Development and evaluation of *Rhizobium pusense* and *Ciceribacter thiooxidans*-based biofertilizer formulation for growth of *Vigna radiata*

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Abstract

The present study aims to evaluate the biofertilizer potential of microbes in promoting the growth of Vigna radiata. Soil was collected from the rhizosphere soil and root nodules of Vigna unguiculata. After molecular characterization, identified strains were cultured and incorporated into vermicompost and vermiwash carriers to formulate biofertilizers. Small-scale experiments were performed to compare the compatibility of vermicompost and vermiwash to act as carrier material for microbes. Pilot-scale evaluations were conducted to identify the best biofertilizer formulation to improve plant growth. The most effective formulation was assessed against Fertiliser Control Order standards to ensure safety and quality. Molecular characterisation and phylogenetic analysis confirmed the presence of Rhizobium pusense (Accession no: MN-460364) and Ciceribacter thiooxidans (Accession no: NR-159178). Small-scale experiments noted that vermicompost worked better than vermiwash and vermicompost containing C. thiooxidans (ST4), recording a 50% rise in shoot, root and total plant length compared to the control.

Pilot study recorded that consortium of R. pusense and C. thiooxidans (1.0 mL each) with vermicompost yielded the greatest improvements in total plant length (+66%) and root development (+233.3%) relative to controls. Physical, chemical and nutrient profiles of biofertilizer formulation (PT10) containing the consortium were found to be comparable to kitchen compost as per FCO.

Keywords: Biofertilizer, *Rhizobium pusense*, *Ciceribacter thiooxidans*, *Vigna radiata*, vermicompost, vermiwash, Plant Growth-Promoting Rhizobacteria.

Introduction

Vigna radiata L., commonly referred to as green gram, mung bean, or golden gram, is a short-duration legume crop (65–90 days) cultivated over 6 million hectares globally, predominantly within the Indian subcontinent. This crop is integral to human nutrition due to its high protein and micronutrient content. It also contributes to soil fertility enhancement via biological nitrogen fixation[19]. Despite its economic importance, green gram cultivation encounters major constraints, including vulnerability to pests, diseases and irregular rainfall patterns, which adversely affect yield stability[20].

To mitigate these challenges, the application of biofertilizers composed of microbial consortia is advocated to enhance nitrogen fixation and phosphorus solubilization, ultimately improving crop productivity and soil health while minimizing the negative environmental impacts of chemical fertilizers[5,8,9]. Biofertilizers are prepared by introducing beneficial microorganisms into solid or liquid carriers that support microbial proliferation which are applied to soil where the microorganisms exert growth-promoting effects[16]. Vermicompost, a solid material derived from earthworm castings and vermiwash, a liquid extract, are natural and most popular biofertilizer carriers[3].

Selection of beneficial microorganisms is the most crucial stage of biofertilizer preparation. The integration of beneficial microbes, particularly *Rhizobia*, in agriculture has garnered considerable attention due to their role in enhancing soil fertility and crop productivity. Among these, *Rhizobium pusense* has emerged as a promising biofertilizer candidate, which facilitates nitrogen fixation and nutrient solubilization in soil, directly benefiting the growth and yield of legumes[7]. Inoculation with this bacterium has been shown to significantly improve plant growth and health in various other crops such as rice[17]. Furthermore, *Ciceribacter thiooxidans* exhibits potential in promoting plant growth and soil health due to its sulfur-oxidising capacity and nutrient solubilization properties, which are especially advantageous when co-applied with rhizobial strains in leguminous crops[10].

The synergistic effect of these diverse microbial populations can enhance plant resilience in nutrient-deficient soils, optimising yield while reducing dependency on chemical fertilizers[5]. The symbiotic interactions between *R. pusense* and *C. thiooxidans* with *V. radiata* substantially improve nitrogen fixation and plant nutrition through complex biochemical processes. This symbiosis facilitates the conversion of atmospheric nitrogen into plant-available forms, thereby improving soil fertility and agricultural productivity.

The development of such biofertilizers not only enhances green gram productivity but also promotes sustainable agricultural practices by improving soil health and reducing chemical fertilizer inputs[10]. The primary objectives of this study were to isolate and identify nitrogen-fixing bacteria from green gram root nodules and rhizospheric soil,

formulate biofertilizers using these isolates and evaluate their impact on green gram growth under both small-scale and pilot-scale conditions. The ultimate aim of this research is to develop microbe-based biofertilizers that contribute to sustainable agriculture and societal benefit.

