J.Mycopathol.Res. 63(4) : 853-865, 2025, ISSN : 0971-3719 (Print), 2583-6315 (Online) 10.57023/JMycR.63.4.2025.853

Phosphate-solubilizing microbes from tea rhizosphere of Sikkim as potential biofertilizers and biocontrol agents

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Received : 08.09.2025 Accepted : 15.11.2025 Published : 29.12.2025

Phosphorous is key macronutrient essential for plants growth and development,although abundant in soils in organic and inorganic forms its availability to plants is restricted as it occurs mostly in insoluble forms.Largeproportion of added P fertilizers is immobilized due to the formation of complex with Al or Fe in acidic soils or Ca in calcareous soils. The use of rock phosphate as phosphatic fertilizer is unsustainable (non-renewable) and its efficiency in acidic and alkaline soil pH is low. Given the negative environmental impacts (eutrophication,pollution, disturbing soil microbial diversity) of added fertilizers, an eco-friendly approach to manage P deficiency in soil can be achieved through the exploration and utilization of beneficial soil microbes. In the present study, potential bacterial and fungal strains have been identified as phosphate solubilizing microbes (PSM) and role of these microbes in phosphate solubilization is well reported.The biotechnological opportunities for manipulating specific microorganism to enhance P availability in soil are an emerging trend in sustainable horticultural and agricultural practices. Thisstudy reports *Aspergillus niger*and *Bacillus pumilus*from tea rhizosphere of Temi Tea Estate, Sikkimwith phosphate solubilizing activity used as potential biofertilizer and biocontrol agents against *Ustulina zonata*, causal organism of Charcoal Stump Disease.

Keywords : *Aspergillus niger*, *Bacillus pumilus,* Phosphate sulubilizing microbes, phosphorous deficiency, tea rhizosphere, Sikkim.

INTRODUCTION

Crop production is often limited by low phosphorus (P) availability in soil; thestunted growth, dark leaves, inhibition of flowering, development of root, stalk, stem strength are the attributes associated with phosphorus (P) nutrition. Although P content in an average soil is 0.05% but only a fraction of this (about 0.1% of the total P present in soil) is available to the plants because of its chemical fixation and low solubility. The concentration of soluble phosphorus (P) in tropical and subtropical soil is usually very low, more than 80% of the P becomes immobile and unavailable for plant uptake because of adsorption, precipitation, or conversion to the organic form. A considerable amount of P is rapidly transformed into less available forms by forming a complex with Al or Fe in acid soils or Ca in calcareous soils before plant roots have had a chance to absorb it.

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The use of chemical fertilizers to circumvent the P deficiency in soil also becomes unavailable to the plants due to the rapid immobilization of P soon after its application. These synthetic chemicals are highly toxic, repeated and injudicious applications of chemical P fertilizers, however, lead to the loss of soil fertility by disturbing microbial diversity, and consequently reduces yield of crops (Gyaneshwar *et al.* 2002). Even if the phosphatic fertilizers are being replaced by direct application of naturally occurring rock phosphate (RP) it is economically unsound becausetheir sources are finite.To escape from these problems, the use of biofertilizers or microbial inoculants for replacing the efficacy of chemical fertilizers has been found to be effective in reducing the cost of cultivation and maintaining the natural fertility of soil (Vance, 2001).

Phosphorus solubilizing microorganisms play role in P nutrition by enhancing its availability to plants through release from inorganic and organic soil P pools by solubilization(inorganic P) and mineralization(organic P). Mineralization and

immobilizationof P occur simultaneously and are influenced by structure and compositionsof microbes and physico-chemical characteristics of soils besides the exudatesof various plant genotypes(Rodriguez *et al*. 2006). Chakraborty *et.al.* (2008b) have documented phosphate solubilizing fungal isolates from agricultural soil and forest soil of North Bengal. The mineralization process is mediated bythe enzymes especially phosphatases (Yadav and Tarafdar,2003, Aseri *et al*. 2009) and phytases (Richardson, 2001, Vassilev *et al*. 2007). Once the inorganic or organic P compound is changed tosoluble P, it can be easily be used up as P nutrient by plants, algae, cyanobacteriaand autotrophic bacteria and thereafter could be immobilized into organic cellularmacromolecules, for example, DNA, RNA and ATP.

The main mechanism of mineral phosphate solubilisation, in general accepted, is the action of organic acids, gluconic acid, protons, humic substances, CO_2 and H_2S synthesized by soil microorganisms (Vazquez *et al*. 2000;Ivanova *et al*. 2006;Rodriguez *et al*. 2004).Recent review (Ahash *etal.* 2025) illustrates the diversity of PSM, the mechanisms involved in solubilizing native soil P, its applicability under different environmental conditions, its impact on agricultural productivity, constraints for PSM usage and future thrusts areas of research gaps in reference to rice.

