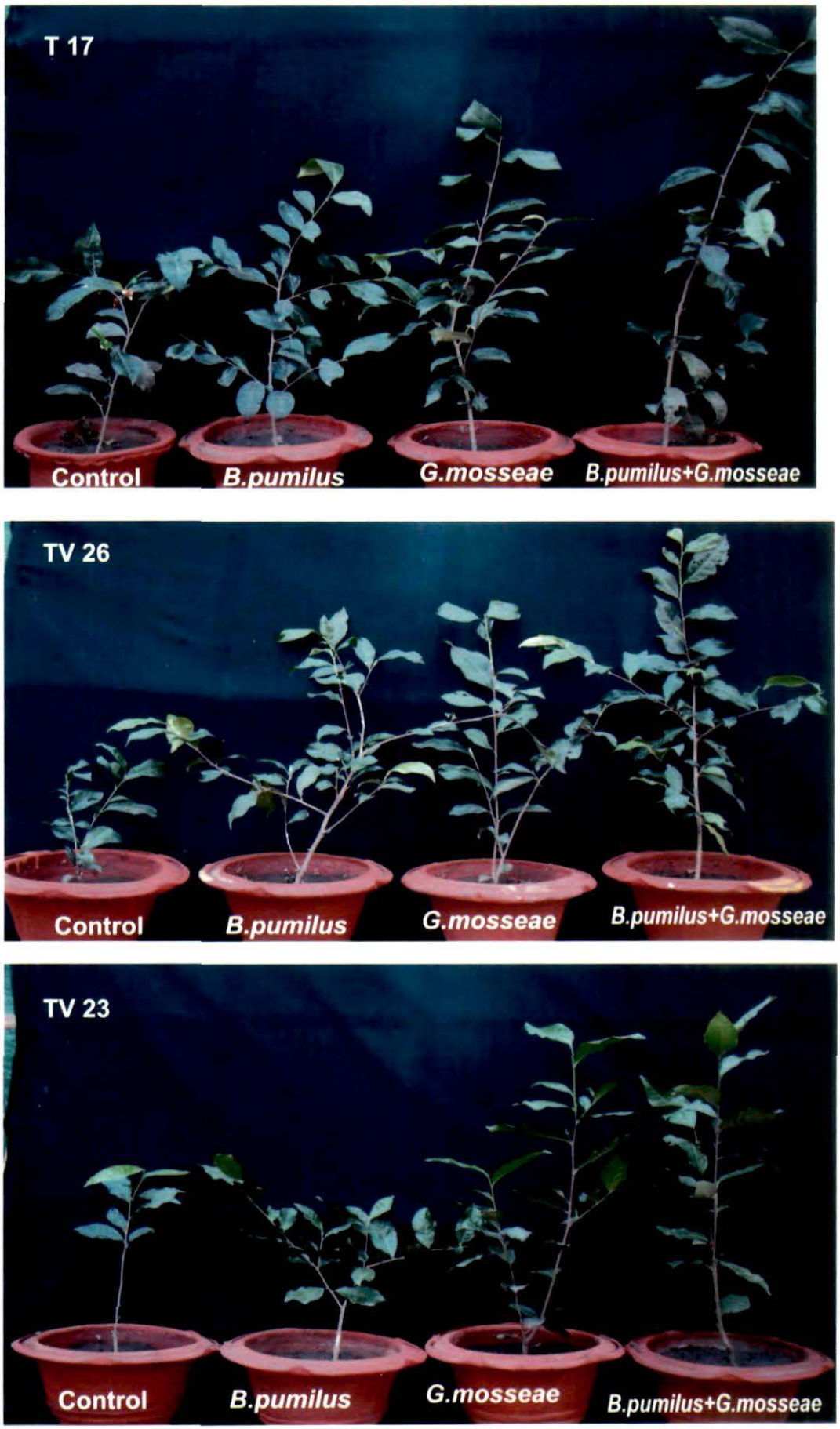


Plate 18: Effect of *B.pumilus*, *G.mosseae* and coinoculation of *B.pumilus* and *G.mosseae* on growth of two year old potted tea plants

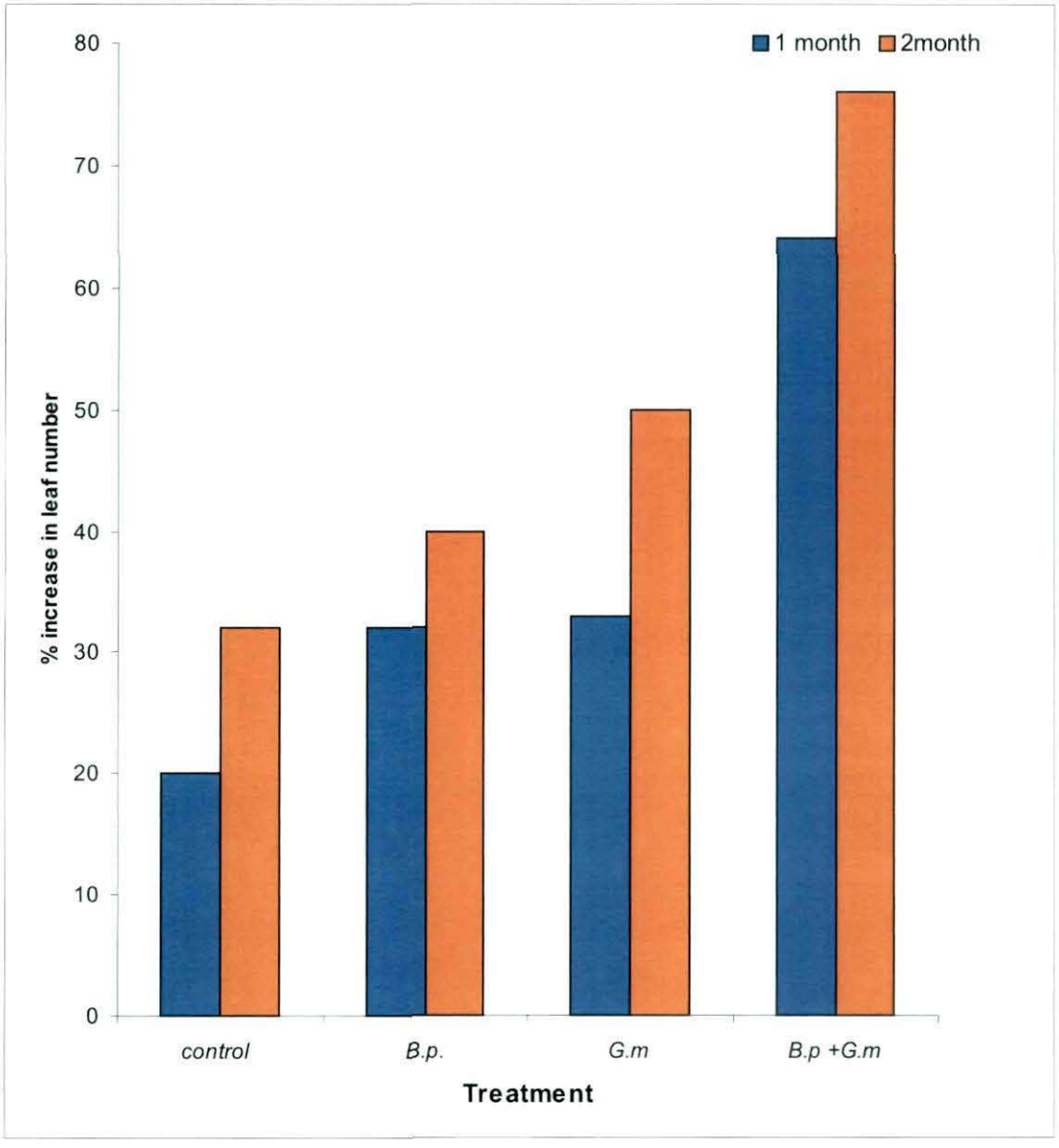


Fig.10: Effect on growth of tea plants in terms of increase in leaf numbers after 1 and 2 months of single as well as combined inoculation with *B.pumilus* and *G.mosseae*

