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Role of Rhizobacteria of Tea Garden in Bioremediation of Pesticide Residues in Tea Soil

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Abstract: ***Endosulfan is a neurotoxin organochlorine insecticide of the cyclodiene family of pesticides. It is an endocrine disruptor, and it is highly acutely toxic. It is a mixture of steroisomers, designated "α" and "β," in a 7:3 ratio. A rhizobacterium capable of metabolizing endosulfan was isolated from one of the tea gardens of South Assam, Silchar, India. Isolate AULS-B3 (Burkholderia sp. AULS-B3) was treated with increasing doses of endosulfan (2.5, 5.0, 10.0, 15.0 and 20.0 µl/ml). After 48 hrs of incubation the sample was analyzed by HPLC, Perkin Elmer Series 200. The results indicated breakdown of endosulfan into other metabolites. Tea seedlings in pots were treated with an increasing dose of endosulfan coupled with Burkholderia sp AULS-B3 and Bacillus sp AULS-BAC to study growth parameters like shoot height, leaf number and root shoot ratio. The present work may provide a basis to study rhizobacteria of tea field soils that may not only degrade pesticides but also promote tea growth and help in establishing a clean and green environment.***

Keywords: Endosulfan, Rhizobacteria, Degradation, tea, soil

1. Introduction

Tea (*Camellia sinensis*) is cultivated in different type of terrains. In the North Eastern plantations, it is planted on the mountain slopes of the eastern Himalayas up to a height of 2000 m in Darjeeling and in undulating flat lands ranging from 20-250 m in the Dooars and Assam regions. Tea garden soils of South Assam are acidic (pH 4.5-5.5) in nature [1]. Certain species of *Penicillium* and *Trichoderma* dominate the rhizosphere of established tea bushes whereas, endophytic bacteria reside within plant tissues and have often been reported to promote plant growth [2]. Rhizobia and *Burkholderia* are particularly known for their symbiotic relationship with leguminous trees planted as shade trees [3].

The tea-growing environment in the North East India is also conducive to a large number of pests and diseases. Studies have been carried out at Tocklai Tea Research Association, Jorhat on the biology and control of tea pests during the last decades [4].

To meet the needs of consumers, tea industry largely relies on use of chemicals in the form of fertilizers and pesticides for better production. The liberal use of synthetic fertilizers and pesticides has led to a global concern for environmental pollution as well as harmful side effects created by their excessive use in tea plantation [5]. Heavy input of chemical fertilizers and pesticides cause disturbance in the plant-microbe interactions [6]. Increased use of agrochemicals for better production has resulted in pollution of garden tea soil, besides rendering native microbiota resistant to these chemicals through development of series of mechanisms that play a major role in the biological transformation of chemical pesticides [7,8].

Ochrobactrum anthropi, isolated from the rhizosphere of healthy tea plants growing at the foothills of Darjeeling and Dooars, was found to be antagonistic to several root rot pathogens of tea plant. Further, a series of in vivo experiments with *O. anthropi* significantly increased the growth and development of tea plants [9].

This study shows the action of an efficient and effective pesticide degrading bacterial strain which could help in maintenance of a balanced ecosystem by improving soil fertility and thereby enhancing the production of tea.

2. Materials and Methods

Study site

The study was conducted at Assam University campus in Cachar district (Barak Valley) of South Assam (92.51ºE longitude and 24.5ºN latitude). The Barak Valley is located at an altitude of 22 meters above sea level, covers an area of 6922 km^2. Average annual rainfall of the place is 280 cms. The maximum temperature ranges from 25.4°C (Jan) - 32.6°C (Aug) while as minimum temperature ranges from 11°C (Jan) - 25°C (Aug). The average humidity of the place varies from 97.5% (max) to 47.5 % (min).

Collection of samples

Rhizospheric soil and root nodules of *Albizia* sp. were collected and maintained at -20°C prior to analysis. The collected samples were screened for bacterial strains by growing on Luria Agar and YEMA (Yeast Extract Mannitol Agar) medium containing 0.0025 % Congo red dye. After incubation at 28±1ºC for 48-72 hrs, isolated colonies were picked up and streaked on fresh plates. Pure cultures were obtained by sub-culturing.

