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Bradyrhizobium japonicum, an efficient microbial consortium for the effective and improved growth of leguminous crops in a polluted soil ecosystem

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Abstract
Maintenance of soil organic matter is very much important for the long-term productivity of agro ecosystems. Legume crop (Vigna radiata L Wilczek. Var CO4) was cultivated to overcome the growing problem of nitrogen fixation of plants grown in pesticides polluted soils. To improve the soil quality for the effective growth of green gram the use of microorganism for bioremediation of organic pollutants was done. Pot culture experiment was performed and the effect of pesticides with Bradyrhizobium japonicum, Bacillus megaterium, and Bacillus mucilaginous was analysed and mineral content of V. radiata L. were observed, studied at various stages of its growth. The morphometric analysis, yield parameters were calculated individually in each pot on 30, 40, 60 DAS. The control pot was characterized by a stronger growth, more abundant green matter, and developed root system with more nodules. Among all treatments the combined inoculation of bio-fertilizers recorded the highest growth and yield of green gram 24.779(mg/ml), inoculation of biofertilizer Bradyrhizobium japonicum only yielded with a protein mass of 23.715 (mg/ml). Bradyrhizobium japonicum favoured a considerable growth, yield for leguminous plants in germination of pot culture experiments that is helpful for development of plant communities and eco-friendly environment.

Keywords: organic matter; legume crop, bioremediation; *Bradyrhizobium*, eco friendly

Introduction

Pulse crops are primarily grown under rain fed condition and on low fertility neglected soils of India. Even then, India is the largest producer and consumer of the world [1, 2]. Green gram has long been a food crop in Asia. Among the pulses, green gram (*Vigna radiata* L.) is one of the most important and extensively cultivated crops in India that is cultivated in arid and semi arid region. Green gram is locally known as "*moong*".

Biofertilizers are living cells of different types of microorganisms, which have an ability to mobilize nutritionally important elements from non-available to available form. The biological nitrogen fixation process, atmospheric nitrogen is changed to ammonia from nitrogen fixing microorganisms by using a complex enzyme system. These bacteria have the nitrogenase enzyme that mediates the mineralization of organic forms of N to ammonium (NH_4^+) and its subsequent nitrification to nitrate (NO^{3-}) by ammonia-oxidizing and nitrite oxidizing bacteria [3]. This process of BNF is of major significance to N availability and has influence on rhizosphere dynamics and proved to be advantageous that, it is economically beneficial and environmental friendly alternative to chemical fertilizers [4]. The biofertilizers have an ability to mobilize nutritionally important elements from non-exchange to readily available form through biological process influencing the nutrient dynamics of rhizosphere soil [5]. Soil is considered as a storehouse of microbes and its activity is more. The addition of organic amendments increased the microbial population of symbiotic bacteria and nitrogen fixation [6]. The microbial activity can also be increased by addition of vermicompost [7,8] reported that the inoculation of *Bacillus licheniformis* clearly enhanced the carbon turnover when composting. The increased microbial activity and soil organic carbon concentration in manure soils might contribute to increased soil stability [9]. Therefore, it is necessary to overcome the limitations of the polluted soil by replacing with the more useful soil bacteria by using it in a more appropriate manner. The present investigation was carried to find out the studies on the effect of pesticides on bacteria involved in nitrogen fixation on Green gram (*Vigna radiata* (L.)Wilczek) and to find out an efficient biofertilizer that has the ability to improve crop yield.

Materials and Methods

Estimation of pesticide residues from the soil sample

Surface (0-15cm) soil samples were collected randomly from 10 different regions of Cuddalore area of Thanjavur, Tamilnadu. The experimental site is located at 23°c-15'.00"N to 23°c-45'-00"N and 88°-45'-00" E to 89°-45'-00"E. Samples were collected from Nellikuppam, Panruti, Chidambaram, Kurinjipadi, Neyveli, Virudhachalam, Kattumannarkoil, Thirunavalur, Parangipettai. All samples were kept in plastic bags and transported to the laboratory and stored at 4°C prior for analysis [10].

a. HPLC Analysis

Desired samples were dissolved in specific solvent (HPLC Grade) and filtered through the 0.22 micron filter [11]. The filtrate was collected and degassed by using sonicator for 50 times at 4°C. Solvent was prepared by desired concentration of Acetonitrile: water (65:35) respectively. The 20µl of sample was injected to the injection head using injection needle. Reserve phase HPLC (Cyberlab, USA) analysis was

carried out in a C $_{18}$ column (250 mm × 4.6 mm) Varian (Lake forest, CA, USA) equipped with a C_{18} guard column. The compounds were eluted with an Isocratic elution of Acetonitrile Vs water at the flow rate 1 ml/min. The effluents were monitored by recording the absorbance at 280 nm.

b. **Separation of bioactive compounds by GC-MS analysis**

The bioactive compounds were identified by using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown compounds was compared with the spectrum of the known compounds stored in the NIST library. The name, molecular weight and the structure of the compounds of the test materials were determined [12].