Plate 19(A&B): Untreated (Control) Tea plants (A) and plants inoculated with *G.mosseae* and treated with *B.pumilus* (B)

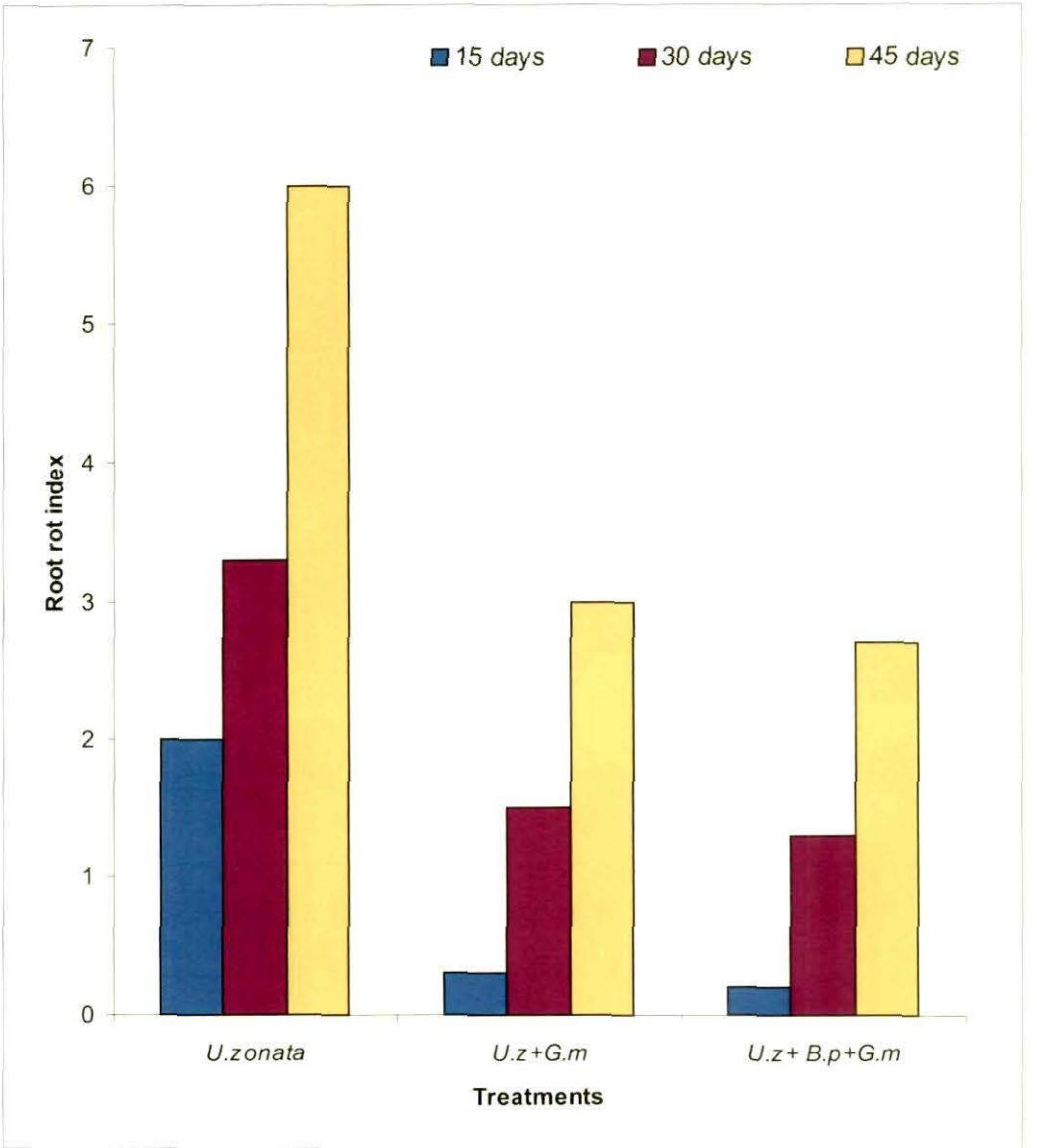


Fig. 11: Disease development in tea roots followed by treatments with *G.mosseae* and *B.pumilus* and inoculated with *U.zonata*

Table 50: Phosphate utilization by tea plants following treatment with *G.mosseae* and *B. pumilus*

Treatment	Soil phosphate (µg/g tissue)
Control	$49.37^{a} \pm 1.18$
B.pumilus	$33.12^{b} \pm 0.57$
G. mosseae	$31.25^{b} \pm 3.60$
B. pumilus+ *G. mosseae*	23.75^{c}

Average of 3 replicates; ± = standared Error; Difference between values significant at P= 0.01 where superscript is different; not significant where superscript is same

4.20. Disease assessment following treatments

Tea plants (TV-18 and T- 17) inoculated with *G. mosseae* and *B. pumilus* singly or jointly and subsequently inoculated with *U. zonata* to assess disease development. Disease intensity was assessed as root rot index It was observed that both the treatments reduced the disease incidence in relation to untreated inoculated control. (Figure 11). Another experiment was performed using all the potent microorganisms (*A. niger*, *B. pumilus* and G. mosseae) as well as VAM product Josh applied to the plant singly and in combinations and then challenged with the pathogen (*U. zonata*). Disease assessment was made by calculating root rot index. Results (Figure 12) revealed that reduction in disease intensity was always more in joint inoculation with PGPR (*B. pumilus*) and *G. mosseae* or Josh (VAM product) and *B. pumilus* (PGPR).

4.21. Biochemical changes in tea plants following application of bioformulations

An increase in phenol content was obtained in all treatments. However, Kalisena treated plants (TV-18 and TV-23) exhibited steady increase of phenolics (Figure-13). Maximum increase in phenolics was noticed in joint treatments of four varieties tested (TV-18, TV-23, T-17 and TV-26) with Josh and PGPR (Figure 14). In another experiment with four varieties (TV-18, TV-26, TV-25 and T-17)were treated with Josh formulation. Glucanase and chitinase activities increased in Josh treated tea plants in relation to control (Figure 15). When the plants were inoculated either with *G. mosseae* and *B. pumilus* separately or jointly and following these treatment the plants were challenge inoculated with *U. zonata,* the level of three defense enzymes (peroxidase, glucanase and chitinase) increased markedly. However, the increased rate of accumulation of these enzymes were noticed when the plants were co-inoculated with both *G. mosseae* and *B. pumilus* and then challenged with the pathogen (*U. zonata*). The results have been presented in Figure 16.