Keeping the above points in view,the present study was undertaken in order to select and screen potential phosphate solubilizing microorganisms from tea rhizosphere of Sikkim Temi Tea Estate, so that one or more of such microorganisms could be exploited asbiofertilizer for sustainable agro-farming system.

MATERIALS AND METHODS

Samples collection and analysis of soil sample

The soil samples were collectedfrom rhizosphere of tea bushes growing in six different blocks of Temi Tea Estate, Sikkim, India were analyzed from Soil Testing laboratory, Institute of Plantation Science and Management, North Bengal University and phosphate solubilizing microbes were isolated.

Isolation of phosphate solubilizing microorganism (PSM) from tea rhizosphere

The screening for phosphate solubilization was done by a plate assay method using Pikovskaya (PVK) agar medium. One gram soil sample was suspended in 9ml sterile distilled water in a tube for serial dilutions, and 1ml aliquots were transferred to PVK medium. The plates were incubated at 28±2ºC for 7 days with continuous observation for colony diameter. Transparent (halo) zones of clearing around the colonies of microorganisms indicate phosphate solubilization. Solubilization index was evaluated according to the ratio of the total diameter (colony + halo zone) and the colony diametermultiplied by 100 (Ngugen *et al.* 1992). Phosphate solubilizing colony was carefully isolated for quantitative determination of phosphate solubilization and pure culture characterization.

Quantitative measurement of phosphate solubilization in liquid medium

Pikovskaya's liquid medium supplemented with Tricalcium phosphate (TCP) and Rock phosphate (RP) was taken in 250ml flasks (50 ml/flask), inoculated with selected microbial culture and incubated at 28±2ºC for 7 days. A control without any inoculation was also maintained at 28°±2ºC for 6-7 days.Cultures were harvestedafter growth periods in order to record the change inpH, concentration of P released in the medium. Microbial culture was filtered through Whatman filter paper No.1 in order to remove insoluble phosphate whereas fungal mycelial mat was filtered through Whatman filter paper No.42. 1g of activated carbon was added and filtrate was centrifuged at 10,000 rpm for 15 minutes. Filtration and centrifugation was repeated until clear solution was obtained. Finally distilled water was added to solution to make a known volume (100ml). In 10ml aliquots of the clear filtrate 25ml of Bartons's reagent was added and final volume was adjusted to 50ml, incubated for 10 minutes, and OD of the solution was measured in a colorimeter at 430nm wavelength. Amount of phosphate solubilized in culture medium was calculated by comparing with standard curve. Change in the pH value during submerged cultivation was also monitored during the growth.

Identification of potential phosphate solubilizing microorganism from tea rhizosphere

Bacterial isolates

Morphological identification,as well as scanning electron microscopic observations followed by biochemical tests, endospore stain, Voges Proskauser reaction, catalase, urea digestion, esculine hydrolysis, casein hydrolysis, starch hydrolysis, indole test , reduction of nitrate to nitrite Bergeys manual of systematic Bacteriology.

Fungal isolates

The isolated fungi were allowed to grow in Petriplates (7cm) containing sterile PDA medium for 7 days, then nature of mycelia growth, rate of growth and time of sporulation were observed. For identification, spore suspension was prepared from individual culture. Drops of spore suspensions were placed on clean, grease free glass slides, mounted with lacto phenol- cotton blue, covered with cover slip and sealed with wax. The slides were then observed under the microscope following which spore characteristics were determined and size of spores measured for identification.

In vitro testing of PSM for antagonism to pathogens

For *in vitro*evaluation of antagonistic activity of rhizobacterial and fungal isolates following root fungal pathogens viz., *Ustulina zonata, Fomes lamaoenses* and *S. rolfsii*were obtained from Immuno-Phytopathology Laboratory, Department of Botany, North Bengal University.The efficacy of individual fungal and bacterial isolates from tea rhizosphere was tested for inhibiting growth of the pathogen in dual culture using PDA or NA.

In vitro plant growth promoting activitycharacterization for PSB
Chitinase production

Secretion of chitinase by the isolateswere determinedas described by Hsu and Lockwood, (1975).

Siderophore production

Production of siderophore was detected following standard method of Schwyn and Neiland (1987).

HCN production

Production of HCN (hydrocyanic acid) was determined using the procedure described by Wei *et al*., (1991) with slight modification.

