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Research Paper

Indigenous plant growth-promoting rhizobacterial consortia greatly reduces fertilizer need for tea nurseries: Characterization and evaluation

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Abstract: A study was conducted to characterize plant growth-promoting rhizobacteria (PGPR) of tea [*Camellia sinensis* (L.) O. Kuntze] and to investigate the potential of using them to enhance growth and nutrient uptake of tea plants, and also to reduce the use of chemical fertilizers in tea nurseries in the mid-country of Sri Lanka. Nitrogen-fixing *Azospirillum* sp. (AZO) and phosphate solubilizing bacteria (PSB) isolated from the rhizosphere of tea grown in three main soil series *i.e.* Kandy, Matale and Ukuwela in Sri Lanka, were screened in an *in vitro* study. Soil series specific dual inoculants were formulated using the most effective strains of N_2 fixer and PSB obtained from each soil series, and they were tested in tea nurseries having respective soil series. In addition, a common consortium was tested across all the three soil series. Dual inoculants were tested along with a modified T 65 fertilizer mixture composed of ½ of N and P replaced with Eppawala Rock Phosphate (ERP) in place of Di Ammonium Phosphate (DAP) in recommended T 65 fertilizer and compared with two non-inoculated controls; modified T 65 fertilizer and T 65 recommended fertilizer, each with ten replicates. Plant growth measurements were taken and N and P uptakes were measured. Application of series-specific consortium to nursery tea plants raised in soil belonging to Ukuwela soil series along with modified T 65 fertilizer mixture improved dry matter contents (12.86 g/plant) and total N and P uptake (786 mg/plant and 155 mg/plant, respectively) compared to that of recommended fertilizer treatment (dry matter contents; 13.24 g/plant, N uptake; 727 mg/plant and P uptake; 166 mg/plant). Results indicated that a 50% reduction of N and replacement of imported DAP with locally available ERP in the present recommendation for tea nursery mixture (i.e. T 65) is possible with the application of dual inoculants formulated with series-specific strains.

Keywords: Biomass, N_2 fixation, nutrient uptake, phosphate solubilization, tea

Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) is consumed worldwide for its characteristic aroma and flavor and it's a major cash crop in Sri Lanka. Sri Lanka spends millions of rupees annually to import fertilizers for tea. If a part of the nutrient requirement of tea could be supplied by locally available resources, the country could save a substantial amount of foreign exchange. In addition, this dependence is associated with problems such as environmental pollution, health hazards, and destruction of biological communities that otherwise support crop production. Hence, the use of microbial inoculants in crop production is gaining importance.

Nitrogen is the most significant yield-limiting element in tea. Of the various rhizosphere associated N_2 fixing bacteria, *Azospirillum* species

are extensively studied and shown to have a significant potential for non –leguminous crops (Choudhury and Kennedy, 2004; Saubidet *et al.,* 2002; Hassen *et al.,* 2016). They enhance soil fertility by increasing the amount of available N and synthesize several different plant hormones that can act to enhance various stages of plant growth. Improved growth of nursery tea plants has been observed due to the incorporation of indigenous *Azospirillum* during the callusing stage by Baby *et al.* (2002) and Tennakoon *et al.* (2007).

Phosphorus is another major plant nutrient required by nursery tea plants in optimum amounts for proper growth in early stages (Zoysa, 1997). Phosphate solubilizing bacteria (PSB) improve the supply of phosphate to plants as a consequence of their capability of solubilizing insoluble phosphates (Rodriguez *et al.,* 2006). Increases in P uptake in other crops such as corn (Yazdani *et al.,* 2009) and rice (Rajapaksha *et al.,* 2011) have been reported due to PSB inoculants. Significant increases in the number of leaves and branches were observed due to application of *Bacillus megaterium* as a soil drench to the rhizosphere of tea plants in a field by Chakraborty *et al*. (2012).

In this respect, *Azospirillum* and PSB contribute greatly to plant nutrition and therefore to the improvement of plant growth and performance as PGPR. Bio-formulations containing such efficient organisms can be an eco-friendly and cost-effective supplement to chemical fertilizers. In this context, this study was undertaken to characterize indigenous *Azospirillum* and PSB from the tea rhizosphere and investigate the potential of using them to enhance growth and nutrient uptake of tea plants to reduce the use of chemical fertilizers in tea nurseries in the mid-country of Sri Lanka.

Materials and Methods

Nitrogen-fixing *Azospirillum* sp. and phosphate solubilizing bacteria (PSB) were isolated from fifty-two rhizosphere soil along with root samples of tea (*Camellia sinensis* (L.) O. Kuntze) collected from tea estates that represent the three main soil series in the mid-country of Sri Lanka. They are Kandy, Matale, and Ukuwela soil series (for USDA, the former has been classified as Typic Troporthents and the latter two as Typic Rhodustuts). Nitrogen-fixing *Azospirillum* sp. were isolated from the collected tea root samples using a semi-solid N free malate medium (Okon *et al.,* 1977) and PSB were isolated from rhizosphere soil samples by dilution plating on Pikovskaya's agar medium (Pikovskaya, 1948). These isolates were evaluated for N_2 fixation, phosphate solubilization, and production of growth-promoting substances by standard procedures, under *in vitro* conditions as detailed below.

In vitro nitrogen fixation:
A loopful of 48 hours-old culture of each isolate of *Azospirillum* spp was inoculated to 5 ml of semisolid N-free malate medium and incubated for 48 hours. One milliliter (10^8 cells/ml) of this culture was inoculated to 50 ml of semisolid media in 250 ml flasks and three replicates were maintained for each isolate. Uninoculated flasks served as controls. The flasks were incubated at 28 $\pm$ 2 °C for 15 days and 10 ml of this culture was used for estimation of nitrogen by micro Kjeldahl method as described by Jackson (1973) and Bremner (1960). All PSB isolates were also assessed for their ability to fix nitrogen under free-living condition by streaking them on Norris-N free agar medium and observed for growth.

Phosphate solubilizing ability:
All 32 *Azospirillum* bacterial isolates were initially tested for their ability to solubilize insoluble inorganic phosphate on Sperber's agar containing di-calcium phosphate and Pikovskaya's agar media containing tri-calcium phosphate, by spotting overnight grown cultures and incubating the plates for 24 to 48 h. The isolates showing clear zones around the colony were considered as P solubilizers. PSB isolates were tested only on Pikovskaya's agar medium. The diameter of the clearing zone was measured. The isolates showing clearing zones on Pikovskaya's agar medium were further examined for their ability to release inorganic P (Pi) from $Ca_3(PO_4)_2$ in broth medium. One milliliter (10^8 cells ml^{-1}) of an overnight culture of each isolate was inoculated to 50 ml of Pikovskaya's broth (Pikovskaya, 1948) in nine replicates, supplemented with 0.5% $Ca_3(PO_4)_2$. All the inoculated flasks were incubated at 28 $\pm$ 2°C. The amount of Pi released in the broth was estimated from triplicate flasks at 4, 8, and 12 days of incubation with a set of non -inoculated controls. The protocol used for Pi estimation was essentially the same as that of Gaur (1990). The available P in

the supernatant was estimated by the phosphomolybdic blue colour method (Jackson, 1973).