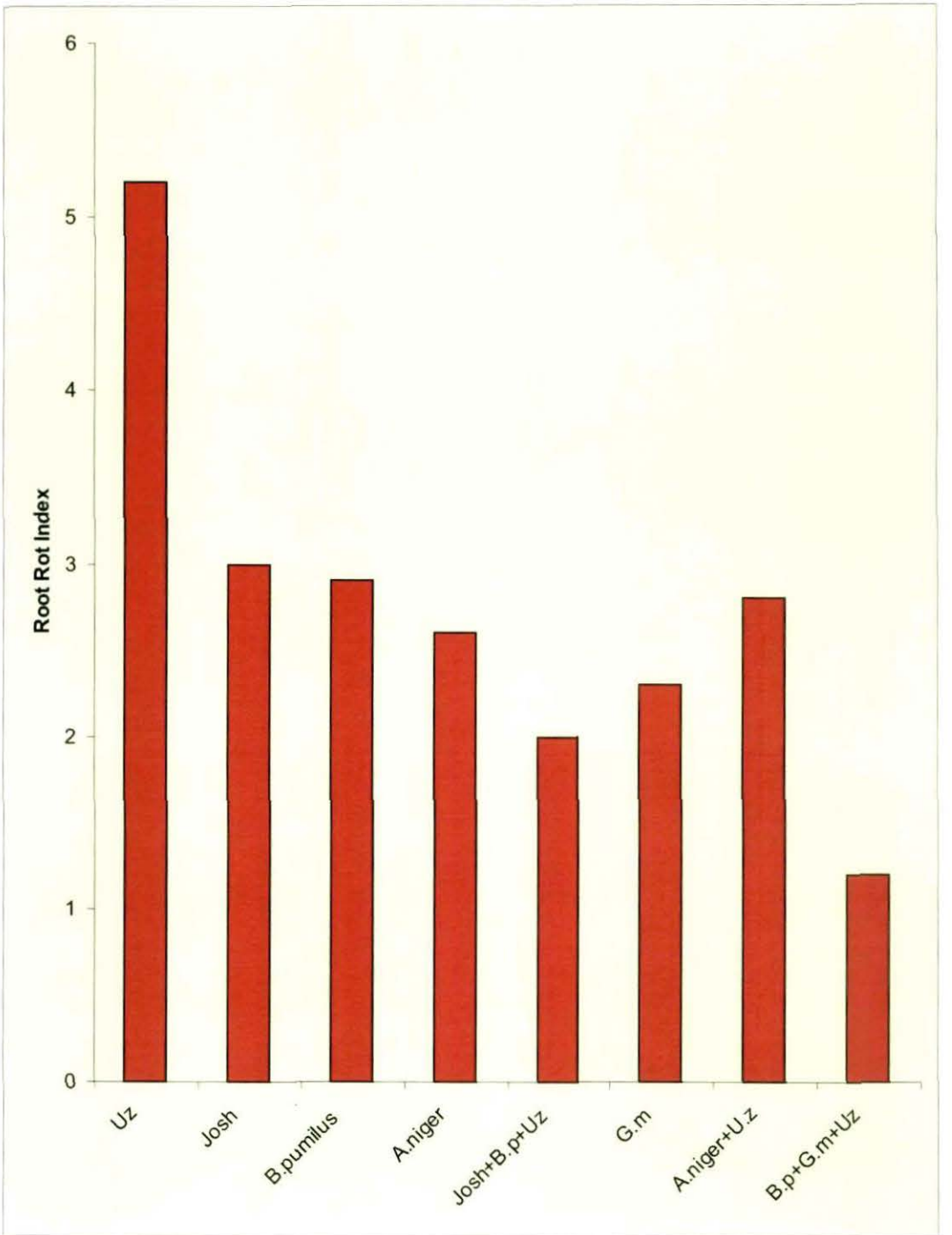


Fig.12: Disease development in tea roots by various treatments and inoculated with *U.zonata*

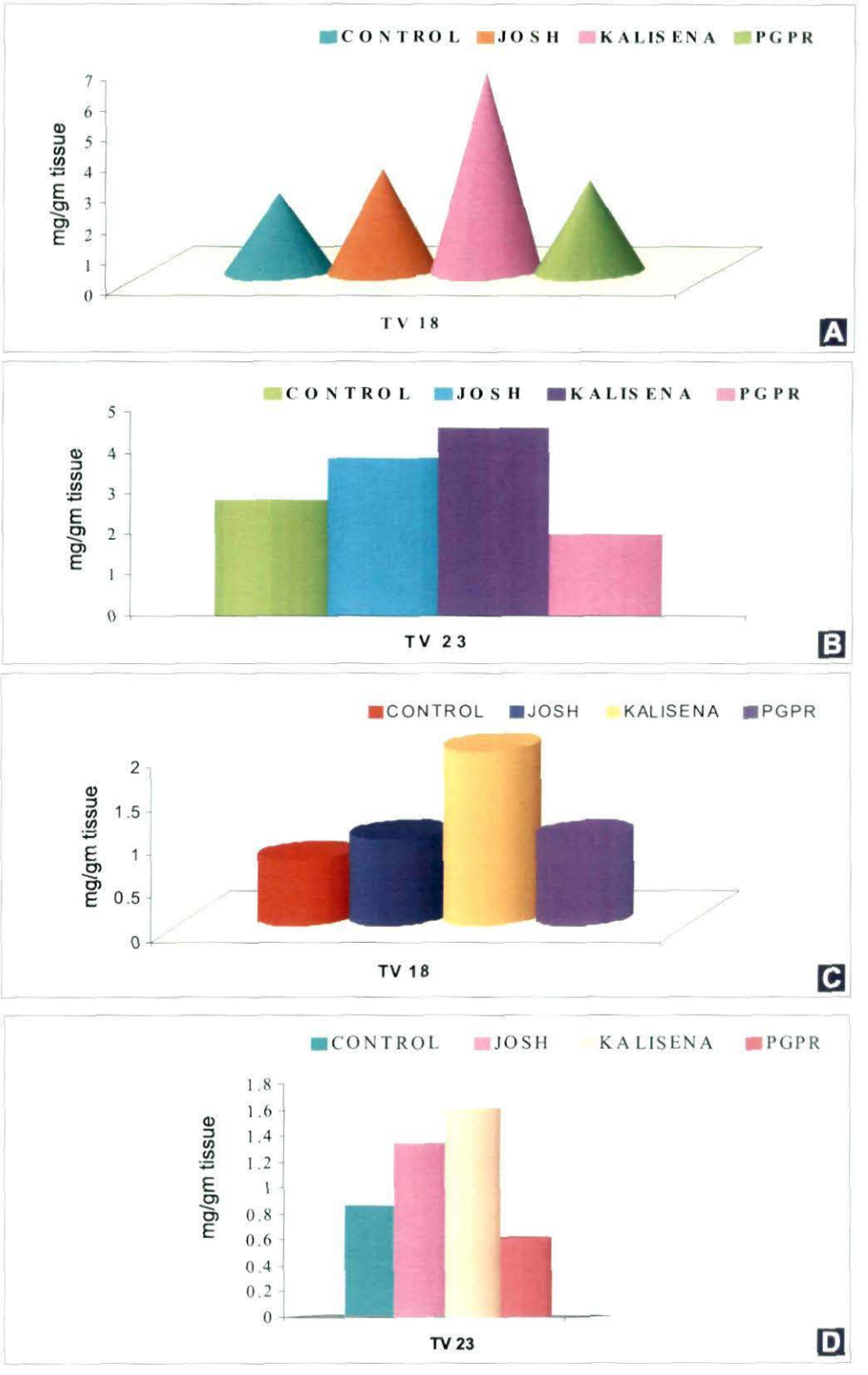


Fig.13(A-D): Changes in phenolics of tea plants following treatments with Josh, Kalisena and PGPR