Authentication of the isolated strains

The isolated bacterial strains were identified by studying their 16S rRNA gene sequences. Nomenclature of the isolates was carried out representing the initials of the University, Department, Generic name and isolate number (numeric figure). For example, AULS-B3 indicates Assam University Life Science *Burkholderia* 3.

Screening of efficient strain

All the isolates were screened for resistance to pesticide endosulfan by subjecting them to growth inhibition study against varied concentration of endosulfan (2.5, 5.0, 10.0, 15.0 and 20.0 µl/ml). Growth was monitored by measuring optical density (OD_{595}) of the aliquots taken after every 24 hr interval by means of spectrophotometer 169 (SYSTRONICS).

Analysis of endosulfan degradation by HPLC method

Isolate AULS-B3 (*Burkholderia* sp. AULS-B3.) was treated with increasing doses of endosulfan (2.5, 5.0, 10.0, 15.0 and 20.0 µl/ml) and kept for growth at 30ºC at 120 rpm for 48 hours. A set of control for each concentration was also prepared. After 48 hrs of incubation the samples as well as the control sets were analyzed by HPLC (Perkin Elmer Series 200) by utilizing a C_{18} reversed-phase column and methanol:water (70:30 v/v) as a mobile phase with a run time of approximately 20 minutes. The maximum absorbance spectrum was obtained at 214 nm.

Bacteria and growth of tea seedlings at different levels of pesticide in pot culture

The inoculation of bacteria on tea seedlings was performed on earthen pots (6 kg capacity). Twenty four numbers of tea seedlings (P1 to P24) provided by P.C. Tea Industry, Silchar were used as test plants. Isolates *Burkholderia* sp. AULS- B3 and *Bacillus* sp. AULS-BAC were inoculated (~10ml) on the tea seedlings where different doses of endosulfan (2.5, 5.0 and 10.0 µl/ml) had been applied. Each treatment was repeated three times to maintain the homogeneity among treatments. Parameters like shoot height and leaf production of the seedlings were recorded on a monthly basis. Biomass and root-shoot ratio measurement were done as a final measurement at the end of the experiment.

Following was the experiment design/treatment combinations followed:

Treatment 1

T	TE_1	TM_1M_2	TM_1
TM_2	TM_1E_1	TM_2E_1	$TM_1M_2E_1$

Treatment 2

T	TE_2	TM_1M_2	TM_1
TM_2	TM_1E_2	TM_2E_2	$TM_1M_2E_2$

Treatment 3

T	TE_3	TM_1M_2	TM_1
TM_2	T M_1E_3	TM_2E_3	$TM_1M_2E_3$

T: Tea seedling; E_1: endosulfan (2.5µl/ml); E_2: endosulfan (5.0µl/ml); E_3: endosulfan (10.0µl/ml); M_1: *Burkholderia* sp.AULS-B3; M_2: *Bacillus* sp. AULS-BAC

3. Results and Discussion

Authentication and screening of efficient strain

Molecular identification and phylogenetic analysis revealed that tea garden soils harbored bacterial species belonging to Rhizobium, *Burkholderia* and Enterobacter.

Isolate AULS-B3 (Burkholderia sp. AULS-B3) was screened out from the other isolates on the basis of its survivability on treatment with increasing doses of endosulfan. *Burkholderia* sp. AULS-B3 tolerated endosulfan upto a concentration of 20 µl/ml unlike others which cannot survive beyond 15 µl/ml [10].