IAA production

Methods as described by Prinsen *et al*., (1993) and Dobbelaere *et al.*, (1999) were followed.

Volatile production

Following the method ofDennis and Webster, (1971) volatile production by microbes were detected .

In vivo tests of plant growth promoting activity of selected fungi and bacteria

The experiment was conducted on tea, under greenhouse condition to assess the efficacy of selected fungal and bacterial isolates to promote plant growth. Tea saplings of different varieties maintained in the Tea Germplasm bank, in the premises of Immuno-Phytopathology Laboratory, Department of Botany, NBU, were used for the experimental purposes. The bacterial and fungal spore suspension was applied to the pots during transplantation of seedling from sleeves. Applications were done @ 0f 100 ml per pot at regular interval of one month for three months subsequently. The rhizosphere of two year's old potted plant was inoculated twice at an interval of 20-25 days. The growth promotion was assessed in seedling by comparing the increase in height, number of leaves of the treated plants to the untreated control plants under the same environmental and physical condition (temperature 30-34 ºC; R.H. 60-80%; 16h photoperiod). The experiments consisted of five replicates in each treatment incompletely randomized design. Similarly, the growth promotion in 2 years plants were also recorded in terms of leaf Area Index (LAI) of individual plant (Watson, 1947) and number of branches of the treated and untreated control plants. Observations were recorded after 2 and 4 months of final application of bacteria or fungus to the soil.

Inoculation of healthy tea plants

In case of potted plant, 2-3 year old potted tea plants were used. Then 100g of *U.zonata* inocula prepared in sand maize meal (Biswas and Sen, 2000) were added carefully in the rhizosphere and ensured that inocula were attached to healthy tea roots.

Biochemical analysis

Chlorophyll was extraction and estimation according to the method described by Harbone, (1973).Total phenol andO-dihydroxy phenol were extracted and estimated fromthe fresh tea leaves following the method of Mahadevenand Sridhar (1982).Extraction method of catechin was based on following the protocol of Obanda and Owuor (1994) with slight modification.

HPLC analysis of catechins

Catechin analysis of the extract was carried out on HPLC (Shimadzu Advanced VP Binary Gradient) using C-18 hypersil column with linear gradient elution system as follows- mobile phase A100% acetonitrile: mobile phase B2% acetic acid in water. Elution: 88% B for 6 minutes then linear gradient to 75% B over 5 minutes. The elution was complete after a total min of 25 min. flow rate was fixed as 1ml min^{-1} with sensitivity of 0.5 aufs. Injection volume was 20ìl and monitored at 278nm.

Extraction and assay of defense enzymes
ß-1, 3- glucanase

Extraction and estimation of ß-1,3- glucanase (EC.3.2.1.39) was done following the method described by Pan *et al.* (1999)

Chitinase

Extraction and estimation of chitinase (EC. 3.2.39) was done by following the method described by Boller and Mauch (1988) with modifications.

Phenylalanine ammonia lyase (PAL)

Extraction of PAL (EC.4.3.1.5) was done by following the method described by Chakraborty *et al.* (1993) with modifications.

Peroxidase

For the extraction of peroxidase (EC.1.11.1.7) (Chakraborty *et al*. 1993) and activity was measured by the method of Mahadevan and Sridhar *et al*. (1982)

Polyphenol oxidase

Extraction of polyphenol oxidase (EC.1.1.14.18.1) from the tea leaf tissues was done following the method described by Mahadevan and Sridhar (1982) with modifications.

RESULTS

Soil sample collection and analysis

The soil samples were collected from the rhizosphere of the healthy tea plants of different age growing in different sites of the Temi Tea Garden, South Sikkim (27.2367^0 N, 88.4222"E), only tea estate in state of Sikkim and widely regarded as one of the finest tea producing gardens in India and Internationally. The Temi Tea Estate (Fig. 1 A&B) currently covers an area of 177 hectares (440 acres) and yields an average annual production of about 100 tonnes. Soil samples were brought to the laboratory for isolation of rhizospheric microorganisms (Fig.1C-E). Moisture content, pH for soil type, soil texture, carbon and nitrogen ratio, K and P available etc were determined fordifferent samples of the Temi Tea garden. On an average the moisture contents is 19 %, pH is 4.3, organic carbon 1.1, nitrogen, soil type is clay and sandy, K_2O138.8 (ppm) and P_2O_5 40 (ppm). Availability of phosphorus in these forms depends on the mineralization or breakdown by the PSM.