Production of growth promoting substances:
To determine the amounts of Indole Acetic Acid **(**IAA) produced by each isolate, a colorimetric technique was performed using the Van Urk Salkowski reagent (Bric *et al.,* 1991). For estimation of the Gibberellic Acid (GA) produced by each isolate, one milliliter (10^8 cells ml^{-1}) from the overnight grown cultures of the isolates was inoculated to 50 ml of sterilized Czapeck's solution and incubated at 37 °C for seven days in dark. After incubation, the cultures were centrifuged at 6000 rpm for 20 minutes. The supernatant was collected in a conical flask and used for the estimation of gibberellic acid (Paleg, 1965). Efficient isolates from each soil series were screened from *in vitro* study. For that, all 32 isolates of *Azospirillum spp.* (AZO) and 32 isolates of PSB were ranked based on their efficiency in *in vitro* nitrogen fixation/phosphate solubilization and production of Indole acetic acid and gibberellic acid. Efficient isolates were further evaluated for their impact on plant growth as single inoculants under nursery conditions for 180 days (results not shown).

Formulation of dual inoculants:
Soil series specific dual inoculants (AZO + PSB) were formulated using the most effective single isolates of N_2 fixer and PSB obtained from each soil series. For that, bacterial species that fixed the maximum amounts of N_2, produced the highest amounts of IAA and GA, and/or solubilized the maximum amounts of insolubilized phosphate under *in vitro* and showed the highest impact on the plant as single inoculants under nursery in the above study were considered. Accordingly, one dual inoculant for each Kandy {AZO-6 (*Azospirillum*sp. strain 6) + PSB-1(*Rhodococcus* sp)}, Matale {AZO-2 (*Azospirillum* sp. strain 2) + PSB-4(*Microbacterium* sp.)} and Ukuwela {AZO-7 (*Azospirillum* sp. strain 7) + PSB-3(*Bacillus cereus*)} soil series were formulated to be tested in respective soil series. In addition, best N_2 fixer and PSB in *in vitro* screening were combined as a common consortium {AZO-6 (*Azospirillum* sp. strain 6) + PSB-3 (*Bacillus cereus*)} to be tested across all the three soil series. Bacterial strains selected for the inoculant formulation were identified using PCR amplification and 16S rRNA sequence analysis (Smibert and Krieg, 1994) at Genetech, Sri Lanka.

Evaluation of dual inoculants for growth promotion and nutrient uptake under nursery conditions at soil series level:
Dual inoculants were tested at two locations in Kandy and Ukuwela soil series. Dual inoculant representing Matale soil series was not tested due to the unavailability of a nursery site belonging to Matale soil series during the experimental period. The average air temperature of the nursery in Kandy soil series during the study period was 23.7 °C and was in an area with a bimodal rainfall pattern and a mean annual rainfall of 2351 mm. The nursery experiment with Ukuwela soil series was carried out in an area with a unimodal rainfall pattern and a mean annual rainfall of 2504 mm. The average air temperature of the area was 26.03 °C during the study period. Three months-old nursery tea plants of clone TRI 4053 were used. Treatment details are given in Table 1.

Table 1: Treatment details

Treatment	Inoculant	Fertilizer
T1	-	½ N+ P (ERP) + K + Mg*
T2	CC (*Azospirillum* sp. strain 6 + *Bacillus cereus*)	½ N+ P (ERP) + K + Mg*
T3	SS (*Azospirillum* sp. strain 6 + *Rhodococcus* sp. for Kandy	½ N+ P (ERP) + K + Mg*
	Azospirillum sp. strain 7 + *Bacillus cereus* for Ukuwela)	½ N+ P (ERP) + K + Mg*
T4	-	N+P (DAP) + K + Mg**

CC and SS are common consortium and series specific consortium, respectively. CC was tested across all the three soil series. SS were tested in respective soil series; ERP = Eppawala Rock Phosphate, which is a locally mined P fertilizer; DAP = Di Ammonium Phosphate; *modified T 65; **T65 recommended fertilizer for nursery tea {composed of 10.5% N (Sulphate of ammonia), 10.6% P_2O_5 (Di Ammonium Phosphate), 11.1% K_2O (Sulphate of Potash) and 3.7% MgO (Commercial Epsom Salt)

For the preparation of inoculants, respective *Azospirillum* spp. were grown in a broth of Okon's medium, and PSB in nutrient broth. Aqueous suspensions of respective broth cultures of *Azospirillum* sp. (10^8 cells mL^{-1}) and PSB (10^8 cells mL^{-1}) at the rate of 15 mL were mixed and applied to the base of the nursery tea plants on the commencement of experiments. Chemical fertilizers were applied as per the treatment schedule, at fortnight intervals along with foliar spraying of $ZnSO_4$. Each treatment was replicated ten times and arranged in a Randomized Complete Block Design (4 treatments * 10 replicates = 40 plants).

Data collection:
Experiments were conducted for a period of 180 days. Plant height, number of leaves per plant, and leaf area per plant were recorded on the day of inoculation as well as after 180 days after inoculation (DAI). Destructive dismantling was done at 180 DAI. Plants were carefully uprooted and root systems washed free of soil. Root and shoot portions were separated from plants and air-dried. Air-dried plant samples were oven-dried at 80 °C to a constant weight. The shoot and root dry weights were recorded and expressed in g per plant.

The oven-dried plant samples were ground to a fine powder and used for estimation of N and P contents. The total N content in the plant samples was determined by the Kjeldahl method (Bremner and Malvaney, 1982) and P content by the vanadomolybdate colorimetric method (Jackson, 1958). The plant nutrient uptake was calculated using Equation 1;

$$\text{Nutrient uptake (g/plant)} = \frac{\text{nutrient content (\%)}}{100} \times \text{dry biomass (g/plant)} \qquad \text{Equation 1}$$

Statistical analysis:
Data were analyzed using the Analysis of Variance (ANOVA) procedure using the SAS statistical software, version 9.1 (SAS Institute, 2002). Treatment means were separated using Duncan's new multiple range test (DNMRT) and the least significant difference (LSD) at P = 0.05.

Results and Discussion

Screening of Azospirillum spp for their beneficial traits *in vitro*:
All the *Azospirillum* isolates (32) were assessed for different functions such as *in vitro* nitrogen fixation, phosphate solubilization, and production of plant growth-promoting substances (Table 2). The amounts of nitrogen fixed by *Azospirillum* isolates over a period of 15 days ranged from 6.30 to 26.37 mg/g of malate added. Among the isolates, AZO-4 showed the highest amount of nitrogen fixation (26.37 mg/ g of malate) over a period of 15 days, which was however comparable to that of AZO-7(24.97 mg/g of malate). AZO-6 and AZO-8 (23.57 mg/g of malate) were significantly superior over the rest of the isolates (Table 2). These results are in conformity with the earlier observations made by several workers (Jolly *et al.,* 2010; Tennakoon *et al.,* 2007; Khan and Akond, 1996).