Degradation of endosulfan by AULS-B3

Isolate AULS-B3 (*Burkholderia* sp.) was treated with increasing doses of endosulfan (2.5, 5.0, 10.0, 15.0 and 20.0 µl/ml) and incubated at 30ºC at 120 rpm for 48 hours and analyzed by HPLC (Perkin Elmer Series 200). The HPLC results were somewhat striking. A major peak was observed between 2.3 to 2.8 minutes in all the control as well as the sample chromatograms. After a thorough study of the pattern and literatures (under the same HPLC parameters) it was concluded that "α" endosulfan gets eluted at ~2-3 mins. And in the sample chromatograms it was observed that around the same elution time, a major peak along with other minor peaks was shown indicating the breakdown of endosulfan into other metabolites.

Effect of *Burkholderia* sp. AULS-B3 and *Bacillus* sp. AULS-BAC on the growth of tea seedlings at different levels of endosulfan in pot culture

Tea seedlings were grown in pots with different treatments of endosulfan and microbial inoculum as per procedure described above. Growth of seedlings was measured in different treatments viz; T, TE_1, TE_2, TE_3, TM_1, TM_2, TM_1M_2, TM_1E_1, TM_1E_2, TM_1E_3, TM_2E_1, TM_2E_2, TM_2E_3, $TM_1M_2E_1$, $TM_1M_2E_2$ and $TM_1M_2E_3$ [T: Tea seedling; E_1: endosulfan (2.5µl/ml); E_2: endosulfan (5.0µl/ml); E_3: endosulfan (10.0µl/ml); M_1: *Burkholderia* sp.AULS-B3; M_2: *Bacillus* sp. AULS-BAC].

The effect of different treatments of endosulfan with or without microbial inoculum on the biomass production, shoot height and root shoot ratio of tea seedlings were observed and the results were presented in Fig.1, 2, 3 and 4 and Table1, 1.1, 2, 2.1, 3, 4 and 4.1.

The biomass production was measured under different treatments at the end of five months. It was observed that all the microbial inoculated endosulfan treated plants showed better biomass production over the endosulfan treated uninoculated ones (Fig. 1). At the first endosulfan level (2.5 µl/ml) the highest seedling biomass was observed in TM_1E_1 combination (8.15g). The control plant (T) had 6.02g. When the seedling was inoculated with AULS-B3 alone without endosulfan (TM_1) it gave 7.78g and when inoculated with AULS-BAC alone (TM_2) it gave 6.32g. The outcome of dual inoculation (TM_1M_2) was 6.91g. This showed that the bacterial inoculum had some effect on the plants. But when the seedling was given endosulfan only (TE_1) the biomass production was just 5.15g. However, in the presence of

microbial inoculum the plant biomass showed improvement over TE_1. At the second and third endosulfan levels, when the endosulfan dose was increased to 5µl/ml and 10 µl/ml respectively, the plant biomass production was decreased. This may be explained by the fact that increasing dose of endosulfan deteriorated the overall health of the seedlings. After a comparative study of different combinations, microbial inoculant *Burkholderia* sp. AULS-B3 improved plant health to a great extend. Mazumdar et al. (2007) reported the ability of Pseudomonas isolates from rhizosphere of tea plants in promoting growth of tea seedlings [5]. Peng et al. (2002) also found that certain strains of rhizobia promoted growth and yield of rice through improved net photosynthetic rate [11].

t- Test result indicated that combination TME1 was highly significant among all the treatments/combinations (Table 1).

Analysis of variance (Table 1.1) of data revealed that variation in biomass production of tea seedlings due to different treatments was significant at 5% level.

The average shoot height and leaf production of the tea seedlings with different treatment/combinations were observed on a monthly basis and the recorded height and leaf number at the end of five months were presented in Fig. 2 and Fig. 3 respectively. TM_1E_1 combination showed the maximum height (72 cm) followed by $TM_1M_2E_1$ (70 cm) Fig. (2). The microbial inoculated plants showed better performances than the control (T). However, in TM_1E_3, TM_2E_3 and $TM_1M_2E_3$ combinations where the endosulfan level was high (10µl/ml), the growth of the tea seedlings was adversely affected though it was better than uninoculated endosulfan treated plant (TE_3). This may be due to the degradation of endosulfan by the efficient microbes and their growth promoting ability. Result of t-test (Table 2) showed that TM_1E_1 was the best combination among all.