Phosphorus solubilization efficiency on PVK plates

The fungal and bacterial isolates were screened for phosphate solubilizing activity in PVK medium. Formation of halo zones around the colony indicated positive results. In case of fungal isolates*Aspergillus* sp. reached to maximum solubilization index with zone of solubilization 0.6 cm on the day 5 then remains constant at the end of the week and among the bacterial

isolates,*Bacillus* sp. showed maximum solubilization indexwith zone of solubilization 0.6 cm on 4th day and remains constant. The zone of inhibition formed by both fungal isolates and bacterial isolates has been presented in (Fig.2A,B). Ability of bacteria and fungi to solubilize phosphate is an important criterion when considering their use as biofertilizers.

Phosphorus solubilization in PVK liquid medium

The fungal isolates *Aspergillus niger*, *Aspergillus nidulae* , *Aspergillus fumigates*and bacterial isolates *Bacillus pumilus* and *Bacillus megatarium*were further evaluated in PVK liquid media amended with tricalcium phosphate (TCP) and rock phosphate (RP) to assess their phosphorus solubilization capacity. The pH of the cultural broth samplesdropped significantly in the tested microbes as compared to the control whereit remained constant around pH 7. The testedisolates reached their maximum biomass level afterseven days of incubation. Culture media with no TCPproduced poor growth. Such result indicated theability of the strains to solubilize P and changeit to available form. The increase of P concentration in thelater stages might be due to the action of the microbes onthe substrate for demands of nutrients, thus releasingmore P from insoluble sources.Results have been presented in Table 1.

Identification of selected PSM

The potential phosphate solubilizing fungal and bacterial isolates were identified based onmacroscopic and microscopic morphological characterization for fungi and bacterial isolates were characterized based onmorphological and biochemical tests.The fungal isolate was initially white, fast-growing, cottony to powdery turns black due to abundant conidial heads, reverse side pale yellowish to colorless. Hyphae - septate, hyaline (transparent) when young, later brownish and conidiophores Long, smooth-walled, hyaline to brown, arising directly from the hyphae, 1.5-3.0mm, Swell at the tip forming a vesicle,Phialides - Radiate from vesicle, Biseriate arrangement (metulae '! phialides '! conidia). Conidia - globose, black, form chains, gives powdery appearance to colonies andwas identified as*Aspergillus niger*.The selected bacterial isolate was identified as *Bacillus pumilus* which was also confirmed by CABI- Bioscience, UK. Identification keys based on morphology and biochemical analysis is represented in Fig.3.

In vitro antagonistic effect of PSF and PSB isolates against root rot pathogens of tea

The disease suppression and inhibitory effects of the isolates viz*. Aspergillus niger* were confirmed by dual culture methods in solid and liquid media.In solidmedium the results revealed that *Aspergillus sp.* inhibitedthe growth of test pathogens *viz*. *Ustulina zonata, F.lamaoensis*with diameter of inhibition zone (cm) for*U. zonata* 1.6± 0.03 cm and *F. lamaoensis* 1.0± 0.01 *cm.* In liquid medium after 7 days of growth, mycelia were harvested; dried and mycelial dry weight was taken, revealed similar observation as in solid medium with 74.4% of reduction in growth of *U. zonata* and *F. Lamaoensis* with 74.76% reduction against the control (Table 2).

Antagonistic activity of selected bacterial isolate viz. *Bacillus pumilus* was confirmed by dual culture method both in solid and liquid media. The results in solid media revealed that the bacteria inhibited *Fomes lamaoensis* with Diameter of inhibition zone 1.6± 0.08 cm, followed by *Ustulina*

Table 1 : Evaluation of phosphorus solubilization by the fungal and bacterial isolates in liquid media amended with tricalcium phosphate (TCP) and rock phosphate (RP)

Isolates	TCP (mg/l)	pH	RP (mg/l)	pH
Aspergillus niger	875	3.8	361	3.7
Aspergillus nidulae	886	3.6	357	3.37
Aspergillus fumigatus	894	3.5	366	3.15
Bacillus pumilus	874	4.05	356	3.6
Bacillus megatarium	900	3.32	374	2.92

TCP = Tricalcium phosphate (P=997mg/l); RP= Rock phosphate (P=500mg/l)

Table 2 : *In vitro* test of phosphate solubilizing fungus (*Aspergillus niger*) against tea root pathogens

Interacting microorganism (mg)	Mycelial dry weight in growth	% of reduction
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	

± = standard error; * Average of 3 replicates.

*zonata*1.4± 0.03 cm and *Sclerotium rolfsii*0.6± 0.05cm (Fig. 4). Liquid medium it was the *B. pumilus* inhibited all the test pathogens (Table 3) with varying reduction in mycelyal dry weight of pathogens.