Out of 32, only eleven (AZO-2, AZO-5, AZO-6, AZO-7, AZO-8, AZO-9, AZO-12, AZO-15, AZO-20, AZO-23, and AZO-29) were able to show haloes on Sperber's agar containing Di-Calcium Phosphate, in the range of 0.3 to 0.6 cm in diameter, indicating their ability to solubilize insoluble phosphate (Table 2). However, none of the isolates showed halo zones on Pikovskaya's agar medium containing Tri-Calcium Phosphate (TCP). The earlier observations that the nitrogen-fixing bacteria like *Azospirillum* can solubilize insoluble inorganic phosphate (Gnanachitra and Govindarajan, 2002) was reconfirmed in this study. One of the principal mechanisms of promoting plant growth by *Azospirillum* spp is related to the production of plant growth-promoting substances (Akbari *et al.,* 2007). Twenty-five out of 32 isolates produced both IAA and GA (Table 2).

The amount of IAA produced by the isolates ranged from 13.1 – 62.5 mg L^{-1}. Among the isolates, AZO-2 produced the highest amount of IAA (62.5 mg L^{-1}) and followed by AZO-6 (62.4 mg L^{-1}). Both isolates were significantly superior compared to the rest of the isolates in this regard.

Table 2: Beneficial characters of *Azospirillum* isolates

Treatment No.	Isolate	Nitrogen fixation (mg/g of malate) over 15 days	Mineral Phosphate solubilizing ability*	Production of IAA (mg/L)	Production of GA (µg/L)
1	AZO - 1	20.53^{c}	NZ	54.5^{b}	66.0^{c}
2	AZO - 2	16.80^{de}	0.3	62.5^{a}	80.3^{a}
3	AZO - 3	18.43^{cd}	NZ	41.3^{de}	64.7^{c}
4	AZO - 4	26.37^{a}	NZ	43.9^{cd}	71.7^{b}
5	AZO - 5	18.43^{cde}	0.5	35.6^{ef}	53.3^{de}
6	AZO - 6	23.57^{ab}	0.6	62.4^{a}	65.0^{c}
7	AZO - 7	24.97^{a}	0.3	49.5^{bc}	19.7^{hijk}
8	AZO - 8	23.57^{ab}	0.5	46.1^{cd}	57.7^{d}
9.	AZO - 9	15.40^{ef}	0.3	31.2^{fgh}	24.3^{fghi}
10	AZO - 10	18.20^{cde}	NZ	-	-
11	AZO - 11	18.20^{cde}	NZ	25.3^{hij}	14.0^{k}
12	AZO - 12	18.20^{cde}	0.3	28.2^{ghi}	50.3^{e}
13	AZO - 13	13.07^{fgh}	NZ	-	-
14	AZO - 14	6.53^{j}	NZ	-	-
15	AZO - 15	18.20^{cde}	0.4	19.9^{jkl}	17.7^{jk}
16	AZO - 16	10.03^{hi}	NZ	26.0^{ghij}	22.0^{ghij}
17	AZO - 17	18.29^{cde}	NZ	22.3^{ijk}	22.3^{fghij}
18	AZO - 18	17.73^{cde}	NZ	-	-
19	AZO - 19	17.97^{cde}	NZ	26.8^{ghij}	17.7^{jk}
20	AZO - 20	20.77^{bc}	0.3	33.2^{fg}	19.7^{hijk}
21	AZO - 21	11.67^{gh}	NZ	20.6^{jk}	25.3^{fgh}
22	AZO - 22	10.50^{igh}	NZ	-	-
23	AZO - 23	10.27^{hi}	0.4	19.7^{jkl}	23.0^{fghij}
24	AZO - 24	7.93^{ij}	NZ	21.1^{ijk}	21.0^{hij}
25	AZO - 25	10.73^{igh}	NZ	30.3^{fgh}	14.3^{k}
26	AZO - 26	11.90^{gh}	NZ	20.4^{jk}	28.0^{fg}
27	AZO - 27	8.17^{ij}	NZ	13.1^{l}	19.0^{hijk}
28	AZO - 28	16.10^{def}	NZ	32.1^{fgh}	17.0^{jk}
29	AZO - 29	15.63^{ef}	0.6	25.5^{hij}	28.3^{f}
30	AZO - 30	10.97^{igh}	NZ	-	-
31	AZO - 31	13.53^{fg}	NZ	17.3^{kl}	18.0^{ijk}
32	AZO - 32	6.30^{j}	NZ	-	-

Within a column, means followed by the same letter are not significantly different by the DNMRT at P=0.05; IAA = indole acetic acid; GA = gibberellic acid; * Diameter of clearing zone of colonies after 48h in Sperber's agar (cm), NZ: No Zone.

The amount of gibberellic acid produced by the isolates ranged from 14 – 80.3 µg L^{-1} of broth (Table 2). Among the isolates, AZO-2 produced the highest amount of gibberellic acid (80.3 µg L^{-1} of broth), which was significantly superior over all the other isolates (Table 2). While eight isolates produced more than 50 µg L^{-1} of broth. These results suggest that a greater variability exists among the isolates to produce indole acetic acid and gibberellic acid and thus variable potentials to improve plant growth. Similar observations on the production of indole acetic acid and gibberellic acid by *Azospirillum* have been made earlier (Malik *et al.,* 1997; Akbari *et al.,* 2007 and Tennakoon *et al*., 2007).

Screening of Phosphate solubilizing bacterial (PSB) isolates for their beneficial traits *in vitro*:

The amount of Pi released from tri-calcium phosphate in the Pikovskaya's broth by the PSB isolates was estimated at 4, 8, and 12 days after inoculation. Table 3 shows that the amount of net soluble P released from tri-calcium phosphate by all the isolates increased with advance in incubation time and was maximum at 12 days after inoculation. The amount of net soluble P released from tri calcium phosphate by the isolates at 12 days after inoculation ranged from 101 to 369 mg L^{-1}. Among the isolates, PSB-3 released the maximum amount of Pi from tri-calcium phosphate (369 mg L^{-1}) which was significantly higher over

the rest of the isolates (Table 3). The two isolates showed more than 300 mg L^{-1} of soluble P content in broth medium, whereas 18 and 12 isolates recorded 200 -300 mg L^{-1} and 100 – 200 mg L^{-1} of Pi respectively, in broth (Table 3). Significant differences existed among the isolates with respect to the amount of Pi released from tri-calcium phosphate. Such differences among the strains to solubilize inorganic phosphate have been reported earlier with different crop plants (Kundu *et al.,* 2002; Anu and Kundu, 2005).