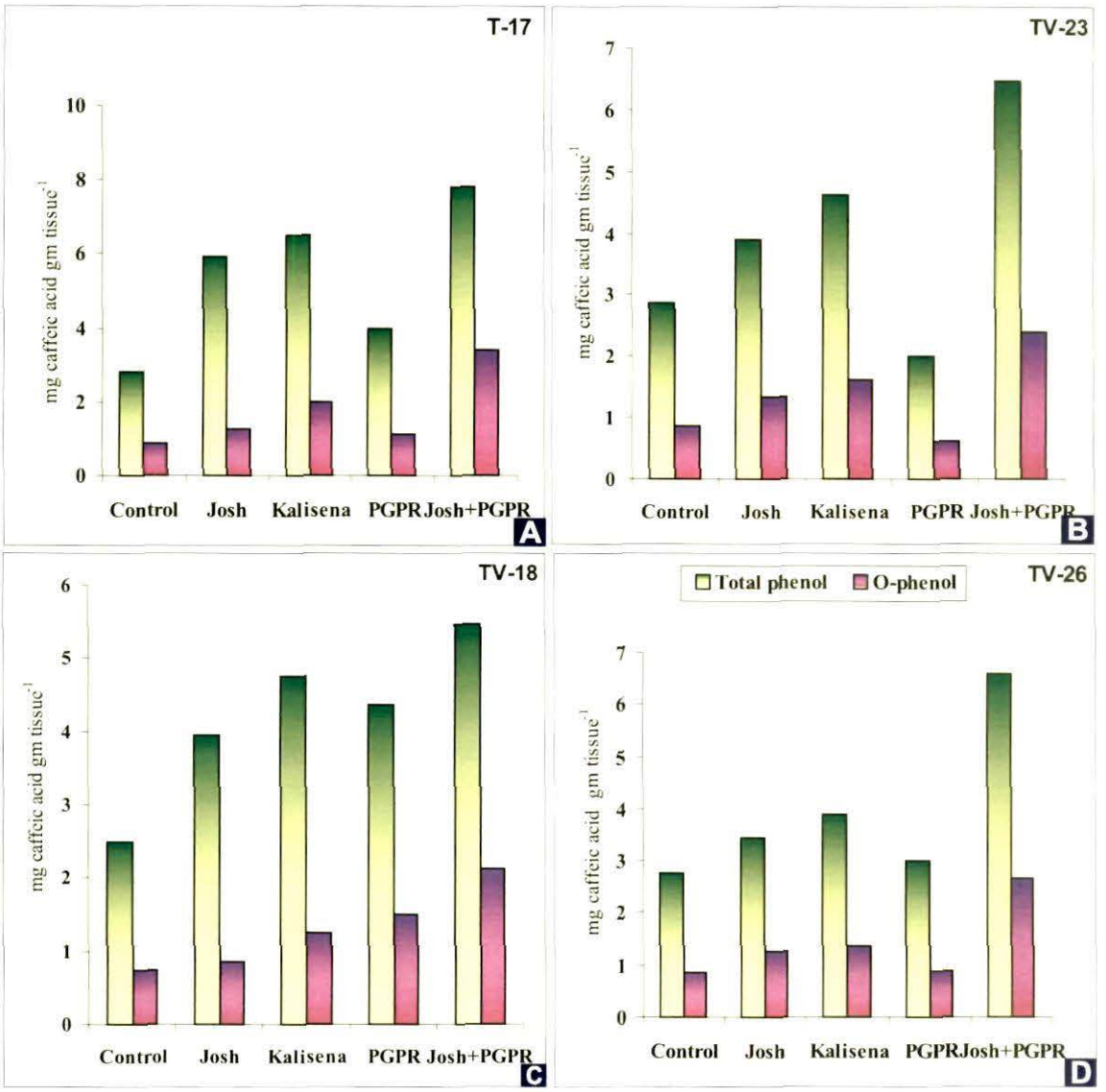


Fig.14 (A-D): Changes in phenolics in tea roots after dual inoculation with VAM (Josh) and PGPR (*B.pumilus*) in comparison with Kalisena

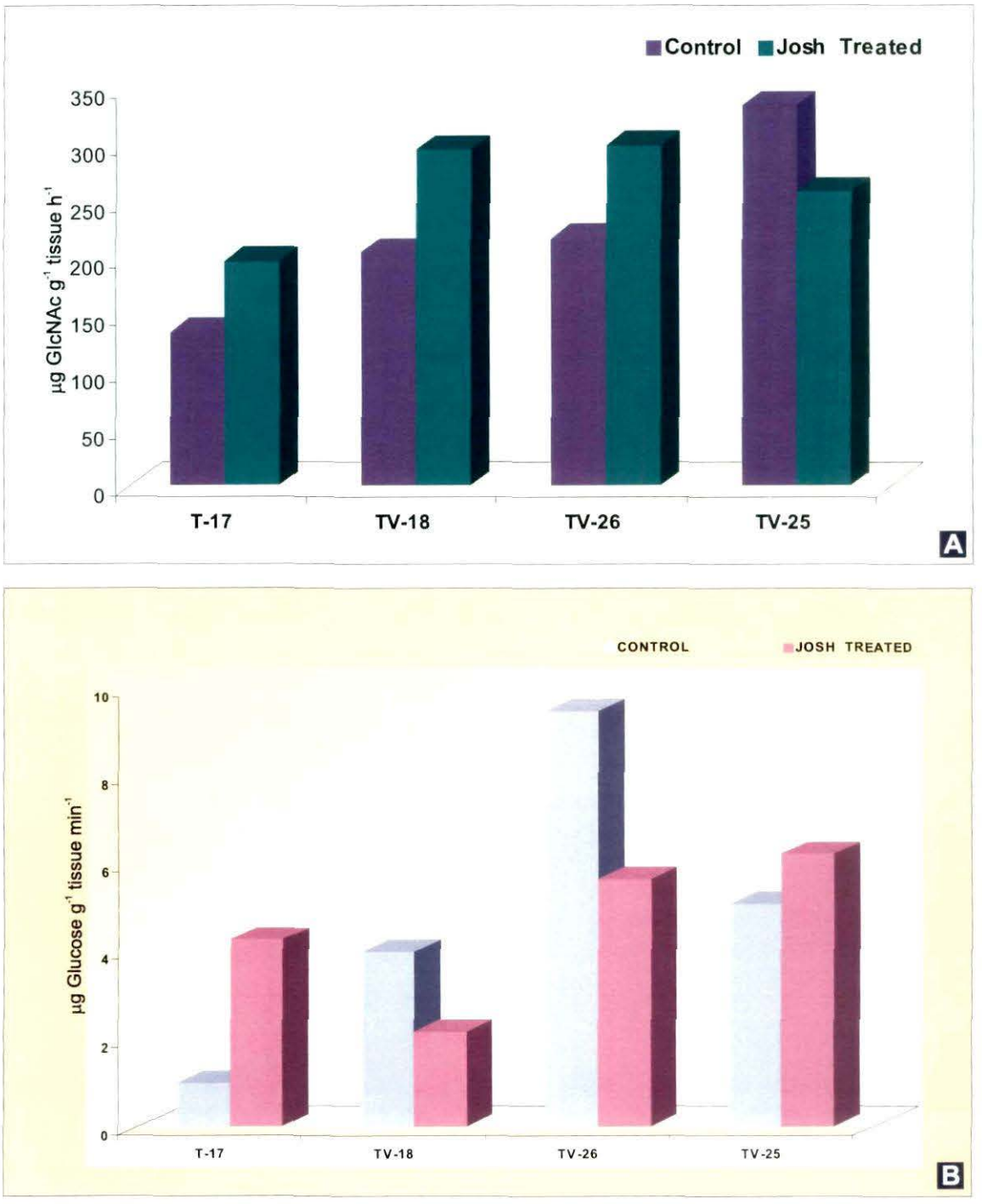
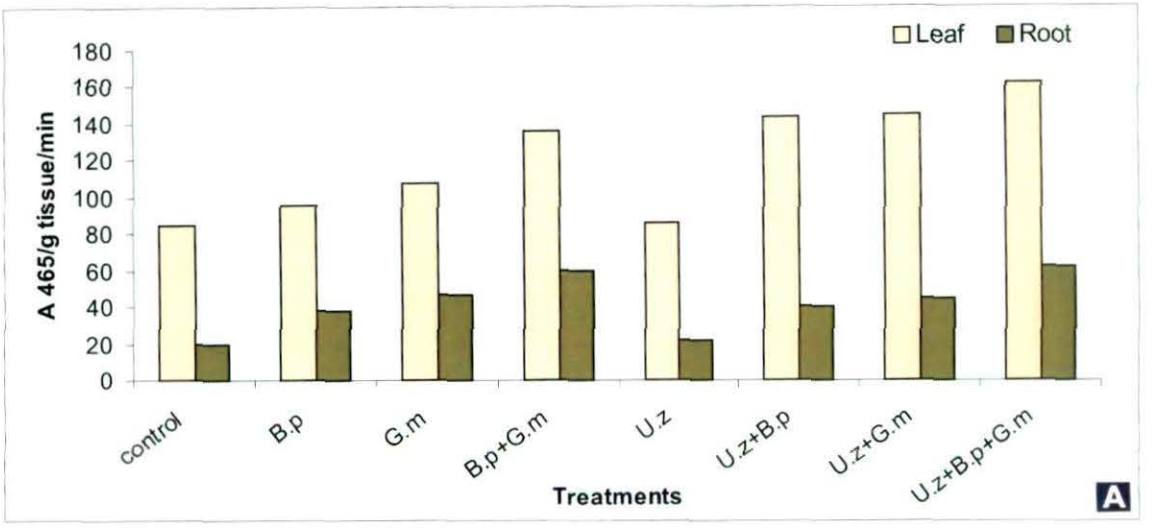
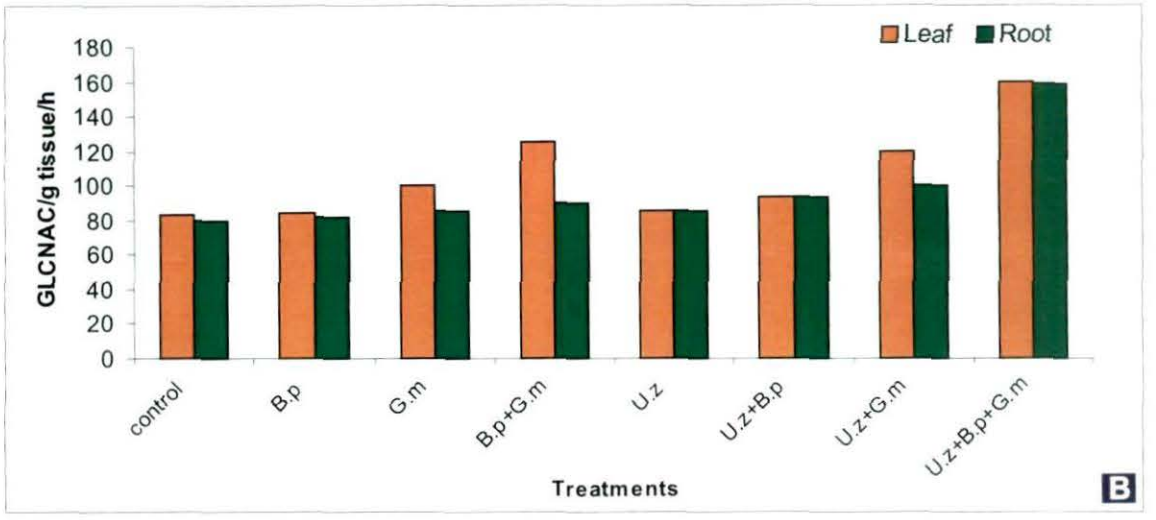