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), Siderophore, HCN, Volatile and Chitinase that trigger responses in a growing plants. Formation of clear zone around the colony grown in

Table 3. *In vitro* test of *B.pumilus*againsttea root fungal pathogens in liquid medium

Interacting microorganisms (mg)	Mycelial dry weight over control	% reduction
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

Pikovskaya's medium is an indication of phosphate solubilization by rhizobacteria. *B.pumilus* recorded the IAA production of 42mg/ L.Siderophore production by the antagonistic rhizabacteria, is confirmed by the appearance of yellow halo region was observed around *B.pumilus* which indicated a bacterium is able to chelate Fe3+ from Chrome Azurol Sugar. *B. pumilus* could solubilize phosphate followed by drop in pH, and produce siderophores and volatiles as well as sufficient amount of IAA. However no HCN was produced.Plant growth promotion activity by *B. pumilus*is summarized in Table 4.

EffectsPSF (A.niger) on growth of tea plants

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 5). The results

Table 4: 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.

showed that the PSF efficiently promoted growth in tea plants irrespective of their variety.

Effect of PGPR (Bacillus pumilus) on the growth of tea plants

When bacterial inoculum 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. Bacterial inoculation led to as much as 125% increase in growth, as against 15-25% in control. Treatment with the bacteria increased the rate of growth of the plants in relation to untreated control. As tea is cultivated mainly for its leaves, the induction of new shoots and more leaves have great impact in considering plant growth promotion (Fig. 5A,B).

Table 5: Effect of Phosphate Solubilizing Fungus (A. niger) on the growth of tea seedling.

Tea variety	Treatment	2 months after treatment % increase in height	% increase in no. of leaves	4 months after treatment % increase in height	% increase in no. of leaves
TV-18	Control	6.6 ±1.20	15.0 ± 1.71	16.0± 1.21	25.2 ± 1.93
	A. niger	35.4 ±1.32	30.0±6.01	55.0± 2.46	49.0± 1.00
T -17	Control	7.0± 1.16	10.0± 1.50	15.4±1.92	20.5± 5.20
	A. niger	36.0± 0.80	36.0± 1.20	50.3± 0.92	48.2± 1.74
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	23.2±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.

Total phosphorous in rhizosphere soil of tea plants

Soil sample from rhizosphere of the treated tea saplings were collected after 60 days treatment and phosphorous content in soil was analyzed for the amount of phosphorous depletion on mobilization after treatment with phosphate solubilizing fungi and bacteria. Phosphorous content in soil has been lowered in the treated block due to excess phosphorous mobilization or utilization by those plants treated with *A.niger* and *B.pimilus* (Table6). Phosphate availability in soil is greatly enhanced through microbial production of metabolites leading to lowering of pH and release of phosphate from organic and inorganic complexes. The organic phosphates will slowly release inorganic phosphate through the process of mineralization caused by micro organism.

Table 6 : Total phosphorous in rhizosphere soil of tea plants subjected to various treatments

Variety		Treatments	Concentration (mg/lit)
TV-18		Control	0.302
			0.190
B.pumilus		*A.niger*	0.185
TV-26		Control	0.354
			0.218
B.pumilus	0.191	*A.niger*	

Biochemical changes in tea following application of B. pumilus

The major components analyzed in tea leaves in present study were polyphenolics, chlorophyll and catechins. In all tested varieties the components increased significantly. Many studies have demonstrated the importance of phenolic compounds in plant defense.

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 (Table7). Phenols are antimicrobial compounds, the increment of phenols determined growth promoting property.

The quantitative assayof cholorophyll(Table 8) revealed that all treatments led to an increase in chlorophyll content both Total as well as chlorophyll a and chlorophyll b.

Catechins are major flavor flavonoid components of tea and their quantitative changes with respect to different isomeric forms were analyzed by HPLC. Catechins derived from leaves of plants whose rhizosphere was soil drenched with bacteria *B. pumilus* 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 (Fig. 6). It was observed that the treatment with the bacteria induced some new isomeric forms.

Table 7: Effect of *B. pumilus* on the biochemical components of tea

Tea varieties	Treatment	Total phenol (mg g^{-1} tissue)	O-dihydroxy phenol (mg g^{-1} tissue)
TV 18	Control	27.2±2.65	7.2±0.76
	B. pumilus	42.1±2.05	10.4±0.85
T-17	Control	22.2±2.75	6.5±0.58
	B. pumilus	37.2±2.67	13.5±1.23
TV-26	Control	23.5±3.84	7.8±0.92
	B. pumilus	38.5±2.63	12.0±1.04

Table 8 : 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

Effects of B. pumilus application on disease development in tea

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 charcoal stump rot disease (Table 9).