Table 3: Release of P from tri calcium phosphate (TCP) by phosphate solubilizing bacterial isolates and change in pH of the growth medium

Treatment No	Isolate	Net soluble P released from TCP (mg L^{-1})			pH of growth medium		
		4 DAI	8 DAI	12 DAI	4 DAI	8 DAI	12 DAI
1	PSB - 1	175^{a}	261^{b}	277^{bcd}	3.75^{hi}	3.28^{jk}	3.43^{ijklm}
2	PSB - 2	146^{ab}	274^{b}	254^{bcdef}	3.33^{j}	3.22^{k}	3.17^{m}
3	PSB - 3	175^{a}	351^{a}	369^{a}	3.02^{k}	2.93^{l}	2.71^{n}
4	PSB - 4	76^{bcdef}	297^{ab}	275^{bcd}	3.74^{hi}	3.62^{efghi}	3.55^{hijk}
5	PSB - 5	87^{bcdef}	195^{cdef}	288^{bcd}	3.85^{hi}	3.48^{ghijk}	3.51^{hijklm}
6	PSB - 6	39^{ef}	238^{bcde}	293^{bc}	4.32^{g}	4.30^{d}	4.09^{fg}
7	PSB - 7	26^{f}	248^{bcde}	316^{b}	3.40^{j}	3.52^{fghij}	3.58^{hijk}
8	PSB - 8	135^{abc}	244^{bcde}	283^{bcd}	3.40^{j}	3.26^{jk}	3.21^{lm}
9	PSB - 9	59^{def}	242^{bcde}	249^{bcdef}	3.75^{hi}	3.59^{efghi}	3.53^{hijklm}
10	PSB - 10	81^{bcdef}	197^{cdef}	213^{cdefgh}	4.03^{h}	4.18^{e}	3.84^{gh}
11	PSB - 11	125^{abcd}	242^{bcde}	249^{bcdef}	3.93^{hi}	3.82^{e}	3.80^{ghi}
12	PSB - 12	191^{a}	195^{cdef}	233^{bcdefg}	3.70^{i}	3.81^{efg}	3.69^{hij}
13	PSB - 13	94^{bcdef}	193^{def}	267^{bcde}	5.42^{def}	3.89^{e}	3.70^{hij}
14	PSB - 14	58^{def}	72^{i}	101^{j}	5.90^{ab}	5.44^{b}	5.41^{bc}
15	PSB - 15	104^{bcde}	192^{def}	264^{bcde}	5.36^{f}	3.63^{efghi}	3.43^{ijklm}
16	PSB -16	104^{bcde}	182^{def}	275^{bcd}	6.03^{a}	3.84^{e}	3.60^{hijk}
17	PSB - 17	85^{bcdef}	141^{fghi}	173^{fghi}	5.75^{abc}	4.60^{c}	4.51^{de}
18	PSB - 18	94^{bcdef}	152^{fgh}	176^{fghij}	5.76^{abc}	3.70^{efgh}	3.57^{hijkl}
19	PSB - 19	85^{bcdef}	173^{efg}	209^{defg}	5.46^{cdef}	3.61^{efghi}	3.50^{hijklm}
20	PSB - 20	93^{bcdef}	179^{efgl}	283^{bcd}	5.42^{def}	3.43^{hijk}	3.33^{jklm}
21	PSB - 21	71^{cdef}	124^{fghi}	143^{hij}	5.74^{abc}	5.76^{a}	5.59^{abc}
22	PSB - 22	88^{bcdef}	187^{def}	269^{bcde}	5.38^{ef}	3.83^{e}	3.69^{hij}
23	PSB - 23	91^{bcdef}	196^{cdef}	267^{bcde}	5.47^{cdef}	3.82^{ef}	3.63^{hij}
24	PSB - 24	91^{bcdef}	171^{efg}	178^{fghij}	5.68^{bcde}	5.86^{a}	5.72^{ab}
25	PSB - 25	58^{def}	91^{hi}	101^{j}	5.70^{bcd}	5.93^{a}	5.79^{a}
26	PSB - 26	124^{abcd}	251^{bcde}	294^{bc}	5.37^{f}	3.35^{ijk}	3.25^{klm}
27	PSB - 27	85^{bcdef}	176^{efg}	192^{efghi}	5.88^{ab}	3.82^{e}	3.70^{hij}
28	PSB - 28	67^{cdef}	116^{fghi}	143^{hij}	5.72^{bcd}	5.76^{a}	5.76^{a}
29	PSB - 29	56^{def}	99^{ghi}	121^{ij}	5.87^{ab}	5.94^{a}	5.81^{a}
30	PSB - 30	78^{bcdef}	151^{fgh}	162^{ghij}	5.62^{bcdef}	4.30^{d}	4.27^{ef}
31	PSB - 31	77^{bcdef}	129^{fghi}	176^{fghij}	5.82^{ab}	5.66^{ab}	5.36^{c}
32	PSB - 32	77^{bcdef}	152^{fghi}	181^{fghij}	5.68^{bcde}	5.80^{a}	4.72^{d}

Within a column, means followed by the same letter are not significantly different by the DNMRT at P=0.05

In general, Ca - phosphate solubilization appeared to be linked with a pH decrease of the medium (Rodriguiz *et al.,* 2006). During the present study, acidification of broth media was accompanied by the release of soluble P from $Ca_3(PO_4)_2$. The highest pH reduction occurred by 4.29 units from initial pH (pH 7.0) on the 12 days after inoculation reaching 2.71 in broth medium inoculated with PSB-3 (Table 3). Twenty isolates resulted in a gradual reduction in pH by about 3 to 4 units at 12 days after inoculation and the remaining 11 isolates reduced pH by about 1- 3 units at 12 days after inoculation. The pH drop in liquid cultures has been reported in several research investigations that supports the pH change in the present study (Srinivasamurthy and Dayamani, 2014; Rodriguiz *et al.,* 2006).

Many of the phosphate solubilizing bacteria are capable of producing physiologically active auxins that may have pronounced effects on plant growth.