Fig.15 (A&B): Chitinase (A) and β-1,3 glucanase (B) activities in tea roots following treatment with Josh





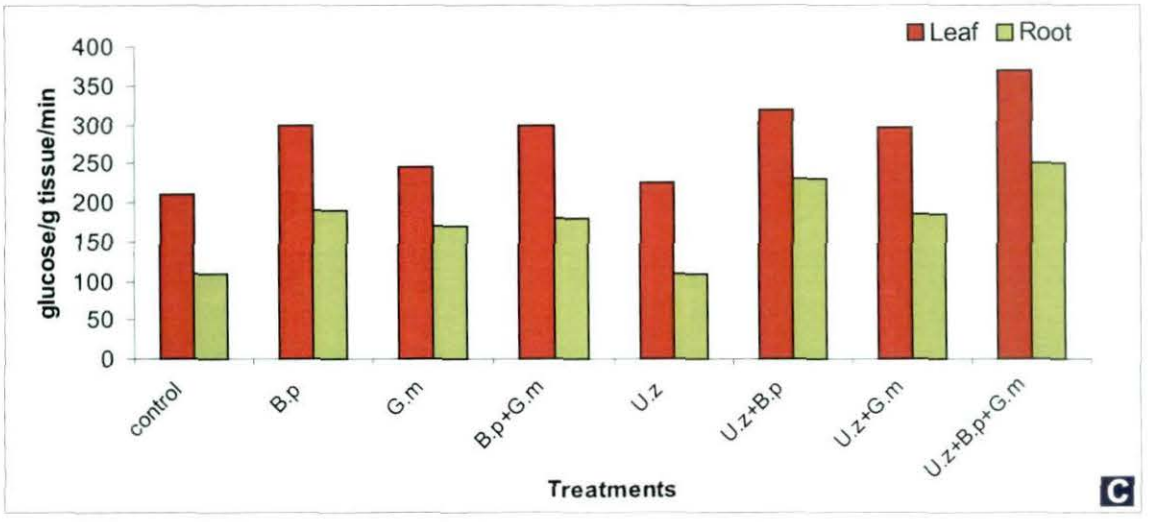


Fig.16(A-C): Changes in peroxidase(A), glucanase (B) and chitinase (C) activities in tea plants following treatments with *G.mosseae, B.pumilus* and inoculated with *U.zonata*

4.22. Field application of *G. mosseae* and *B. pumilus* and plant growth

Finally field application of AM fungus (*Glomus mosseae*) and PGPR (*B. pumilus*) singly or jointly was made in one selected plot of Tea Germplasm Bank where twelve different tea varieties are being maintained. Tea plants were treated with both AMF and PGPR after pruning of the tea leaves. Excellent leaf growth was prominent in the field for those plants treated either with AMF or PGPR. However dual application resulted maximum growth (Plate 20). In another experiment in the field trial marigold plant was inoculated with *G. mosseae* and *B. pumilus* separately. Flower bud initiation was maximum along with the growth pattern of plants in both the treatment. However, PGPR application resulted in best flowering conditions (Plate 21)

4.22.1 Catechins

Catechins derived from leaves of plants whose rhizosphere was soil drenched with bacteria *B. pumilus* and inoculated with *G. mosseae* individually as well as dual application of both were analysed in HPLC. Results revealed in the *B. pumilus* treated tea leaves peaks intensity is high and few new peaks can be observed when compared to control (figure 17, 18; Table 51,52) Similar results can be observed in the *G. mosseae* inoculated tea leaves, peaks intensity is high and one very prominent peaks can be observed (Figures19; Table 53). Major changes in the peaks following bacterization and *G. mosseae* when inoculated together, here all the peaks are of high intensity and few new peaks can be observed with respect to control. (Figure 20; Table 54).

Table 51: HPLC analysis of Catechins extracts from leaves of untreated tea plants

Pick 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
13		14.690	6993.7403	258.686	2.235	3.910
14		16.120	32019.2394	981.295	10.232	14.830

Plate 20(A-D): Field application of *B.pumilus* and *G.mosseae* on growth of tea plants. Control plots (A).Plants inoculated with *G.mosseae* (B); Plants treated with *B.pumilus* (C) and dual application of *G.mosseae* and *B.pumilus* (D)

Plate 21(A-F): Field application of *B.pumilus* and *G.mosseae* on growth of marigold plants. Untreated control (A&B). Plants inoculated with *G.mosseae* (C-E) and *B.pumilus* (D-F)

Table 52: HPLC analysis of Catechins extracts from leaves of *B. pumilus* treated tea plants

Pick No.		Ret time	Area (mv.S)	Height (mv)	Area (%)	Height (%)
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

Table 53: HPLC analysis of Catechins extracts from leaves of *G. mosseae* inoculated tea plants

Pick No.		Ret time	Area (mv.S)	Height (mv)	Area (%)	Height (%)
1	Inoculated with *G. mosseae*	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

Table 54: HPLC analysis of Catechins extracts from leaves of *B. pumilus* and *G. mosseae* treated tea plants

Pick No.		Ret time	Area (mv.S)	Height (mv)	Area (%)	Height (%)
1	Treated with *B.pumilus* & *G. mosseae*	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

4.23 Immuodetection of *U. zonata* and *B. pumilus*

Mycelial antigen prepared from the pathogen (*U. zonata*) and antigen prepared from selected PGPR (*B. pumilus*) were analysed initially by SDS-PAGE. The molecular weight of protein bands visualized after staining with coomassie blue were determined from the known molecular weight marker. Mycelial protein exhibited 19 bands in SDS-PAGE ranging in molecular weight (Ca. 97.4 kDa) and bands were of varying intensities and more proteins of lower molecular were present (Plate 22, fig. C) . Antigen prepared from *B. pumilus* was resolved in SDS-PAGE (Plate 23, fig.A) before production of polyclonal antibodies against the tea root pathogen (*U. zonata*) and PGPR (*B. pumilus*). Effectiveness of each antigen in raising antibodies were checked initially using agar gel double diffusion technique. Strong precipitation reactions were noticed in immunodiffusion tests (Plate 22, fig. A; Plate 23, fig. B). Western blot analysis of mycelial antigen of *U. zonata* was perfomed using PAb of the pathogen (Plate 22, fig. C). Dot immunobinding assays using antigens of both *U. zonata* and *B. pumilus* were also standardized (Plate 22, fig. D; Plate 23, fig. C).