Effects of PSF application on 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 10) revealed that plants prior treated with PSF treatment reduced the severity of the disease.

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 11).

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 12).

Higher activities of defense enzymes like peroxidase, PAL, chitinase and β, 1, 3-glucanase were observed in A. niger treated tea plants challenged with a pathogen U. zonata against control. (Table 13).

DISCUSSION

Rhizospheric microbe- plant interactions have a great influence on plant health and soil quality since these root- associated microorganisms are able to help the host plant to deal with drought, nutritional and soil- borne pathogens stress conditions. The increase in the soluble carbon in soil sections close to the root surface is related to the rhizodeposition of root exudates that include low molecular weight organic acids, carbohydrates, nucleic acids derivatives and amino acids. Microorganisms in turn contribute to the availability and mobilization of nutrients, production of growth regulators, phototoxic substances or by suppression of pathogens and

Table 9: 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.

Table 10 : Effects of PSF (*A. niger*) application on the development of charcoal stump rot disease of tea

Varieties	Treatments	Charcoal 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

Table 11. Effect on protein, phenol and chlorophyll content of *A. niger* inoculated tea plants

Biochemical Components (mg g^{-1} tissue)	Treatments	
	Control	PSF
Protein	30.0	46.20
Total Phenol	12.8	34.21
Ortho phenol	2.6	3.56
Chlorophyll a	0.596	0.686
Chlorophyl b	0.289	0.426
Total chlorophyll	0.885	1.112

pollutants added to soil. The role of microorganism in solubilization of inorganic phosphates in soil and making them available to plants is the well known mechanism and such organisms are called phosphate solubilizers. High proportion of phosphate solubilizing microorganisms (PSM) is concentrated in the rhizosphere, and they are metabolically more active than other sources (Vazquez *et al*. 2000). Phosphate availability in soil is greatly enhanced through microbial production of metabolites leading to lowering of pH and release of phosphate from organic and inorganic complexes. The organic phosphates will slowly release inorganic phosphate through the process of mineralization caused by micro organism.The use of efficient phosphate-solubilizing

Table 12 : 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

Table 13: Enzyme activities in PSF treated tea varieties following inoculation with pathogen (*U. zonata*)

Tea Variety	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+ 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 24.3± 1.2	58 ± 0.21	389± 2.1
	A.niger+ U. zonata	9.0± 1.1			112± 0.12	523± 2.1
TV-26	Control	3.7±0.0	5.6±0.2	11.5± 1.4	66 ± 0.23	376±1.1
	A. niger + U. zonata			26.5±0.3	109.5±1.6	525±2.2

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

microorganisms (PSM), opens up a new horizon for better crop productivity besides sustaining soil health.But there is still a gap in our knowledge to fully understand the mechanistic basis of phosphate solubilization.For the development of good quality inoculants with greater efficiency in different field or soil condition, extensive screening needs to be done for getting highly efficient PSM from different sources.

The overall results of the present study have shown that PSM isolated from tea rhizosphere, could enhance growth of tea plants by various means like phosphate solubilization, siderophore production, volatile production, IAA production and increased in activity of catechins, phenols and chlorophyll and defense enzymes which are known to play definite roles in a plant defense (Panwar and Vyas, 2002). Siderophores are low molecular weight molecules that are secreted by microorganisms to take up iron from the environment and their mode of action in suppression of disease were thought to be solely based on competition of iron with the pathogens . Productions of volatile compound by bacteria have also been shown to be an important mechanism of plant growth promotion (Kloepper *et al*.2004).Interestingly siderophores have also been shown to induce systemic resistence (Bakker *et al*. 2003). In this regard, the studies of Gull *et al*. (2004) and Zaidi (2006) have conclusively shown that PSM solubilizes the fixed soil P and applied phosphates, resulting in higher crop yields (Vance *et. al*.2003).They also act as bioprotectants against root pathogens (Chakraborty *et al*. 2007; 2008a).

Chakraborty *et.al*. (2010) have evaluated phosphate solubilizer from soil of North Bengal and their diversity have also been analysed. PSM have recently gained importance in view of sustaining farming as it avoids P accumulation in soil by reducing P fertilizers, avert nutrient imbalances, decreasing environmental pollution and augmenting agricultural productivity (Ahash *et.al.* 2025). Enhancing P solubilization will pave the way for utilizing this non-available P reserve from the soil. Development of better formulations to ensure survival activity in the different field condition and through understanding of their contribution to the cycling of P in soil-plant systems is needed for the successful application

Fig.1: (A&B) Temi Tea Estateshowing tea products and factory,(C-E) Rhizosphereic microorganisms from temi tea garden. (A,B)Fungal isolates and (C) bacterial isolates.