It was found that 17 out of 32 isolates produced both indole acetic acid and gibberellic acid but differed significantly in the amount of indole acetic acid and gibberellic acid produced (Table 4). The amount of indole acetic acid produced by the isolates ranged from 9.67 – 32.7 mg L^{-1} and that of gibberellic acid ranged from 15.7 – 67.7 µg L^{-1} of broth (Table 4). Among the isolates assessed, PSB-1 recorded the maximum production of indole acetic acid (32.7 mg L^{-1}) which was however comparable to that of PSB-3 (30.3 mg L^{-1}). It was also noted that the maximum quantity of gibberellic acid (67.7 µg L^{-1} of broth) was also produced by the isolate PSB-1 which was significantly superior over all other isolates. PSB-4 (59.3 µg L^{-1} of broth) and PSB-7 (51.7 µg L^{-1} of broth) were the next better performing isolates and both were significantly superior over the rest of the isolates (Table 4). De Frietas *et al.* (1997) and Srinivasamurthy and Dayamani (2014) made similar observations with different P solubilizing bacteria. All the 32 isolates of PSB were spotted on the Norris – N–free agar plates to test the nitrogen-fixing ability of the isolates. Among them seven isolates, PSB-1, PSB-3, PSB-5, PSB-10, PSB-22, PSB-24, and PSB-32 grew in N –free medium showing their ability to fix atmospheric N_2 (Table 4). Similar results were observed by Rajapaksha *et al.* (2011) for P solubilizing bacterial isolates of the rice rhizosphere.

Table 4: *In vitro* nitrogen fixation and production of IAA and GA by phosphate solubilizing bacterial isolates

Treatment No.	Isolate	Nitrogen fixation	Production of IAA	Production of GA
		Qualitative	(mg L^{-1})	(µg L^{-1})
1	PSB - 1	+	32.7^{a}	67.7^{a}
2	PSB - 2	NG	25.3^{b}	43.3^{d}
3	PSB - 3	+	30.3^{a}	45.0^{d}
4	PSB - 4	NG	24.3^{b}	59.3^{b}
5	PSB - 5	+	23.0^{b}	29.7^{f}
6	PSB - 6	NG	11.3^{ef}	40.7^{d}
7	PSB - 7	NG	17.7^{cd}	51.7^{c}
8	PSB - 8	NG	21.7^{bc}	45.3^{d}
9	PSB - 9	NG	-	-
10	PSB - 10	+	-	-
11	PSB - 11	NG	-	-
12	PSB - 12	NG	18.3^{cd}	15.7^{h}
13	PSB - 13	NG	-	-
14	PSB - 14	NG	-	-
15	PSB - 15	NG	12.3^{ef}	28.0^{fg}
16	PSB - 16	NG	-	-
17	PSB - 17	NG	11.7^{ef}	35.3^{e}
18	PSB - 18	NG	-	-
19	PSB - 19	NG	-	-
20	PSB - 20	NG	23.0^{b}	23.7^{g}
21	PSB - 21	NG	-	-
22	PSB - 22	+	15.3^{de}	29.7^{f}
23	PSB - 23	NG	-	-
24	PSB - 24	+	-	-
25	PSB - 25	NG	9.67^{f}	31.7^{ef}
26	PSB - 26	NG	11.7^{ef}	17.7^{h}
27	PSB - 27	NG	-	-
28	PSB - 28	NG	-	-
29	PSB - 29	NG	15.3^{de}	24.0^{g}
30	PSB - 30	NG	-	-
31	PSB - 31	NG	-	-
32	PSB - 32	+	12.7^{ef}	28.0^{fg}

Within a column, means followed by the same letter are not significantly different by the DNMRT at P=0.05

Overall performance of Azospirillum spp and PSB under *in vitro*:

In the *in vitro* evaluation, four isolates showed significantly higher nitrogen fixation (AZO-4, AZO-6, AZO-7, and AZO-8) and 2 isolates in the production of growth-promoting substances (AZO-2 and AZO-6). Among them, AZO-2 and AZO-4 were originated from Matale soil series and AZO-7 from Ukuwela soil series. Both AZO-6 and AZO-8 were from Kandy soil series. Among the PSB isolates, PSB-3 and PSB-7 were superior in solubilization of insoluble inorganic phosphate and PSB-1, PSB-3, PSB-2, and PSB-4 were superior in the production of growth-promoting substances while PSB-1 and PSB-3 have also shown ability to fix atmospheric N_2, showing their potential for the formulation of inoculants. Among them, both PSB-1 and PSB-2 were originated from Kandy soil series, PSB-3, and PSB-7 from Ukuwela and PSB- 4 from Matale soil series, respectively. Consequently, dual inoculants (AZO + PSB) were formulated using the most effective isolates originated from respective soil series *i.e.,* AZO-6 (*Azospirillum*sp. strain 6) + PSB-1 (*Rhodococcus* sp.) for Kandy, AZO-2 (*Azospirillum* sp. strain 2 + PSB-4 (*Microbacterium* sp.) for Matale and AZO-7 (*Azospirillum*sp. strain 7)+ PSB-3 (*Bacillus cereus*) for Ukuwela soil series. In addition, best performed N_2 fixer and PSB in *in vitro* screening were combined as a common consortium {AZO-6 (*Azospirillum* sp. strain 6) + PSB-3 (*Bacillus cereus*)} to be tested across all the three soil series.

Performance of dual inoculants under nursery conditions at soil series level:

Application of treatment T3 with soil series specific consortium (SS) along with 50 % N and P replaced with ERP in place of DAP in recommended fertilizer to nursery tea plants raised in soil belonging to UIkuwela soil series, improved dry matter contents (12.86 g/plant), leaf N contents (5.04%), total N and P uptake (786 mg/plant and 155 mg/plant respectively) to that of treatment T4 with recommended fertilizer (13.24 g dry matter/plant; 727 mg N/plant and 166 mg P/plant). In contrast, dry matter contents (10.58 g/plant), total N and P uptake (558 mg N/plant ant and 130 mg P/plant respectively) were significantly low in the inoculated treatment T2 with the common consortium (Figures 1, 2, 3 and Table 5).