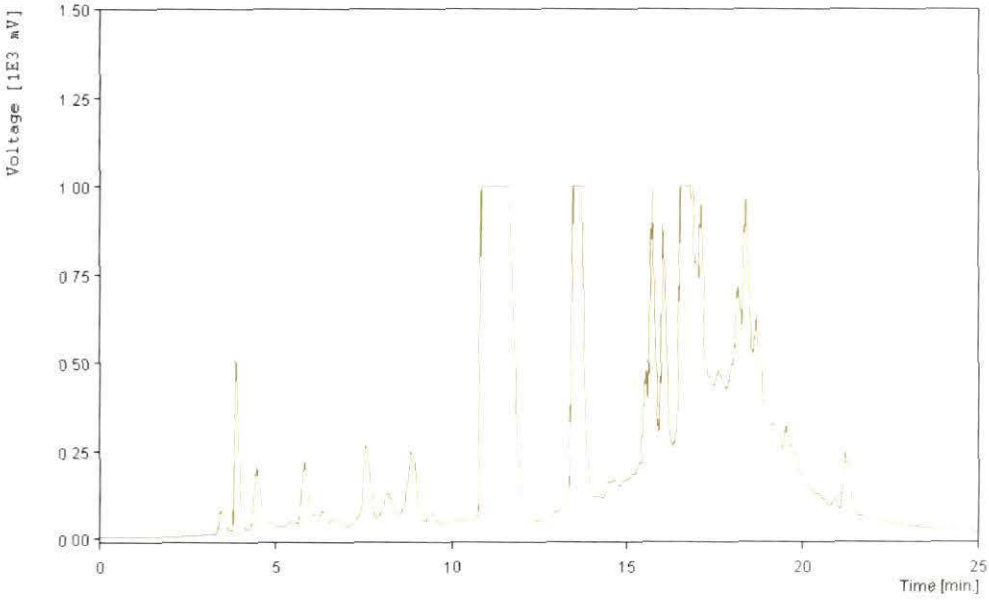


Fig.17: HPLC profile of catechins extracted from tea leaves of untreated (control) plants.

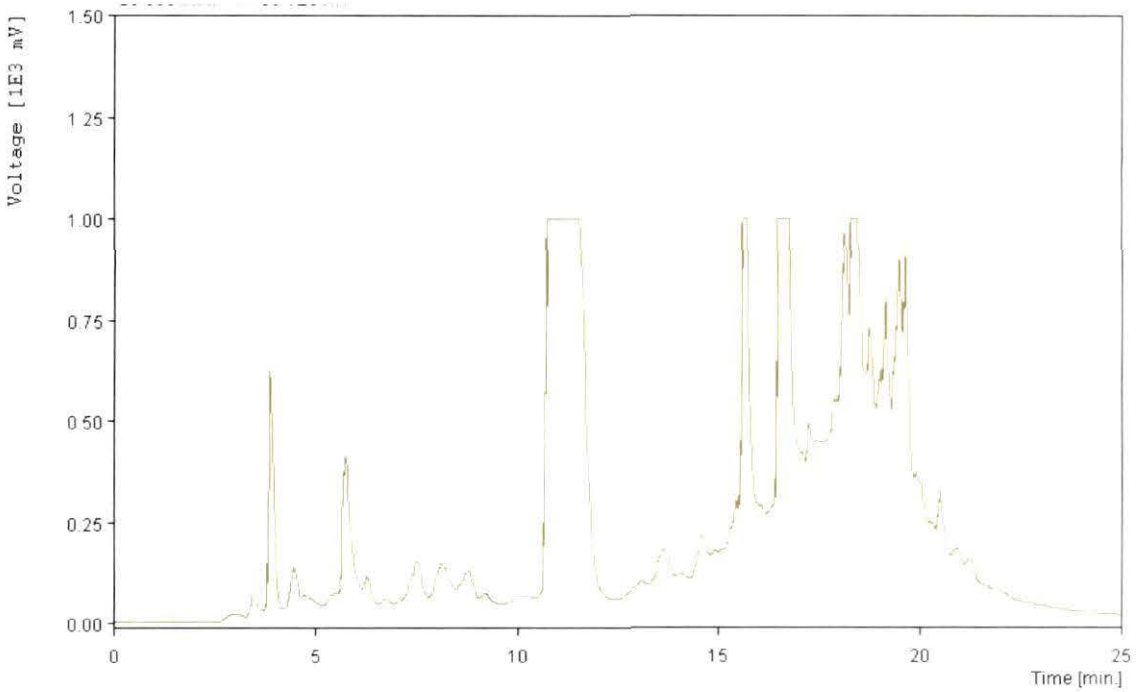


Fig.18: HPLC profile of catechins extracted from tea leaves from treated plants PGPR (*Bacillus pumilus*)

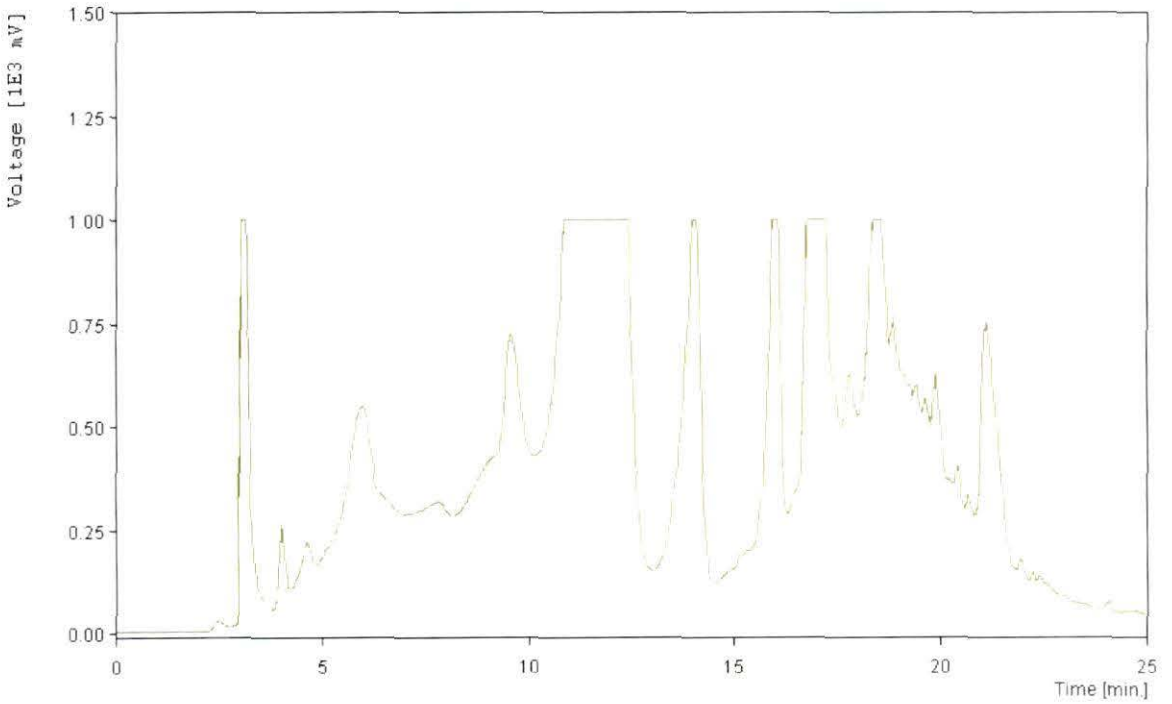


Fig.19: HPLC profile of catechins extracted from tea leaves from *Glomus mosseae* inoculated plants