Regarding leaf numbers, the microbial inoculated plants have an overall better leaf production than the control plants (Fig. 3). When the dose of endosulfan was raised it leads to decrease in leaf number.

Analysis of variance (Table 2.1 and 3) data revealed that variation in shoot height and leaf production of tea seedlings due to different treatments was significant at 5% level.

The root shoot ratio of the tea seedlings were studied under different treatments and measured at the end of the experiment. Table 4 revealed the results of R: S ratio. It was observed that TM_1E_1 inoculated bacterial combination showed 0.17 which was within the range of a normal healthy plant. However, as the endosulfan dose increased there was a profound change in the root shoot ratio. This may be explained by the stunted/ regarded growth of the tea seedlings as a result of which there was less of shoot weight. This was in agreement with the report of Harris (1992). Except for injury to the roots, a reduction in the root-shoot ratio was almost always in response to more favorable growing conditions. An increase in the root-shoot ratio, on the other hand, indicated that a plant was probably growing under less favorable conditions.

Table 4 showed that in TM_1E_1 inoculated seedlings, the result was highly significant. Analysis of variance (Table 4.1) data showed that variation in root shoot ratio of tea seedlings due to different treatments was significant at 5% level.

Experimental data revealed that endosulfan was toxic to the health of tea seedlings. However, efficient microbes along with the pesticide reduced the degree of toxicity to a certain extend.

4. Conclusion

Keeping in view of the fact that pesticides are unavoidable in the present scenario, it would be of immense value to apply microbial inoculum technology having the potential of pesticide degradation. This would most definitely help to solve more than 50% of the problem of pesticide contamination in soil, water and food and help in establishing a safe tea beverage and a clean soil environment. Therefore studies on microbial diversity and development of consortium technology for their application in tea agro-ecosystem should be developed. This will help the tea growers to enhance the tea productivity and reduce the soil and water pollution caused by the pesticides.

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Conflict of Interest: NIL

Financial Support: NIL

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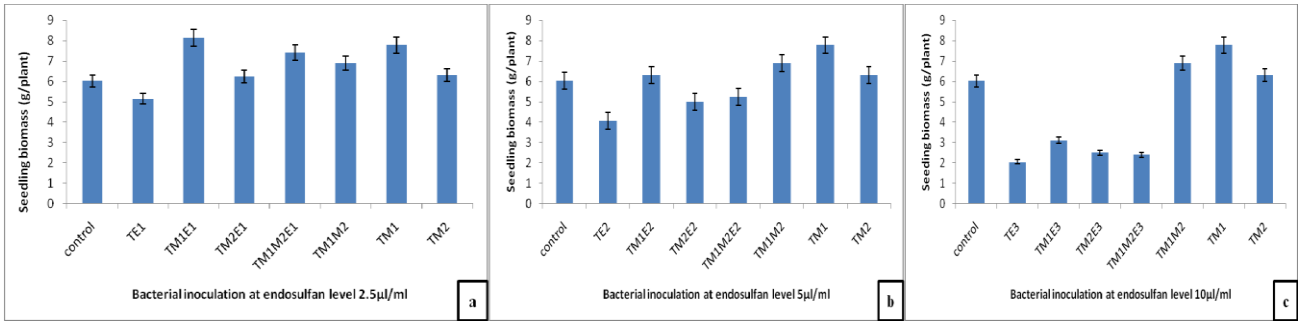

Fig. 1: Biomass production of tea seedlings under different combinations and different endosulfan level

Table 1: Biomass production of tea seedlings with and without treatment

Biomass production	Control	TM1E1	Control	TM1E2	Control	TM1E3
Mean ± S.E.	6.02 ± 0.002	8.15 ± 0.005	6.02 ± 0.002	6.31 ± 0.003	6.02 ± 0.002	3.1 ± 0.006
t Stat (paired)	18971.21* (0.001)		4. 31*(0.001)		5.04(0.008)	

*Difference in mean are significant at P<0.0

Table 1.1: ANOVA table showing effect of different treatments on biomass production of tea seedlings