Isolation of ammonifying and nitrogen fixing bacteria from the various polluted soil samples:

Microbial population was estimated by plate count method. Innoculum from serial dilutions were plated. Bacteria representative of the predominant morphological types present on the plates was selected at random and purified on Yeast extract mannitol agar and Ashby's Mannitol Agar media for isolation and identification of Ammonifying and nitrifying bacteria. Later the isolates were identified following Bergey's manual for bacteriology.

a. Measurement of Urease Activity: Bacteria were grown in Christensen's urea broth [13]. Thecultures were incubated at 28°C with shaking at 150 rpm for 3-5 days until cultures reached the mid-log phases. Then the cultures were centrifuged at 10,000 rpm for 10 min. The supernatants were used to measure extracellular urease activity. 3.0 ml of citrate buffer (pH 6.7) and 5.0 ml of 10% urea substrate solution were added to 1.0 ml of supernatant or sonicated cell suspension and the sample was incubated for 3 h at 37°C. The absorbance at 578 nm was measured. Controls without addition of urea were also included.

Ammonia concentrations without and with addition of urea were used to determine the released ammonia resulting from urease activity. The concentration of ammonia was determined by comparison with a standard curve.

b. Measurement of Ammonification: Bacteria were propagated in peptone water and incubated at 28°C with shaking at 150 rpm for 3-5 days until cultures reached the mid-log phases. 0.1 ml of Nessler's reagent was added to 2 ml of the culture. The absorbance at 400 nm was immediately measured. Ammonium chloride in the range of 0-500 µm was used in the reaction to prepare a standard curve. The concentration of ammonia produced was determined by comparison with a standard curve [13].

c. Nitrate and Nitrite respiration: Isolates were grown in YEM broth on a rotary shaker at 28 ºC until late log phase. A loop full of culture suspension was inoculated into sterile screw-cap tubes containing 10 ml of sterile YEM broth amended with 10 mM KNO_3 and a Durham tube for gas collection. As a control, triplicate tubes of YEM broth without KNO_3 was inoculated. Sterile liquid paraffin (0.5 ml) was layered over the liquid and then the tubes were capped. Inoculated tubes were incubated at 28 ºC.Optical density was measured OD at 660 nm [15].

d. Collection of seed materials for the growth of *V.radiata* L: The seeds of green gram (*Vigna radiata* (L.) Wilczek.) variety CO4 were obtained from Regional pulses Research Institute, Regional Research Station, Cudalore, Cudalore District of Tamil Nadu, India.

Germination studies

Germination, the critical phase in the lifecycle of a crop plant is subjected to numerous environmental factors [25].Germination studies were conducted with green gram (*Vigna radiate* var. CO4) of 2 kg seeds and they were allowed to grow up to ten days in plastic pots filled with different dosage of 2,4,6g/kg seeds and were added to each pot.The morphological growth parameters such as germination percentage, growth and dry weight of seedlings were analysed**.** The combined application were also done within the biofertilizers and were used for the pot culture experiment.

a. Germination ability: The germination ability was counted, after the tenth day of seed sowing. Three replicates were maintained for each treatment. The total germination percentage was calculated.

Germination percentage =No. of seed germination with inoculated×100/No. of seed germinated in control

b. Seedling growth (cm/seedling)

Twenty seedlings were randomly selected from each treatment for recording the seedling growth. The growth of the 10 day old green gram seedlings were measured by using a centimeter scale and the values were recorded.

c. Dry weight (g/seedling)

The same seedlings used for seedling growth measurement were kept in a hot air oven at 80 ºC for 24 hr. Then, the samples were kept in desiccator for some time. Their dry weight was taken by using an electrical single pan balance and the average values were expressed in g/seedling.

d. Pot culture experiment

Pot culture experiment [17] were conducted with green gram seeds to know the effect of various biofertilizers on growth and yield performance of green gram.

Split Pot Design

Table 1

T1	T3	T5
T6	T4	T2
T7	T1	T8
T5	T6	T7
T4	T5	T1
T3	T2	T6
T2	T8	T3
T8	T7	T4

T1-Control T2-*Bacillus megaterium* T3- *Bradyrhizobium japonicum* T4- *Bacillus mucilaginous* T5-*Bradyrhizobium + B. Mucilaginous* T6 – *B. Mucilaginous + B.megaterium* T7-*Bradyrhizobium japonicum + B. megaterium* T8- *B. megaterium + B. japonicum+ B. mucilaginous*

The experimental soil i.e., pesticide effected mung bean soil is taken from Kurinjipadu and Katturmankoil region, thoroughly ploughed, leveled. The pot was filled with five

kilogram soil by split pot design method with three replicates. A pre-sowing irrigation was given to ensure sufficient soil moisture.

Biochemical analysis

The photosynthetic pigments such as total chlorophyll and the biochemical contents such as carbohydrate, protein, were analyzed in the plants grown in the pot culture experiments, the test crop were randomly collected periodically on 20th, 40th, and 60th DAS.

a. Chlorophyll

Five hundred mg of fresh leaf material was grounded with a mortar and pestle with 10 ml of 80 % acetone. The homogenate was centrifuged at 800 rpm for 15 minutes. The supernatant was saved and the residue was re-extracted with 10 ml of 80 % acetone. The supernatant was saved and the absorbance values were read at 645 and 663 nm in a UV-spectrophotometer [18]. The chlorophyll a, chlorophyll b and total chlorophyll contents were estimated and expressed in mg/g fresh weight basis.