Material and Methods

Isolation and Identification of Microorganisms: The experiments were conducted between July 2024 and June 2025. Soil samples (1 gram) were collected from a farming field in South Bangalore (coordinates: 12.8564° N, 77.5888° E), Karnataka, India, in July 2024. These samples were serially diluted and plated onto Jensen's nitrogen-selective medium, then incubated at 28 °C for five days to promote microbial growth. Concurrently, root nodules of the leguminous plant *Vigna unguiculata* were carefully excised. To remove surface contaminants and aid in DNA removal, nodules were initially immersed in 95% methanol, followed by treatment with 3% (v/v) hydrogen peroxide.

Following thorough rinsing with sterile distilled water, nodules were homogenized by crushing in sterile distilled water to obtain a suspension. A loopful of this homogenate was streaked onto Congo Red Yeast Extract Mannitol Agar (CRYEMA) plates and incubated at 38 °C for 3–4 days. Pure microbial colonies obtained were subsequently subcultured for further analysis[12].

Characterization of Isolates: Genomic DNA was extracted from the pure bacterial cultures and its integrity was confirmed by electrophoresis on a 1.0% agarose gel, showing a prominent high-molecular-weight band. The 16S rRNA gene (~1400 bp) was amplified using universal primers 27F and 1492R. PCR amplification success was verified via gel electrophoresis. The resulting amplicons were purified and subjected to bidirectional sequencing using primers 785F and 907R with the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730xl Genetic Analyzer. The obtained sequences were analyzed by BLAST against the NCBI GenBank database to identify closest homologs. Multiple sequence alignment of the top ten homologous sequences was performed using Clustal W. A phylogenetic tree was constructed by the neighbour-joining method in MEGA 11 software with 1000 bootstrap replicates. The finalised 16S rRNA sequences were submitted to the NCBI GenBank for public accession.

Preparation of *Rhizobium pusense* and *Ciceribacter thiooxidans*

Vermicompost and vermiwash were sourced from Biocentre and Lalbagh Botanical Garden, Bangalore, India. Both carrier materials were sterilized by autoclaving at 121 °C for 15 minutes before use. Pure cultures of *R. pusense* and *C. thiooxidans* were inoculated into 100 mL of Jensen's broth and CRYEMA broth and incubated for 5–7 days at 34 °C (*R. pusense*) and 28 ± 2 °C (*C. thiooxidans*). For large-scale propagation, 10 mL of actively growing inoculum was transferred into 1,000 mL of the respective medium and incubated in a shaker incubator for 5 days (*R. pusense*) and 7 days (*C. thiooxidans*). The final biofertilizer formulations were assessed for physico-chemical characteristics and texture[8].

Physicochemical Analysis of Biofertilizer Formulation: Physico-chemical properties of the formulated biofertilizers were characterized including pH, electrical conductivity (EC), bulk density, color, odor, particle size and the presence of pathogens. pH and EC measurements were conducted using calibrated pH and conductivity meters. Bulk density was determined following the core method using a graduated cylinder and balance. Particle size distribution was assessed by sieving samples through 4 mm mesh in accordance with Fertilizer Control Order (FCO) guidelines[11]. Microbial safety was evaluated by streaking samples on nutrient agar and selective media to detect pathogenic organisms.

For nutrient composition analysis, samples underwent acid digestion following standard protocols and were analyzed via Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) to quantify macro- and micronutrients including N, P, K, C, S, Ca, Fe, Mn, Ni, Cr, Cu, Zn, Cd and Pb. These findings were benchmarked against the standards prescribed by the FCO order (Ministry of Agriculture and Farmers Welfare, Government of India)[11].

Small-Scale Experimentation: *V. radiata* seeds were cultivated under ambient conditions in seed trays, each comprising of 50 compartments. Sterilized cocopeat was used as the growth substrate and mixed with vermicompost in a 1:1 ratio (5 g each). Two seeds were sown per compartment. In a parallel preparation, 10 g of cocopeat was supplemented with 20 mL of vermiwash. 1 mL inoculum (10^9 CFU) of *R. pusense* and *C. thiooxidans* was applied to the compartments separately. Additionally, a microbial consortium comprising of *R. pusense* and *C. thiooxidans* (0.5 mL each) was incorporated into both carrier materials. These treatments, nine in total, were replicated thrice, with each replicate containing 10 plants (Table 1).

Pilot-Scale Experiment: Pilot experiments were conducted using 11 pots arranged in triplicate. Ten pots contained a mixture of soil, cocopeat and vermicompost in a 1:1:1 ratio (20 g each), while one pot contained soil alone as the control. Inoculation concentrations of *R. pusense* and *C. thiooxidans* were varied at 1 mL, 2 mL and 3 mL (10^9 CFU/mL). Moreover, a consortium of both strains was applied at volumes of 0.5 mL, 1 mL and 1.5 mL per strain (Table 2).