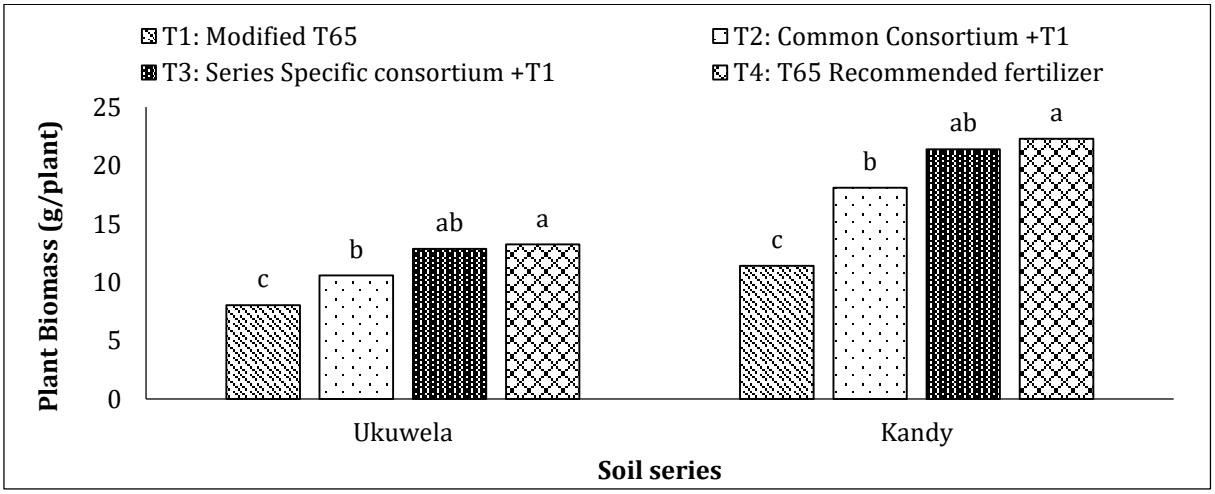

Figure 1: Effect of application of dual inoculants on plant biomass of nursery tea plants raised in Ukuwela and Kandy soil series. Means with the same letters are not significantly different at P = 0.05 for a given location. Common consortium: *Azospirillum* sp. strain 6 +*Bacillus cereus* and series specific consortium: *Azospirillum* strain 7 +*Bacillus cereus* for Ukuwela and *Azospirillum* sp. strain 6 + *Rhodococcus* sp. for Kandy soil series respectively. The T65 is recommended fertilizer for nursery tea plants.

In the study on Kandy soil series too, only the treatment T3 with series-specific consortium produced comparable total dry matter (21.4 g/plant) and total P uptake (124 mg P/plant) to that of treatment T4 with recommended fertilizer (22.3 g/plant and 138 mg P/plant, respectively). Comparable total N uptake by the inoculated treatments T2 and T3 to that of treatment T4 with recommended fertilizer in Kandy soil series suggest that *Azospirillum* spp. common to both inoculants (AZO-6) had fixed N_2 efficiently, resulting in N levels comparable to that of N level in recommended fertilizer.

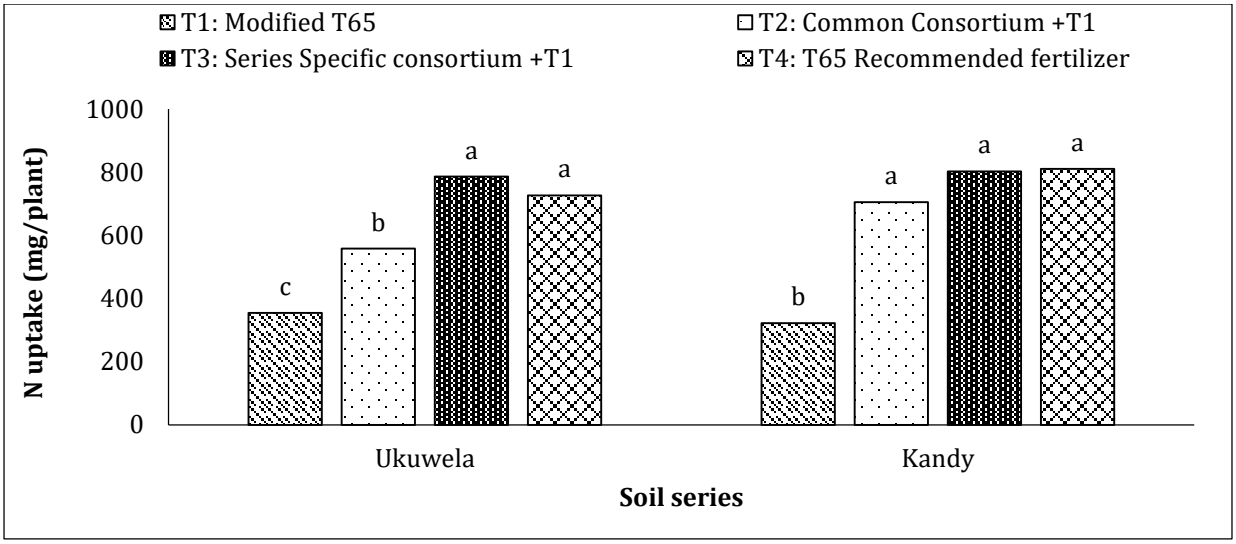

Figure 2: Effect of application of dual inoculants on total N uptake of nursery tea plants raised in Ukuwela and Kandy soil series. Means with the same letter are not significantly different at P = 0.05 for a given location. Common consortium: *Azospirillum* sp. strain 6 +*Bacillus cereus* and series specific consortium: *Azospirillum* strain 7 + *Bacillus cereus* for Ukuwela and *Azospirillum* sp. strain 6 + *Rhodococcus* sp. for Kandy soil series respectively. The T65 is recommended fertilizer for nursery tea.

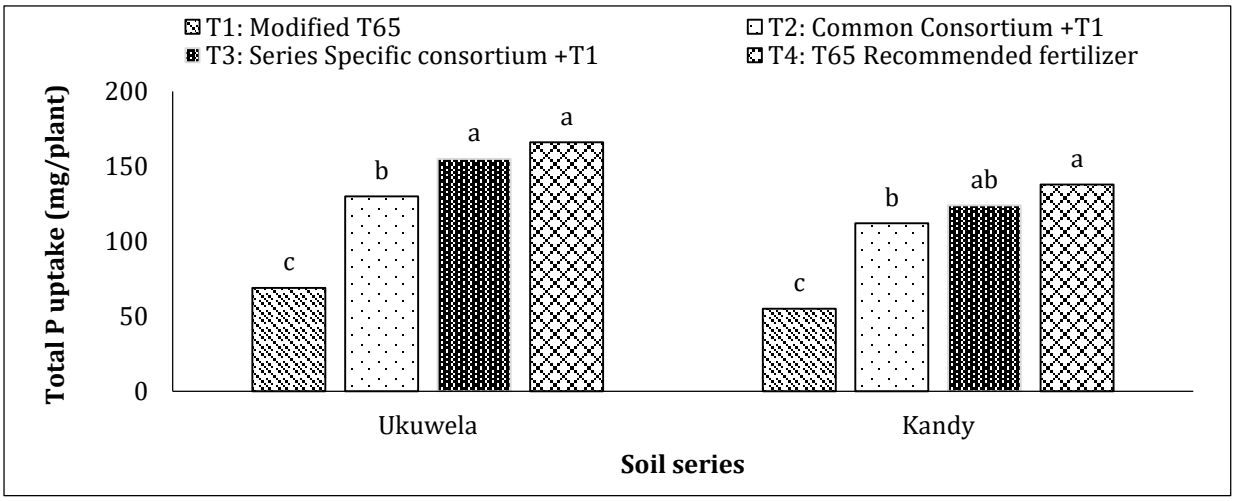

Figure 3: Effect of application of dual inoculants on total P uptake of nursery tea plants raised in Ukuwela and Kandy soil series. Means with the same letters are not significantly different at P = 0.05 for a given location. Common consortium: *Azospirillum* sp. strain 6 + *Bacillus cereus* and series specific consortium: *Azospirillum* strain 7 + *Bacillus cereus* for Ukuwela and *Azospirillum* sp. strain 6 + *Rhodococcus* sp. for Kandy soil series respectively. The T65 is recommended fertilizer for nursery tea.