Chlorophyll 'a' = (0.0127)x(O.D 663)-(0.00269)x(O.D 645)
Chlorophyll 'b' = (0.0229)x(O.D 645)-(0.00488)x(O.D 663)
Total chlorophyll = (0.0202)x(O.D 645) + (0.00802)x(O.D 663)

b. Estimation of carbohydrates

One gram of plant materials was taken in a test tube and hydrolysed with 2 ml of conc. H_2SO_4 for 30 minutes at 100ºC. To 0.5 ml hydrolysate, 1 ml of 5 per cent phenol and 5 ml of conc. H_2SO_4 were added and mixed thoroughly. The color developed was measured at 490 nm in a UV-spectrophotometer. Standard graph was prepared by running standard glucose (conc. 10 g to 100g). The amount of carbohydrate was calculated by using standard graph and the results were expressed as mg carbohydrate/g of the sample [19].

c. Estimation of protein

(i) Extraction

Five hundred mg of plant materials (root, stem and leaf) were weighed and macerated in a pestle and mortar with 10 ml of 20 per cent trichloro acetic acid. The homogenate was centrifuged for 15 minutes at 600 g. The supernatant was discarded. To the pellet 5 ml of 0.1 N NaOH was added and centrifuged for 5 minutes. The supernatant was saved and the volume was made upto 10 ml of 0.1 N NaOH. This extract was used for protein estimation [20].

(ii) Estimation

Protein was estimated by adding 0.5 ml Folin-phenol reagent to the extract. The sample was read at 660 nm in a UV-spectrophotometer [20].

d. Enzymatic activity

The activities of catalase and peroxidases in green gram were estimated and recorded in the plants grown at all the sampling days.

i. Catalase

Catalase activity was measured [21] by taking one gram of leaf sample was homogenized in 10 ml of 0.1 M phosphate buffer (pH 7) and centrifuged at 4 ºC for 10 minutes at 10,000 rpm. An aliquot of 1 ml of the supernatant of the enzyme extract was added to the reaction mixture containing 1 ml of 0.01 M H_2O_2 and 3 ml of 0.1 M phosphate buffer. The reaction was stopped after incubation of 5 minutes at 20ºC by adding 10 ml of one per cent H_2SO_4. The acidified medium without or with the enzyme extract was titrated against 0.005 N $KMnO_4$ and catalase activity was expressed as 'n' moles of H_2O_2 utilized (units min/mg/protein).

ii. Peroxidase

One gram of fresh plant material was homogenized with 20 ml of ice-cold extraction buffer. The homogenate was strained through two layers of cheesecloth and centrifuged at 10,000 rpm for 15 minutes. The supernatant was made upto 20 ml with the same buffer and it was used as the source of enzyme [21, 26]. The activity was expressed in unit = 0.1 absorbance mg/protein/min.

e. Estimation of leghaemoglobin content of root nodules

i. Leghaemoglobin Assay

Leghaemoglobin concentration was determined by the cyanmethemoglobin method [22].

ii. Nitrogenase activity by Acetylene Reduction Assay

The process is accessed by using (Thermo scientific Chemito Ceres 800 plus) gas chromatography [23].

Nitrogenase activity in root nodules of test crop was estimated at various sampling days by using Acetylene reduction assay method. One gram of freshly collected root nodules were washed thoroughly in distilled water. They were placed in 65 ml serum vials and closed with rubber stoppers. 6.3 ml of air from the serum vial was evaluated at 28 + 1 ºC for one hr with the sterile disposable syringe. At the time of assay, 6.3 ml of acetylene gas was injected by using the sterile disposable. These bottles were incubated at 28 + 1 ^{0}C for one hour. At the time of assay, 0.5 ml of the gas sample was withdrawn after flushing twice and injected into gas chromatography and tested for ethylene production. The factor 0.006 was arrived by injecting pure ethylene gas. The nitrogenase activity was expressed as a mole of ethylene produced per gram of nodules per hour.

The statistical analysis of experimental results was carried out as per the procedure [24]. Split analysis was done to find out the interaction between the varieties by using the statistical package IRRI STAT.

Results and discussion

Analysis of pesticide residue by colorimeter method of various sampling were recorded. Among the ten sample area, Kurinjipadi soil has maximum pesticide residue 1.23(mg/kg) and minimum pesticide residue from Chidambaram soil 0.27(mg/kg).