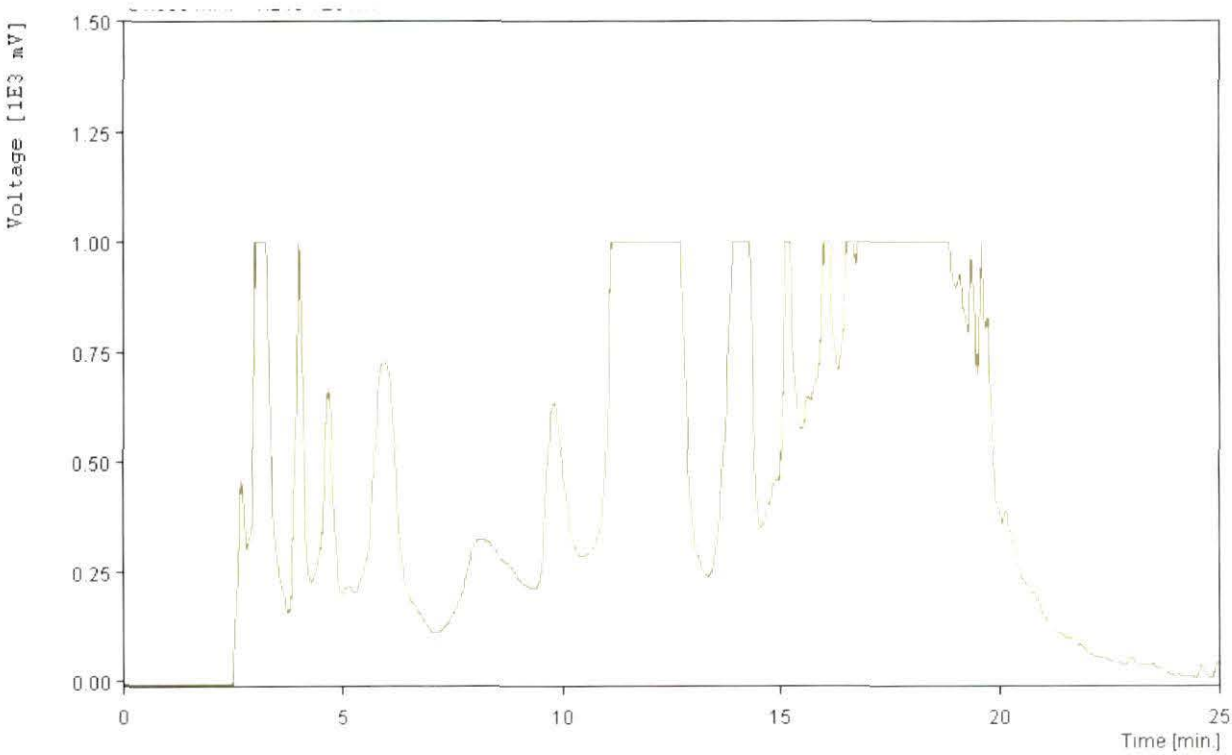


Fig.20: HPLC profile of catechins extracted from tea leaves of *B.pumilus* treated and *G.mosseae* inoculated plants

4.23.1. Immunodetection of *U. zonata* in soil

In order to determine whether PAb of *U. zonata* could detect the pathogen in soil, samples were collected from various locations including several tea estates. Antigens were also prepared from amended soil infested with the propagules of *U. zonata* either in field condition or in pot condition, in this investigation, antigens were prepared from samples from root rhizosphere soil and tested against the PAb of *U. zonata*. These were serologically assayed using PTA-ELISA formats. Results have been presented in Table 55. Soil samples 4 and 8 gave high absorbance value indicating the presence of pathogen.

Table 55: PTA- ELISA responses of different soil antigens with PAb of *U. zonata*

Soil sample	absorbance at 405
0	0.485
1	0.362
2	0.552
3	0.443
4	0.845
5	0.584
6	0.790
7	0.468
8	0.922
9	0.534
Mycelia of *U. zonata*	2.567

PAb concentration (mycelia) - 40 µg/ml

Soil antigen-0= control, 1= sterile soil, 2-9= soil collected from different polts.

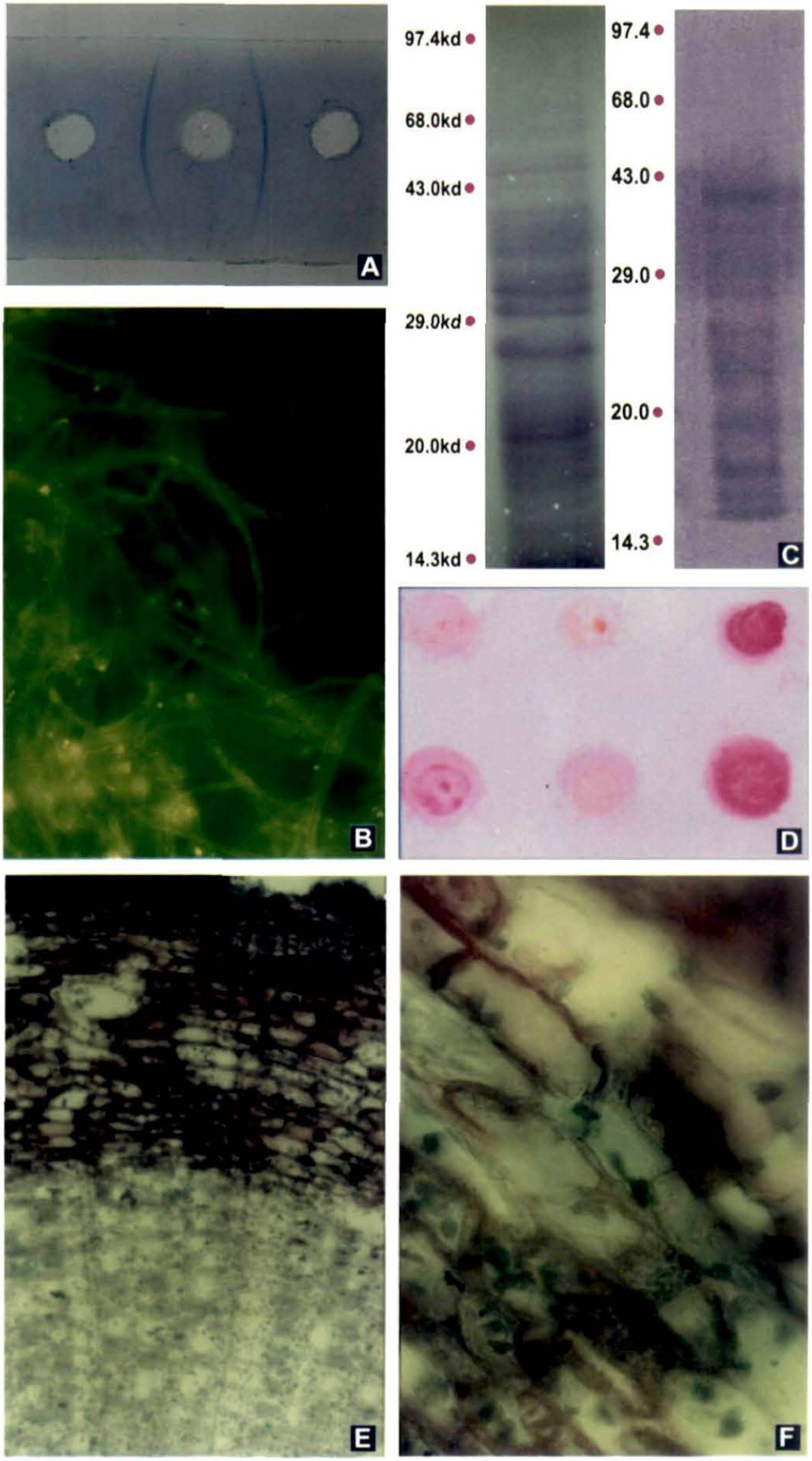


Plate 22(A-F): Serological assay using PAbs of *Ustilina zonata.* Agar gel double diffusion(A), Undirect immunofluroscence of mycelia using FITC(B), SDS-PAGE and western blot analysis of mycelial antigen(C), Dot immunobinding assay(D) and immunocytochamical staining of tea root tissue infected *U.zonata* probed with PAbs of the pathogen(E&F)