Source of variation	Degrees of freedom	S.S.	M.S.S.	Calculated value of F	Calculated value of F at 5% level of significance
Between treatments	2	182058.09	91029.045	3.48*	3.47
Within treatments	21	549523.75	26167.7976		

*Significant at 5% level of significance

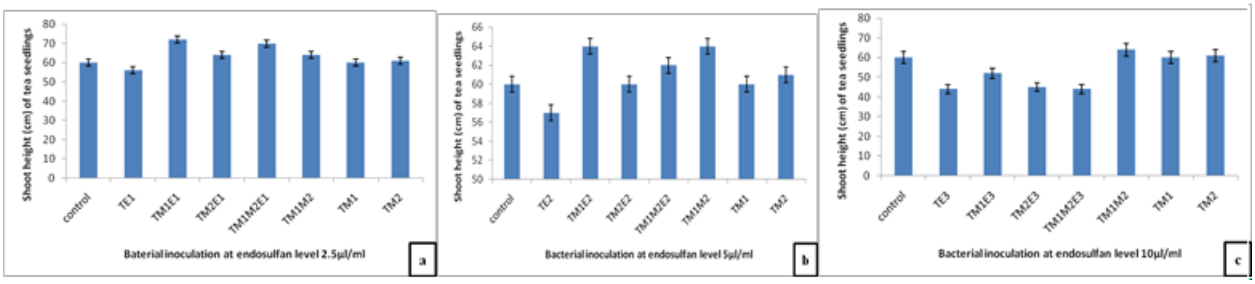

Figure 2: Shoot height of tea seedlings under different treatments

Table 2: Shoot height of tea seedlings with and without treatment

Shoot height (cm)	Control	TM1E1	Control	TM1E2	Control	TE3
Mean ± S.E.	60.01 ± 0.03	72.04 ± 0.01	60.01±0.03	70.05 ± 0.06	60.01±0.03	44.02± 0.02
T Stat	0.00673*(0.003)		0.00169*(0.0002)		0.00049 (0.0009)	

*Difference in mean are significant at P<0.0

Table 2.1: ANOVA table showing effect of different treatments on shoot height of tea seedlings

Source of variation	Degrees of freedom	S.S.	Estimated value of variance	Calculated value of F	Calculated value of F at 5% level of significance
Between months	4	2085.18	521.295	67.61*	2.52
Between treatment/combination	15	2476.36	165.10	21.41*	Between 1.86 & 1.81
Residual	60	460.42	-	-	-
Total	79	5021.99	-	-	-

*Significant at 5% level of significance

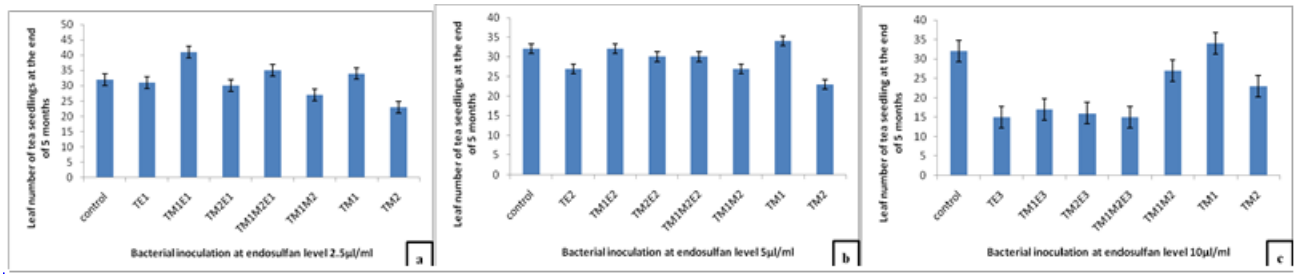

Figure 3: Leaf number of tea seedlings under different treatments.

Table 3: ANOVA table showing effect of different treatments on leaf numbers of tea seedlings.