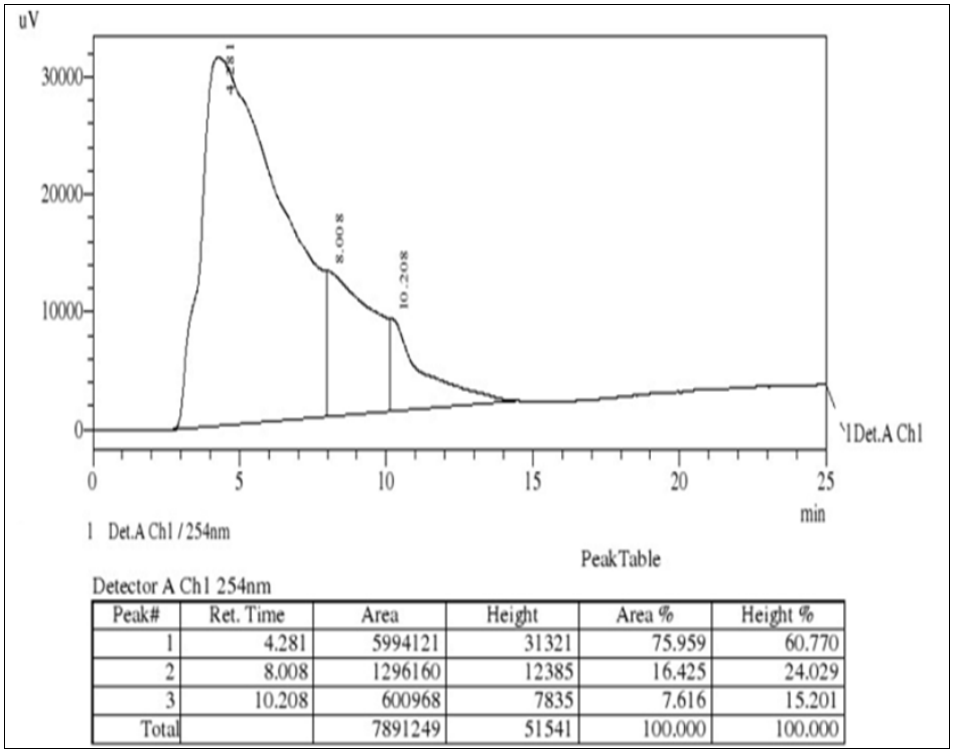
Table 2: Estimation of pesticide residue by colorimetric method of various sampling places

S. No	Name of the places	Pesticide residue (mg/Kg)
S_1	Nellikuppam	0.30
S_2	Panruti	0.29
S_3	Chidambaram	0.27
S_4	Kurinjipadi	1.23
S_5	Neyveli	0.52
S_6	Virudhachalam	0.42
S_7	Kattumannarkoil	1.12
S_8	Thirunavalur	0.41
S_9	Parangipettai	0.61
S_{10}	Vadalur	0.98

Estimation of pesticide residue by using HPLC analysis was done. The maximum retention time in Kurinjipadi and Kattumannarkoil soil was recorded. Minimum retention time in Virdhuchalam soil was recorded.

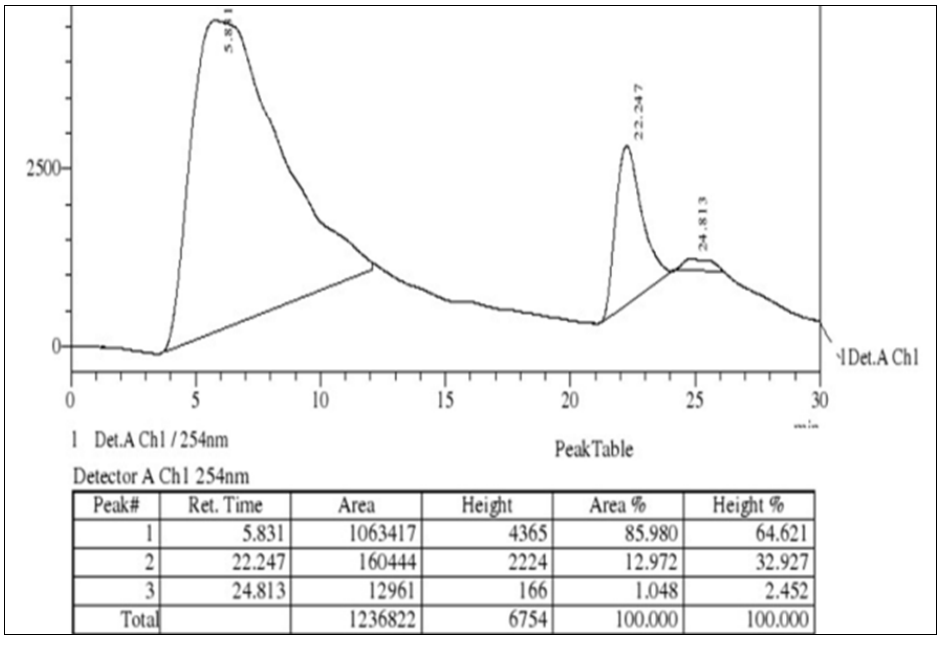
Table 3: HPLC analysis of pesticide polluted soil sample at different places

S. No	Name of the places	Retention time
	Nellikuppam	4.209, 6.113, 12.056
S_2	Panruti	3.805, 7.889
S_3	Chidambaram	5.550, 6.134, 6.704
S_4	Kurinjipadi	4.281, 8.008, 10.208
S_5	Neyveli	4.616, 9.585
S_6	Virudhachalam	3.649
S_7	Kattumannarkoil	5.831, 22.247, 24.813
S_8	Thirunavalur	3.535, 5.300
S_9	Parangipettai	4.561, 10.567
S_{10}	Vadalur	3.557, 5.613



Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.281	5994121	31321	75.959	60.770
2	8.008	1296160	12385	16.425	24.029
3	10.208	600968	7835	7.616	15.201
Total		7891249	51541	100.000	100.000

Fig 1: HPLX analysis of pesticide polluted soil sample karinjipadi



Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.831	1063417	4365	85.980	64.621
2	22.247	160444	2224	12.972	32.927
3	24.813	12961	166	1.048	2.452
Total		1236822	6754	100.000	100.000

Fig 2: HPLC analysis of pesticide polluted soil sample of kattamannarkoil

The GCMS analysis of pesticide were analysed from the Kurinjipadu site and Kattumannarkoil site, compounds with respective retention time and molecular weight of the compounds were identified.

Table 4: GCMS analysis of pesticide polluted soil sample of Kurinjipadi sites

S. No	Retention time	Molecular weight	Compound
1	4.243	222	Diethyl Phthalate
2	4.312	296	1-Iodo-2-methylundecane
3	5.269	302	Nonadecane, 1-chloro-
4	5.622	290	2,5-Octadecadiynoic acid, methyl ester
5	5.858	296	1-Iodo-2-methylundecane
6	7.568	296	1-Iodo-2-methylundecane
7	8.231	524	alpha.-d-Xylopyranoside, methyl-2,3,4-tris-O-[9-borabicyclo[3.
8	9.359	901	1-Iodo-2-methylundecane

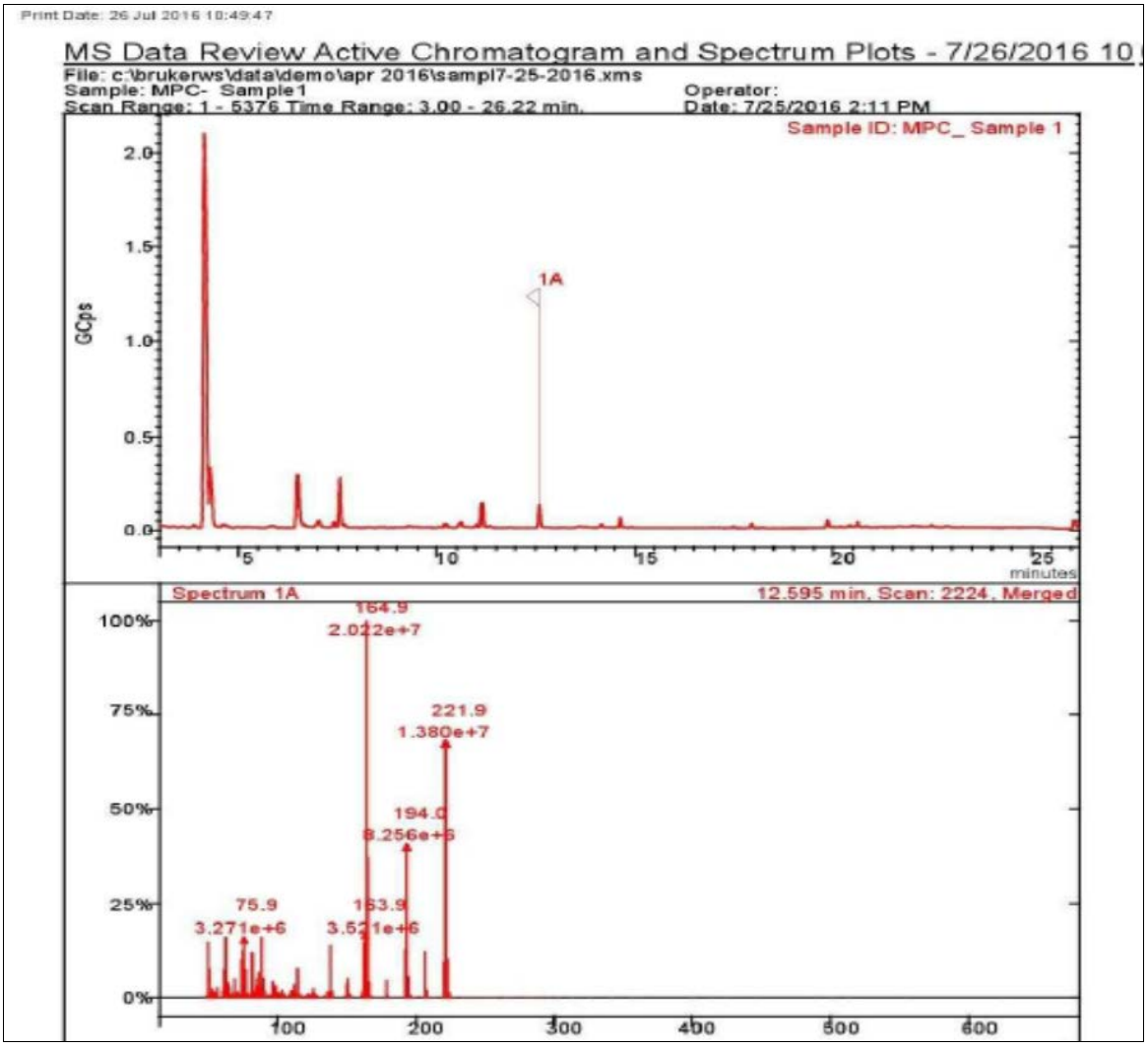


Fig 3: GCMS chromatogram of Kurinjipadu site

Table 5: GCMS analysis of pesticide polluted soil sample of Kattumannarkoil sites