Increased cell elongation and multiplication due to enhanced nutrient uptake by plants following inoculation of *Azospirillum* spp. and P solubilizing bacteria probably enhanced the growth parameters (Ahemad and Kibret, 2014). The increased dry matter could be attributed to the increased growth that mainly contributes to plant biomass. Increased nutrient uptake due to inoculation may be ascribed to increase in biomass.

Plant growth-promoting substances produced by the isolates as observed under *in vitro* conditions might have increased the root growth (Bhattacharjee *et al.,* 2008; Ahemad and Kibret, 2014) thereby creating more absorptive area for the uptake of other nutrients as well. Due to effective nutrient increase, in this study, with the addition of respective series specific dual inoculants, about 50 % reduction of N and replacement of imported DAP with locally available ERP in the recommended mixture for tea nurseries

appeared to be possible. This agrees with Adesemoye *et al.* (2009) who showed that supplementing 75% of the recommended fertilizer rate with inoculants composed of a mixture of plant growth-promoting bacteria including *Azospirillum* and *Bacillus* sp. produced plant growth, yield, and nutrient (N and P) uptake that were equivalent to the full fertilizer without inoculants in a greenhouse study with tomato. In another study, Alagawadi and Gaur (1992) have observed a significant increase in grain and dry matter yields and N and P uptake of sorghum over the non-inoculated fertilizer treatment by the combined use of *Azospirillum brasilence* and *Bacillus polymyxa* with low-level applications of nitrogenous fertilizers (40 kg N ha^{-1}), along with cheaper phosphatic nutrients such as rock phosphate (60 kg P_2O_5 ha^{-1}).

Table 5: Effect of application of dual inoculants on leaf nutrient contents of nursery tea plants raised in Ukuwela and Kandy soil series.

Treatment	Soil Series			
	Ukuwela		Kandy	
	N (%)	P (%)	N (%)	P (%)
T1 (Modified T 65)	2.89[c]	0.27[b]	2.91[c]	0.23[b]
T2 (CC +T1)	3.63[b]	0.45[a]	3.72[b]	0.36[a]
T3 (SS +T1)	4.04[a]	0.47[a]	3.96[a]	0.36[a]
T4 (T 65)	3.68[b]	0.45[a]	3.68[b]	0.38[a]
LSD (*P*=0.05)	0.36	0.09	0.23	0.05

Within a column, means followed by the same letter are not significantly different by the DNMRT at P=0.05; CC and SS denote the common consortium (*Azospirillum* sp. strain 6 and *Bacillus sereus*) and series specific consortium *Azospirillum* strain 7 and *Bacillus cereus* for Ukuwela and *Azospirillum* sp. strain 6 and *Rhodococcus* sp. for Kandy soil series respectively.
* modified T 65; ** The T65 is the recommended fertilizer for nursery tea plants

Though only a few studies have been conducted to test microbials for tea, Saikia *et al.* (2011) have also reported a synergistic effect on plant growth by inoculation of *Azospirillum* sp. with PSB. They have reported a significant increase in growth and yield of young tea inoculated with indigenous species of *Azospirillum* and phosphate solubilizing bacteria irrespective of cutting down of 25% of N and 50% P.

In a nursery trial of tea, Jayasekera *et al.* (2008), have observed higher shoot/root ratio, and high photosynthetic rate compared to full fertilizer treatment by using two bacterial biofilms along with 50% of the recommended fertilizer. In field experiments with immature tea, De Silva *et al.* (2013) have reported similar yield responses and improved P, K and Mg uptake over recommended chemical fertilizer treatment by the application of fungal – bacterial biofilms along with ½ the recommended fertilizer.

Potentials of indigenous strains over introduced strains:

The indigenous bacterial strains are likely to perform better than the introduced strains in promoting growth due to their superior adaptability to the environment (Tennakoon *et al.,* 2019; Akbari *et al.,* 2007; Vikram *et al.,* 2007). This was clearly demonstrated in this study. Dutta *et al.* (2015) have also shown the potential of indigenous PGPR isolates to use as microbial inoculants for the growth promotion of tea crops in West Bengal and Assam, India. This reiterates the importance of preparing inoculants with indigenous microorganisms a fact repeatedly demonstrated previously (Tennakoon *et al.,* 2019; Akbari *et al.,* 2007; Vikram *et al.,* 2007).

Conclusion

Added inoculants showed specificity towards soil at the series level and a clear synergistic effect in improving the growth of tested tea cultivars under nursery conditions. Results provided clear evidence of 50 % reduction of N and replacement of imported Di-Amonium Phosphate (DAP) with locally available Eppawala Rock Phosphate (ERP) in there recommended T 65 mixture for nurseries is possible with the application of dual inoculants formulated with respective series specific strains.

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References

Adesemoye A.O., Torbert H.A. and Kloepper J.W. (2009): Plant growth promoting rhizobacteria allow reduced application rates of chemical fertilizers. *Microb. Ecol.*, 58: 921-929.

Ahemad M. and Kibret M. (2014): Mechanisms and applications of plant growth promoting rhizobacteria. Current perspective. *J. King Saudi Univ. Sci.*, 26: 1-20.

Akbari G.A., Arab S.M., Alikhani H.A., Allahdadi I. and Arzanesh M.H. (2007): Isolation and selection of indigenous *Azospirillum* spp. and the IAA of superior strains effects on wheat roots. *World J. Agric. Sci..* 3(4): 523-529.

Alagawadi A.R. and Gaur A.C. (1992): Inoculation of *Azospirillum brasilense* and phosphate solubilizing bacteria on yield of sorgum (*Sorghum bicolor* L. Moench) in dry land. *Tropi. Agric.,* 69: 347-35

Anu and Kundu B.S. (2005): Development and characterization of lac Z marked strains of phosphate solubilizing bacteria. *Indian J. Microbiol.,* 45(4): 261-263.

Baby U.I., Baliah N.T., Ponmurugan P. and Premkumar R. (2002): Effect of *Azospirillum* on nursery grown tea plants. In: Sreedharan K., Vinodkumar P.K. and Jayarama C.B.M. (Eds). *Proc. 15th Plantation Crops Symp*. pp. 369 374. Mysore, India.

Bhattacharjee R.B., Singh. A. and Mukhopadhyay. S. N. (2008): Use of nitrogen fixing bacteria as biofertilizer for non-legumes: prospects and challenges. *Applied Microbiology Biotechnology,* 80: 199-209.

Bremner J.M. (1960): Determination of nitrogen in soil by the Kjeldahl method. *Journal of Agricultural Sciences,* 55: 11-13.

Bremner J.M. and Malvaney C.S. (1982): Total nitrogen. In: Page, L. (Ed.). *Methods of Soil Analysis*. Part 2(2). pp 595–622. American Society of Agronomy. Madison, Wisconsin, U.S.A.