Identification of *U. zonata* propagules in artificially infested and non infested root rhizosphere soil was also carried out through dot immunobloting reaction. Soil antigens were prepared from soil samples from *U. zonata* amended soil and different locations including tea gardens and reacted with PAb of *U. zonata*. Among the collected soil samples only two samples 4 and 8 showed positive reactions, though dots were of low intensity in another two samples, either no dots could be detected or the reactions were very weak (Table 56; Plate 22 fig. D)

Table 56: Dot-immunobinding assay of different soil antigens with PAb of *U. zonata*

Soil antigen	color intensity
1	-
2	-
3	±
4	+
6	-
7	-
8	+
9	-
10	±

a= Fast red color intensity: + Pinkish red; ± Faint; – no reaction; IgG conc. 40 µg/ml
Soil antigen-1= control, 2= sterile soil, 3-10= soil collected from different plots

4.23.2. Detection of *U. zonata* in infected tea root tissues

To detect the pathogen in host tissues a number of immunodetection assays have proved effective where antisera raised against the pathogen reacted with antigens of infected material to give high absorbance values. These include ELISA, Western Blot and Dot Blot analysis. To detect *U. zonata* in infected tea root tissues indirect immunofluorescence was done. Cross section of the infected tea root tissues were probed with PAb of *U. zonata* and immunocytochemical staining was done. Microscopic observation was made. Fungal infection in epidermal and cortical tissues were evident (Plate 22, figs E&F).

4.23.3 Dectection of *B. pumilus* in soil

The sustainability of applied bacteria in the tea rhizosphere was determined by ELISA. Different soil antigen was 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, through even after 3 months, these were still detectable at fairly high concentrations (Table 57). It was also noted that bacteria survived equally well in rhizosphere when applied individually or in combination.

Table 57: Detection of survival of *B.pumilus* in soil after direct application using PTA-ELISA format

Antigen source Rhizosphere soil from	Absorbance at 405nm			
	Uninoculated	Time after bacterial application		
		Immediate	15 days	3months
TV-18	0.350±0.06	1.131±0.03	1.023±0.07	0.906±0.06
TV-26	0.442±0.08	1.274±o.09	1.052±00.9	0.911±0.02
T17	0.340±0.03	1.165±0.08	1.112±0.17	0.998±0.11

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

Dot immunobloting technique is rapid and sensitive method for detection of survival of bacteria in the 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 antigen was prepared soon after bacterial application (Plate23,figE).With time, the intensity decreased markedly (Plate 23, figs G-I). Dot-blot was also used for detection of pathogen in soil. When the antigen prepared from the rhizosphere soil amended with *B. pumilus* and inoculated with pathogen (*U. zonata*) and probed with PAb of *B. pumilus,* violet coloured dots were visible indicating the presence of PGPR (Plate 23,fig.F). Survival of *B. pumilus* as evident on dot blots clearly indicates the inability of pathogen to survive in presence of PGPR. Colony blot transfer from isolated bacterial colonies grown on NA plates and probed with PAb of *B. pumilus* confirmed the identity of microrganism (Plate 23, fig D).

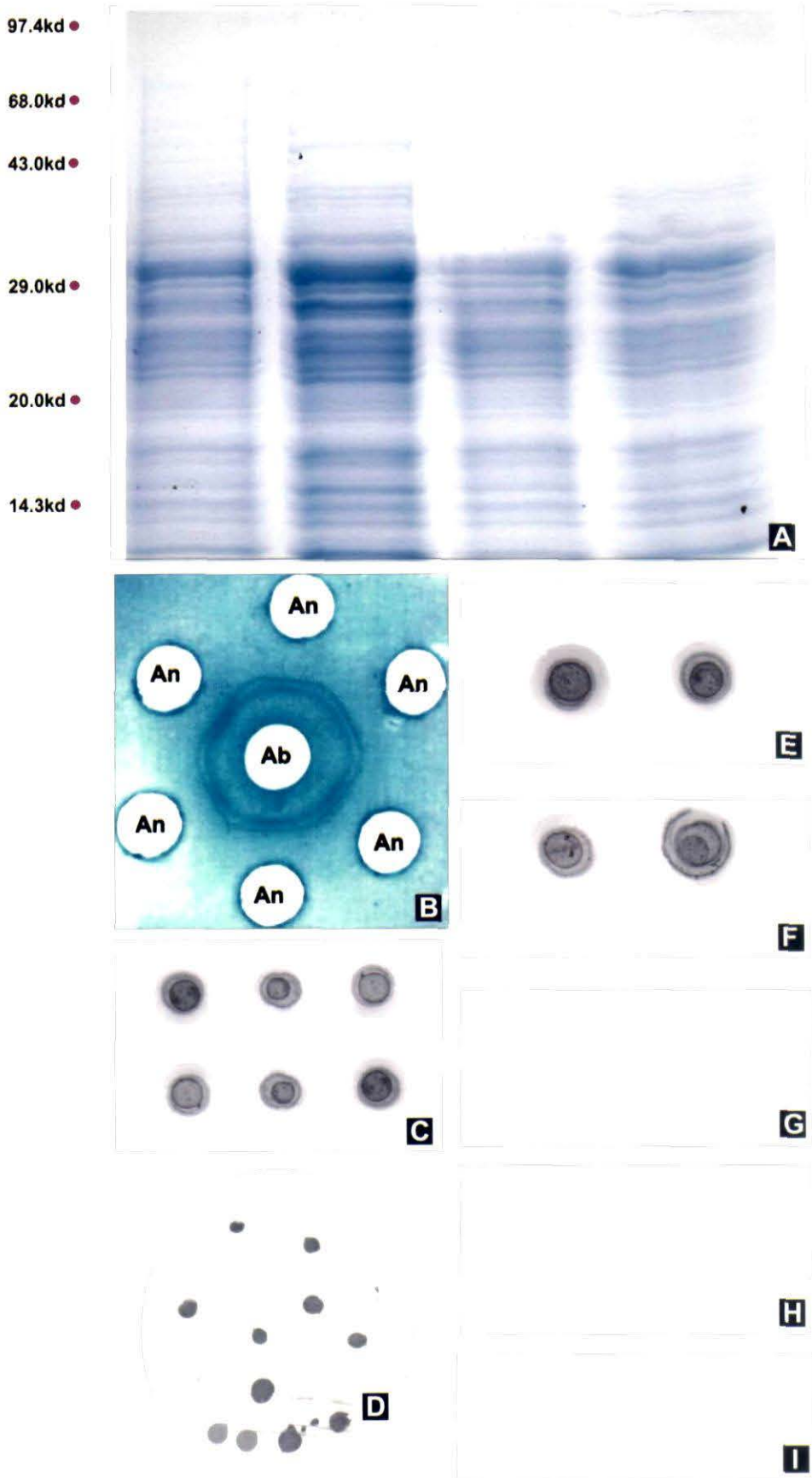


Plate23(A-I): SDS-PAGE analysis of soluble proteins (A) and serological assay of *B.pumilus* (B-I), Agar gel double diffusion (B), DOT immunobinding assay(C), Colony blot hybridization of (D), DOT immunobinding assay of rhizosphere soil of tea plants treated with *B.pumilus* and probed with PAbs of *B.pumilus*