S. No	Retention time	Molecular weight	Compound
1	4.276	296	1-Iodo-2-ethylundecane
2	4.310	296	1-Iodo-2- ethylundecane
3	8.280	604	Tetracontane, 3,5,24-trimethyl-
4	9.464	408	2-methyloctacosane
5	9.794	284	Hexadecanoic acid, 15-methyl-, methyl ester
6	10.227	390	Di-nitro-octylphthalate
7	13.036	296	Phytol

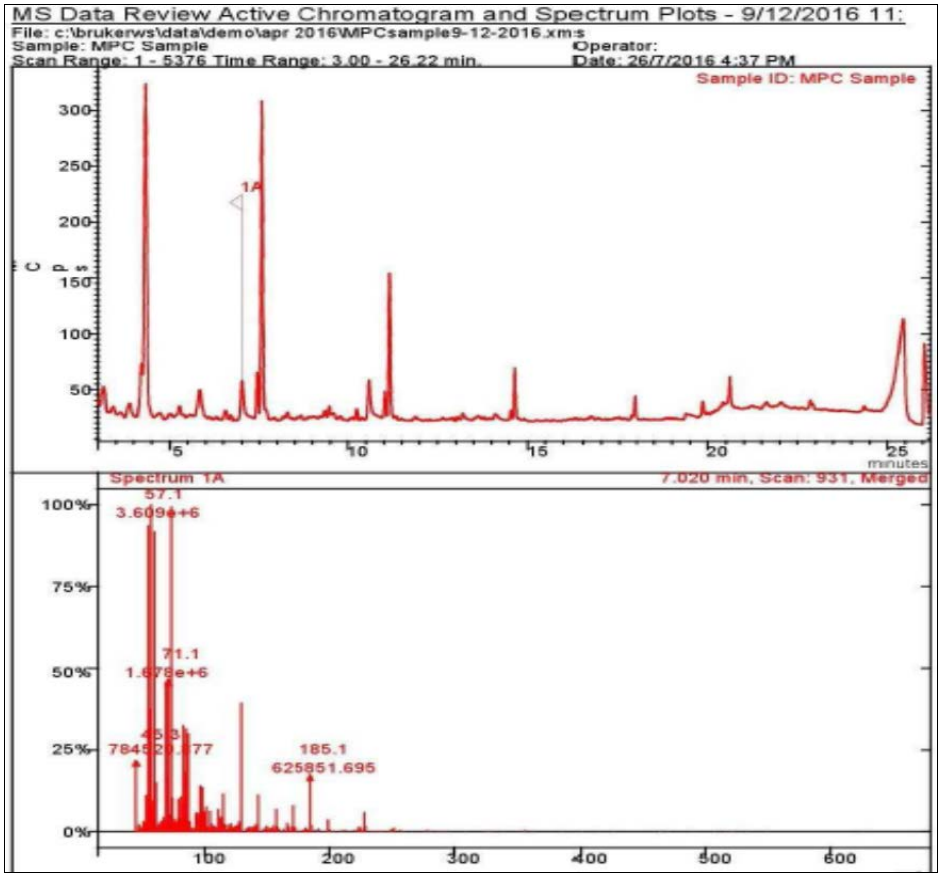


Fig 4: GCMS chromatogram of Kattumanarkoil site

The bacterial isolates such as *Pseudomonas*, *Bacillus megaterium*, *Nitrosomonas* sp and *Streptococcus* sp, *Nitrobacter* sp, *Bradyrhizobium* sp, *Azetobacter* sp were isolated from soil polluted with pesticide from the cultivated leguminous plants. From this study we can estimate that the pesticides have differential effect on the growth of nitrogen fixing bacteria

Fig 5: Isolation of nitrogen fxing bacteria from green gram soil by Jensen's medium.

The biochemical examination of the four isolates clearly indicated that these belong to the genera Nitrosomonas and Nitrobacter, so screening of microorganisms for conversion of ammonia to nitrite and nitrate was important and was done based on the results obtained.

Table 6: Quantification of bacterial isolates

S.no	Name of bacteria	Ammonification (%)	Urease activity (%)	Catalase activity (%)
1	*Nitrobacter sp*	21.8	9	9.4
2	*Nitrsomonas sp*	34.5	10	9.1
3	*Azotobacter sp*	37	11.8	10.5
4	*Bradyrhizobium sp*	50.6	17.4	13.4

Among all these bacteria, *Bradyrhizobium sp* showed high percentage of ammonification, urease activity and catalase activity which predominantly understood to contribute the major function for fixing nitrogen in plant, so *Bradyrhizobium japonicum* was taken as one of the major consortium among the three biofertilizers.