Source of variation	Degrees of freedom	S.S.	Estimated value of variance	Calculated value of F	Calculated value of F at 5% level of significance
Between months	4	1035.56	258.89	25.08*.	2.52
Between treatment/combination	15	3224.2	214.95	20.83*.	Between 1.86 - 1.81
Residual	60	619.24	10.32	-	-
Total	79	4879	-	-	-

*Values represented in the table are mean of 3 replicates.

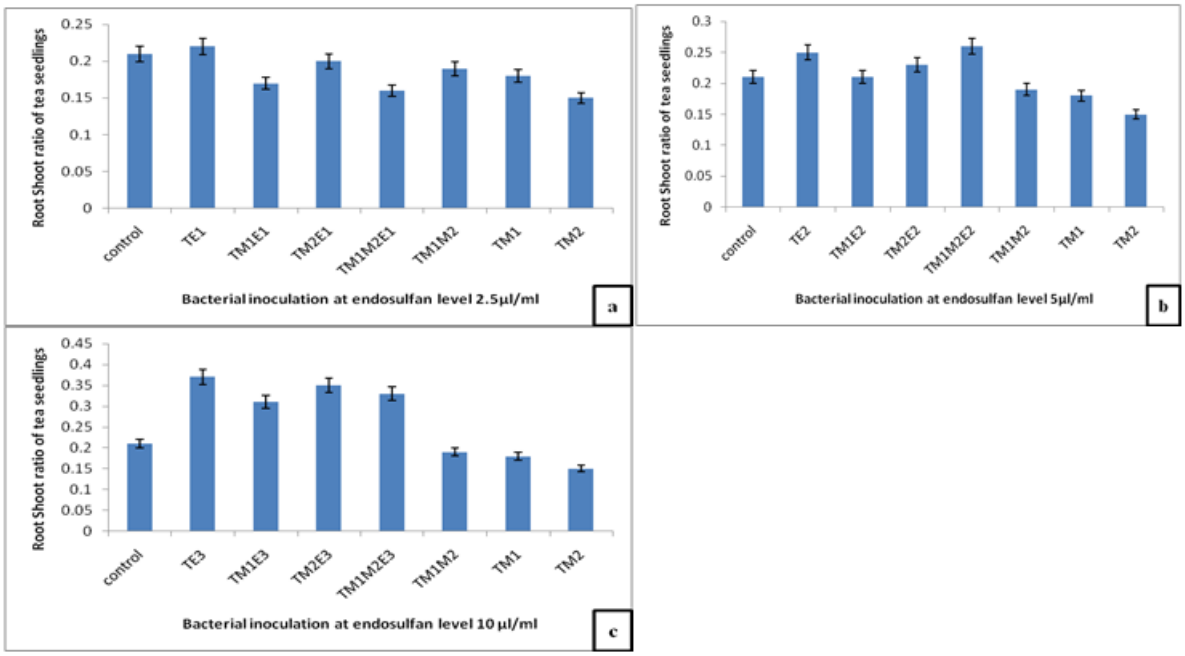

Figure 4: Root shoot ratio of tea seedlings under different treatments

Table 4: Root shoot ratio of tea seedlings with and without treatment

R:S ratio	Control	TM1E1	Control	TM1E3	Control	TM2E1	Control	TE3
Mean ± S.E.	0.21 ± 0.002	0.17 ±0.001	0.21 ± 0.002	0.37 ± 0.04	0.21 ± 0.002	0.2 ± 0.004	0.21 ± 0.002	0.35 ±0.01
T Stat	49637.11* (0.002)		0.00109 (0.004)		0.00165 (0.0018)		0.00103 (0.004)	

* Difference in mean are significant at P<0.0

Table 4.1: ANOVA table showing effect of different treatments of on root shoot ratio of tea seedlings

Source of variation	Degrees of freedom	S.S.	M.S.S.	Calculated value of F	Calculated value of F at 5% level of significance
Between treatments	2	241.75	120.87	3.78*	3.47
Within treatments	21	670.87	31.94		

*Significant at 5% level of significance