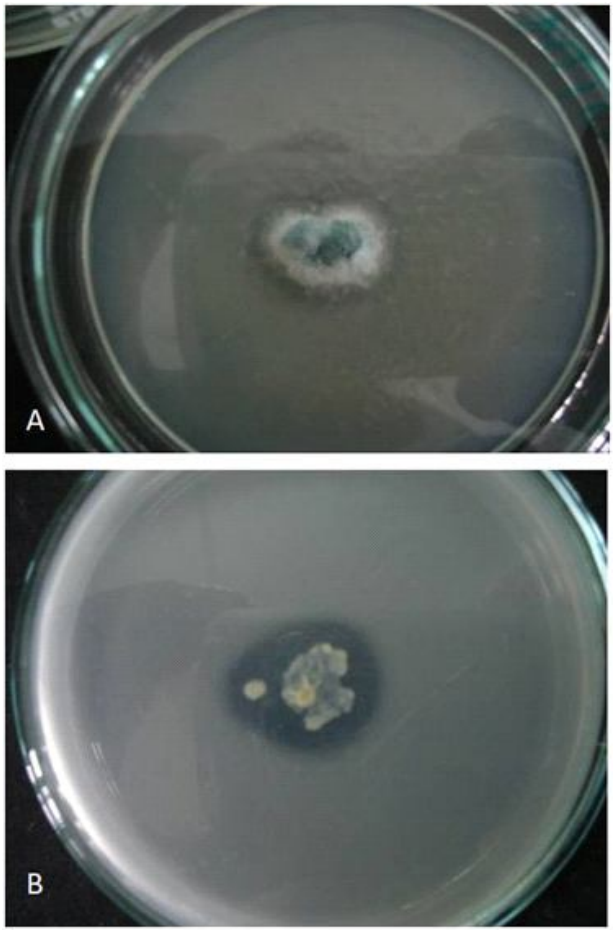

Fig. 2: (A)Fungal isolate (*Aspergillus niger*) (B) Bacterial isolate (*Bacillus pumilus*) showing clear halo zones on PKV medium.

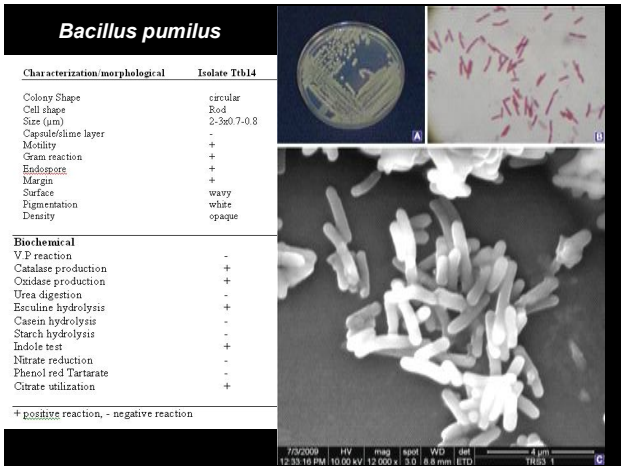

Fig. 3 : Morphology and biochemical characterization of bacterial isolate (*Bacillus pumilus*) (A)Growth pattern on Nutrient agar (NA), (B) Bright filed microscopy with Gram stain (C) Scanning electron microscopic image of *Bacillus pumilus*.

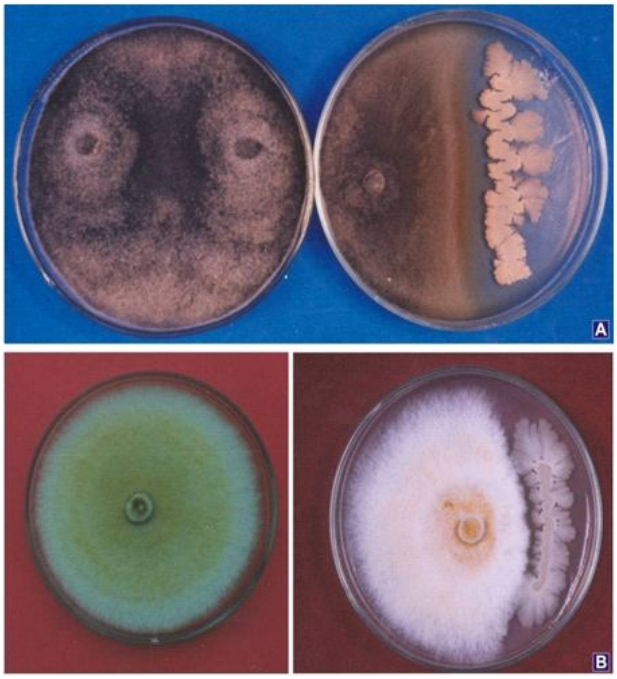

Fig. 4 : *In vitro* antagonism test of *B. pumilus* against tea root fungal pathogens (A) *Ustulina zonata* (B) *Fomes lamaoensis*