The suitability of fertilizers for the best growth at germination level was evaluated. The highest germination percentage was observed in combined application of biofertilizers (*Bradyrhizobium japonicuum, Bacillus megaterium* and *Bacillus mucilaginous).*

Table 7: Analysis of seed germination with biofertilizers at different dosages of 1, 2 and 3 g/kg

Treatment	Fertilizer dosage (g/kg)	Seed germination (%)	Seedling growth (cm)	Dry weight of seedlings (cm)
T_1	-	55±1.65	10.75±0.322	0.485±0.014
T_2	1	25±0.75	6.38±0.191	0.589±0.017
	2	30±0.90	7.54±0.2262	0.132±0.003
	3	65±1.95	14.30±0.429	1.620±0.048
T_3	1	21±0.63	5.42±0.162	0.498±0.014
	2	30±0.90	4.54±0.136	0.200±0.006
	3	32±0.96	6.79±0.203	0.542±0.016
T_4	1	32±0.96	6.76±0.202	0.538±0.016
	2	40±12	5.41±0.162	0.420±0.012

	3	45±1.35	7.98±0.239	0.628±0.018
T_5	1	72±2.16	13.00±0.390	1.110±0.033
	2	76±2.28	14.50±0.435	1.289±0.038
	3	86±2.58	14.99±0.449	1.800±0.054
T_6	1	72±2.16	13.74±0.412	1.295±0.388
	2	80±2.4	12.52±0.375	1.228±0.036
	3	78±2.34	11.72±0.351	1.110±0.033
T_7	1	84±2.52	14.20±0.426	1.600±0.048
	2	80±2.4	12.72±0.381	1.000±0.030
	3	80±2.4	12.69±0.380	1.225±0.036
T_8	1	98±2.94	18.94±0.568	1.882±0.056
	2	96±2.88	18.72±0.561	1.800±0.054
	3	92±2.76	18.38±0.551	1.758±0.052

The maximum no of germination can be seen in the T_8 combination of 98 ± 2.94 which is a mixture of all fertilizers and also T_5 combination which is a mixture of *Bradyrhizobium japonicum* and *B. mucillaginous.*

Biochemical and enzyme activities

The increase in various growth parameters is ultimately increased in various biochemical constituents of crop in both seedling stage and different stages of crop growth under pot cultured conditions. The highest chlorophyll content was recorded in 60 DAS old plants when compared with all other sampling days. The increasing chlorophyll content was due to the presence of microorganisms in bio-fertilizers.

Table 8: Effect of bio-fertilizers on bacteria involved in nitrogen fixation on biometric analysis of green gram (*Vigna radiata* L.) on 10th Days seedling.

S. No	Treatment	Total chlorophyll	Carbohydrates	Protein
1	T_1	1.489±0.044	0.756±0.022	12.153±0.364
2	T_2	1.573±0.047	0.985±0.029	13.748±0.412
3	T_3	1.499±0.044	0.937±0.028	12.679±0.380
4	T_4	1.552±0.046	0.912±0.027	12.548±0.37
5	T_5	2.113±0.063	1.299±0.038	13.880±0.416
6	T_6	1.985±0.059	1.120±0.033	13.841±0.415
7	T_7	1.972±0.059	1.028±0.030	13.811±0.414
8	T_8	2.169±0.065	2.000±0.060	13.990±0.419

Table 9: Effect of biofertilizers on bacteria involved in nitrogen fixation on biometric analysis of green gram (*Vigna radiata* L.) on 20th Days seedling.

Treatments	Total Chlorophyll (µg/ ml)	Carbohydrates (mg/ml)	Protein (mg/ml)
T_1	1.538±0.046	1.368±0.041	13.521±0.405
T_2	1.611±0.048	2.1±0.063	18.9±0.567
T_3	1.600±0.048	1.15±0.034	18.01±0.540
T_4	1.589±0.047	1.983±0.059	16.742±0.502
T_5	2.189±0.065	3.385±0.101	21.742±0.652
T_6	2.173±0.065	3.220±0.096	21.685±0.650
T_7	2.168±0.065	3.185±0.095	20.974±0.629
T_8	2.348±0.070	4.330±0.129	22.748±0.682

Table 10: Effect of bio-fertilizers on bacteria involved in nitrogen fixation on biometric analysis of green gram (*Vigna radiata* L.) on 60th Days seedling.

Treatments	Total Chlorophyll (µg/ ml)	Carbohydrates (mg/ml)	Protein (mg/ml)
T_1	1.711±0.051	1.83±0.054	11.72±0.351
T_2	1.645±0.049	3.57±0.107	19.84±0.595
T_3	1.639±0.049	2.84±0.085	15.9±0.477
T_4	1.611±0.048	2.19±0.657	14.3±0.429
T_5	2.878±0.086	5.984±0.179	23.715±0.711
T_6	2.611±0.078	5.771±0.173	23.600±0.708
T_7	2.710±0.081	5.679±0.170	23.489±0.704
T_8	2.732±0.081	7.384±0.221	24.799±0.734

Table 11: Effect of biofertilizers on bacteria involved in nitrogen fixation on catalase activity of green gram (*Vigna radidata* L.) on 20, 40 and 60th Day seedlings