Bric J.M., Bostock R.M. and Silverstone S.E. (1991): Rapid *in situ* assay for indole acetic acid production by bacteria immobilized on a nitrocellulose membrane. *Appl. Env. Microbiol.*, 57: 535-538.

Chakraborty U., Chakraborty B. N. and Chakraborty A. P. (2012): Induction of Plant Growth Promotion in *Camellia sinensis* by *Bacillus megaterium* and its Bioformulations. *World J. Agric. Sci.*, 8(1): 104-112.

Choudhury A.T.M.A. and Kennedy I.R. (2004): Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. *Biol. Fertil. Soils,* 39: 219-227.

De Freitas J. R., Banerjee M. R. and Germida J. J. (1997): Phosphate solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brasssica napus* L,). *Biol. Fertil. Soils,* 24: 358-364.

De Silva M. S. D. L., Jayasekera, A. P. D. A., Seneviratne, G., Prematunge, E. W. T. P. and Wijesekera, S. N. (2013): Soil fertility improvement through biofilmed biofertilizers: Potentials for field applications*. 223rd Meeting of the Experiments and Extension Forum.* pp 20-31 February. Tea Research Institute of Sri Lanka:

Dutta J., Handique P. J. and Thakur D. (2015): Assessment of culturable tea rhizobacteria isolated from tea estates of Assam, India for growth promotion in commercial tea cultivars. *Front. Microbiol.,* 6: 1252.

Gaur A.C. (1990): Phosphate solubilizing microorganisms as biofertilizers. Omega Scientific Publishers, New Delhi.

Hassen A.I., Bopape F.I. and Sanger L.K. (2016): Microbial inoculants as agents of growth promotion and abiotic stress tolerance in plants. In: Singh, D., Singh, H. and Prabha, R. (Eds). *Microbial Inoculants in Sustainable Agricultural Productivity*. Springer. New Delhi.

Illmer P. and Schinner F. (1995): Solubilization of inorganic calcium phosphates: solubilization mechanisms. *Soil Biol. Biochem.*, 27: 257- 263.

Jackson M.L. (1958): Soil Chemical Analysis. pp 134-182. Prentice Hall, Inc., Englewood Cliffs, N. J., U.S.A.

Jackson M.L. (1973): Soil Chemical Analysis. Prentice Hall of India (P) Ltd., New Delhi.

Jayasekera A.P.D.A., Seneviratne, G., De Silva, M.S.D.L., Jayasinghe L.A.S.P. and Premathunge P. (2008): preliminary investigations on the potential applications biofilmed biofertilizers for tea nurseries. In: *Proc. Second Symp. Plantation Crop Res*., pp 170-175. Colombo, Sri Lanka.

Jolly S.N., Shanta N.A and Khan Z.U.M. (2010): Quantification of Heterotrophic Bacteria and *Azospirillum* from the Rhizosphere of Taro (*Colocasia esculenta* L. Schott.) and the Nitrogen Fixing Potential of isolated *Azospirillium*. *Int. J. Bot.,* 6: 117-121.

Khan Z.U.M. and Akond M.A. (1996): Nitrogen fixation by *Azospirillum brasilense* isolated from rice fields of Bangladesh. In: Rahman, M., Podder, A.K., Van Hove, C., Begum, Z.N.T., Heulin, T., and

Hartmann, A. (Eds). Biological nitrogen fixation associated with rice production Kluwer Academic Publishers, London.

Kundu B.S., Gera R., Sharma N., Bhatia, A. and Sharma R. (2002): Host specificity of phosphate solubilizing bacteria. *Indian J. Microbiol.*, 42: 19-21.

Malik K.A., Rakshanda B., Samina M., Rasul G., Mirza M.S and Ali S. (1997): Association of nitrogen fixing, plant growth promoting rhizobacteria (PGPR) with Kallar grass and rice*. Plant Soil.,* 194: 37-44.

Okon Y., Albrocht S.L. and Burris, R.H. (1977): Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. *Appl. Environ. Microbiol.,* 33: 85-87.

Paleg L.G. (1965): Physiological effects of gibberellins. *Ann. Rev. Plant Physiol.,* 16: 291-322.

Pikovskaya R.I. (1948): Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Microbiol.*, 17: 362-370.

Rajapaksha R.M.C.P., Herath D., Senanayake A.P. and Senevirathne T.L. (2011): Mobilization of rock phosphate phosphorus through bacterial inoculants to enhance growth and yield of wetland rice*. Communic. Soil Sci. Plant Anal.,* 42: 301- 314.

Rodriguez H., Fraga R., Gonzalez T. and Bashan Y. (2006): Genetics of phosphate solubilization and its potential applications for improving plant growth promoting bacteria. *Plant Soil.*, 287(1-2): 15-21.

Saikia D. N., Sarma J., Dutta, P. K and Baruah D. K. (2011): Sustainable productivity of tea through conservation of bio-mass, addition of bio-fertilizers and reduction of inorganic fertilizer. *Two and a Bud.* 58: 109-117.

Saubidet M. I., Fatta N., Barneix A. J. (2002): The effect of inoculation with *Azospirillum brasilense* on growth and nitrogen utilization by wheat plants. *Plant Soil.,* 245: 215-222.

Smibert R. M. and Krieg N. R (1994): Phenotypic characterization. In: Gerhardt, P. (Ed). *Methods for General and Molecular Bacteriology*. pp. 607-654. American Society of Microbiology. Washington D.C.

Srinivasamurthy R. and Dayamani K.J. (2014): Characterization of mineral phosphate solubilizing gram-positive bacteria. *Int. J. Scient. Res. Publ.,* 4 (8): 1-4.

Tennakoon P.L.K., Alagawadi A.R and Jagadeesh K. S. (2007): N_2 fixing bacterial isolates of Tea and their plant growth promotional activities. *Sri Lanka J. Tea Sci.,* 72(2): 44-53.

Tennakoon P.L.K., Rajapaksha R.M.C.P and Hettiarachchi L.S.K. (2019): Tea yield maintained in PGPR inoculated field plants despite significant reduction in fertilizer application. *Rhizosphere,* 10.100146.

Vikram A., Alagawadi A.R., Hamzehzarghani H. and Krishnaraj P.U. (2007): Factors related to the occurrence of phosphate solubilizing bacteria and their isolation in Vertisols. *Int. J. Agric. Res.*, 2: 571-580.

Yazdani M., Bahmanyar M., Pirdashti M and Ali M. (2009): Effect of phosphate solubilization microorganisms (PSM) and plant growth promoting rhizobacteria (PGPR) on yield and.yield components of corn (*Zea mays* I.). *Int. J. Agric. Biosyst. Eng.,* 3(1): 50-51.

Zoysa A.K.N. (1997): The influence of phosphorus fertilizer forms and rhizosphere processes on the phosphorus nutrition of tea. Ph. D. thesis. Massey University, New Zealand.