in sustainable agricultural system. Hence, PSM biotechnology islikely to ensure conservation of our environments and also providesan excellent opportunity to develop environment-friendly alternative for sustainable farming.

ACKNOWLEDGEMENT

Financial assistance received from the State Government of Sikkim is gratefully acknowledged. Necessary facilities for this research received in Immuno-Phytopathology Laboratory, Department of Botany, North Bengal Universityare highly

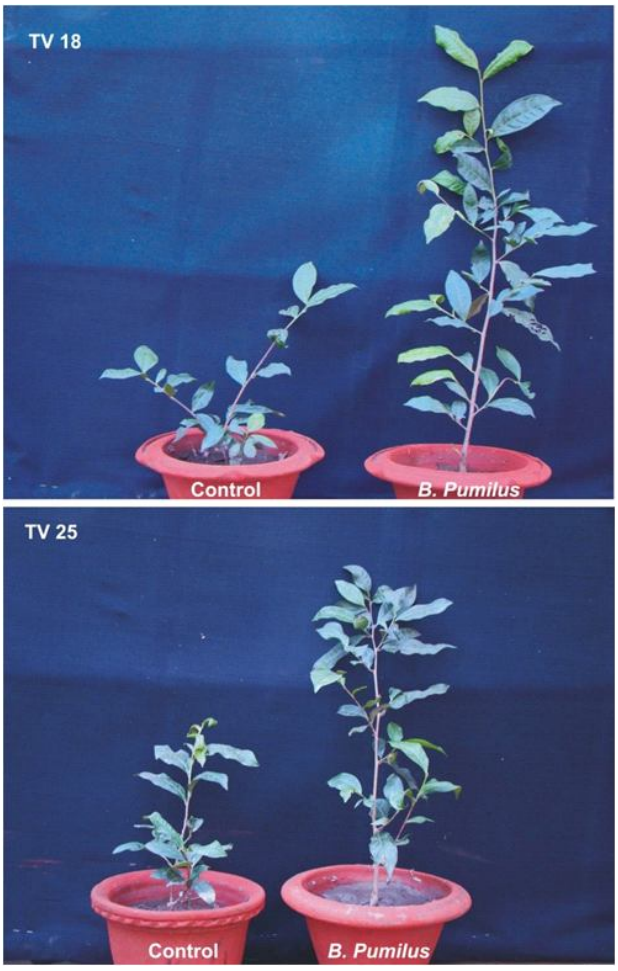

Fig.5 : Effect of foliar application of *Bacillus pumilus* on growth of two year old tea plants (TV-18 and TV-25).

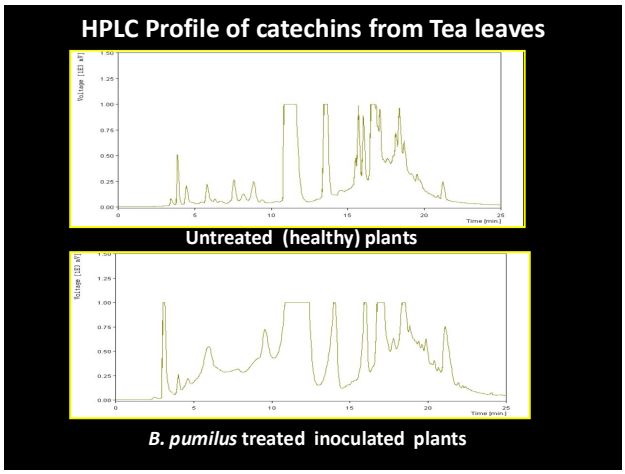

Fig.6: HPLC profile of catechins extracted from tea leaves from untreated (Healthy) and *Bacilluspumilus* treated plant inoculated with *Ustulina zonata.*

appreciated. The cooperation and support extended by the Manager and Assistant Manager of Temi Tea Estate are also sincerely acknowledged.

DECLARATION

Conflict of Interest.Authors have no conflict of interest.

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