Treatments	20 Days	40 Days	60 Days
T_1	5.978±0.179	8.654±0.259	10.543±0.316
T_2	6.542±0.196	9.543±0.286	11.723±0.351
T_3	7.567±0.227	9.102±0.273	11.000±0.330
T_4	8.237±0.247	10.760±0.322	12.928±0.387

T_5	10.542±0.316	11.841±0.355	13.112±0.393
T_6	10.371±0.311	11.652±0.349	13.100±0.392
T_7	10.211±0.306	11.441±0.343	13.000±0.390
T_8	10.984±0.329	12.682±0.380	13.569±0.407

Table 12: Effect of bio-fertilizers on bacteria involved in nitrogen fixation on peroxidase activity of green gram (*Vigna radiata* L.) on 20, 40 and 60th Day seedlings

Treatments	20 Days	40 Days	60 Days
T_1	8.721±0.261	9.628±0.288	10.110±0.303
T_2	10.21±0.306	10.445±0.313	11.320±0.339
T_3	9.561±0.286	10.000±0.300	11.101±0.330
T_4	10.541±0.316	11.589±0.347	12.652±0.379
T_5	12.741±0.382	12.796±0.383	13.651±0.409
T_6	12.65±0.379	12.662±0.379	13.470±0.404
T_7	12.478±0.374	12.540±0.376	13.330±0.399
T_8	13.74±0.412	13.118±0.393	14.110±0.423

Leghaemoglobin content

The highest leghaemoglobin content of green gram was observed in the combined application of biofertilizers (*B. japonicum* + *B. megaterium* + *B. mucilaginous*) when compared with control and other treatments. Moreover, twenty five per cent increase of leghaemoglobin content was observed in individual applications and thirty per cent increase in leghaemoglobin content was observed in combined biofertilizers application. This is because of the N_2-fixing nodules which facilitates O_2 diffusion to respiring bacteria in the infected cells. The physiological relationship between leghaemoglobin concentration and nitrogen fixing efficiency in legume root nodules was initially studied [27]. Similar results were also observed in the earlier studies [28, 29, 30].

Nitrogenase activity

The highest nitrogenase activity of nodules in green gram was recorded in the combined application of biofertilizers, (Nitrogen + Phosphorus + Potassium solubilizing biofertilizers than in control. The nitrogenase uniformly activated the root nodules of green gram at all sampling days and it is the most important factor studied for the efficient growth of the leguminous plant. This may be due to the presence of O_2 concentration within infected cells that is highly regulated by a combination of nodule respiration, leghaemoglobin and an O_2 diffusion barrier [31].The enhanced nitrogenase activity with the addition of biofertilizer might be due to its significant role in energy transfer reactions. The results are in close confirmity with these findings [32, 33].

Table 13: Effect of biofertilizers on bacteria involved in nitrogen fixation on leg hemoglobin contents of root nodule bacterium of *Vigna radiata* L. (20, 40 and 60th Days)

Treatments	20 Days	40 Days	60 Days
T_1	0.03±0.001	0.70±0.021	0.52±0.015
T_2	0.05±0.001	0.29±0.008	0.70±0.021
T_3	0.09±0.002	1.03±0.030	0.9±0.027
T_4	0.06±0.001	0.90±0.027	0.60±0.018
T_5	0.471±0.014	1.354±0.040	1.218±0.036
T_6	0.440±0.013	1.122±0.033	1.100±0.033
T_7	0.412±0.012	1.092±0.032	1.720±0.051
T_8	0.572±0.017	1.671±0.050	1.420±0.042

Table 14: Effect of biofertilizers on bacteria involved in nitrogen fixation on nitrogenase activity of root nodule bacterium of *Vigna radiata* L. (20, 40 and 60th Days)

Treatments	20 Days	40 Days	60 Days
T_1	0.90±0.027	0.72±0.021	1.00±0.030
T_2	0.99±0.029	1.3±0.039	1.25±0.037
T_3	0.97±0.029	1.2±0.036	1.20±0.036
T_4	1.10±0.033	2.1±0.063	1.70±0.051
T_5	2.73±0.081	2.75±0.082	3.50±0.105
T_6	2.61±0.078	2.68±0.080	3.32±0.099
T_7	2.32±0.069	2.51±0.075	3.11±0.093
T_8	2.91±0.087	3.11±0.093	3.92±0.117

Conclusion

The effect of nitrogen, phosphorus, and potassium solubilizing biofertilizers on increased seed germination, seedling growth, biochemical constituents, enzyme activities, mineral contents and yield components of green gram (*Vigna radiata* (L.) Wilczek CO4) was seen. The morphological parameters had a significant effect of increase on the plant with different concentrations when compared to control. The highest morphological growth and yield parameters were recorded in combined status of biofertilizers applied pots. There was a steep increase in the growth of the morphological, biochemical parameters where *Bradyrhizobium japonicum* biofertilizer was used thereby boosted the growth and development of plant communities and ecofriendly environment. Therefore, *Bradyrhizobium japonicum* is considered as a good microbial consortium that favoured the growth of *Vigna radiata*. L.

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