Assessment of Physical Growth Parameters: Root and shoot lengths were measured manually using a ruler, recording the distance from the root tip to the shoot apex. Growth rates were calculated based on length increments over time intervals. Leaf and root morphological traits were qualitatively assessed in both treated and untreated plants. For small-scale experiments, shoot and root measurements

were recorded on day 5 post-sowing. In the pilot-scale study, growth parameters including shoot length, root length, growth rate and morphological characteristics of leaves and roots, were documented after 20 days. Data were collected in triplicate and the mean and standard deviation were calculated. All collected data were subjected to ANOVA to evaluate statistical significance (p-value) using SPSS, version 26.

Results and Discussion

Isolation and identification of microorganisms: Pure cultures were isolated from the soil and identified using molecular methods. High-quality genomic DNA was successfully isolated as indicated by a sharp, high-molecular-weight band on a 1.0% agarose gel. Amplification of the 16S rRNA gene using universal primers 27F and 1492R yielded a single, distinct band of approximately 1400 bp, confirming specific amplification. The purified PCR product was sequenced bidirectionally using primers 785F and 907R and high-quality sequence reads were obtained. The forward and reverse sequences were assembled into a consensus sequence of the 16S rRNA gene, which was approximately 1360 bp in length.

Blast analysis of the consensus sequence against the NCBI GenBank 'nr' database revealed the closest match with *R. pusense* and *C. thiooxidans*, with a maximum identity score of (99.5%). The top ten homologous sequences were selected for multiple sequence alignment using Clustal W. Phylogenetic analysis using MEGA 11 confirmed the close evolutionary relationship of the isolate with the identified species, as the query sequence clustered strongly (bootstrap support >90%) with its closest relative in the phylogenetic tree (Figures 1 and 2). The isolate sequences were submitted to NCBI as *R. pusense* (Accession no. MN-460364) and *C. thiooxidans* (Accession no. NR-159178). The results were similar to Choudhary et al[7].

Small-scale experiments: The results demonstrated that both vermicompost and vermiwash, especially when combined with microbial inoculants, significantly improved the growth performance of *Vigna radiata* compared to the control (Table 3). Notably, microbial treatment along with vermicompost resulted in a well-balanced growth response, compared to vermiwash, demonstrating the benefit of microbial synergy in a nutrient-rich carrier environment[2]. Compared to the control (ST 1: 14.0 ± 1.9 cm total length), vermicompost containing *C. thiooxidans* (ST4) recorded a maximum (50%) rise in shoot, root and total plant length. Treatment ST 5 (combination of both microbes) also showed notable gains in total length (39.3%), highlighting the effectiveness of *R. pusense* and *C. thiooxidans* as growth stimulants (Figure 3). These findings are consistent with prior reports that highlighted the benefits of combining organic substrates with PGPR to enhance nutrient cycling, root development and biomass accumulation[1,23].

Table 1
Microbial concentrations used for small-scale experiments

S.N.	Plant Type	Carrier	Microbial Concentration (ml)
1	ST 1	No Carrier	-
2	ST 2	Vermicompost	-
3	ST 3	Vermicompost	1.0mL *R. pusense*
4	ST 4	Vermicompost	1.0 mL *C. thiooxidans*
5	ST 5	Vermicompost	0.5 mL of both microbial cultures
6	ST 6	Vermiwash	-
7	ST 7	Vermiwash	1.0mL *R. pusense*
8	ST 8	Vermiwash	1.0 mL *C. thiooxidans*
9	ST9	Vermiwash	0.5 mL of both microbial cultures

Table 2
Microbial Concentration used for pilot study

S.N.	Plant Type	Soil +Carrier	Microbial Concentration (ml)
1	PT 1	No carrier	
2	PT 2	Vermicompost	-
3	PT 3	Vermicompost	1.0mL *R. pusense*
4	PT 4	Vermicompost	2.0mL *R. pusense*
5	PT 5	Vermicompost	3.0 mL *R. pusense*
6	PT 6	Vermicompost	1.0 mL *C. thiooxidans*
7	PT 7	Vermicompost	2.0 mL *C. thiooxidans*
8	PT 8	Vermicompost	3.0 mL *C. thiooxidans*
9	PT 9	Vermicompost	0.5 mL of both microbial cultures
10	PT 10	Vermicompost	1.0 mL of both microbial cultures
11	PT 11	Vermicompost	1.5 mL of both microbial cultures

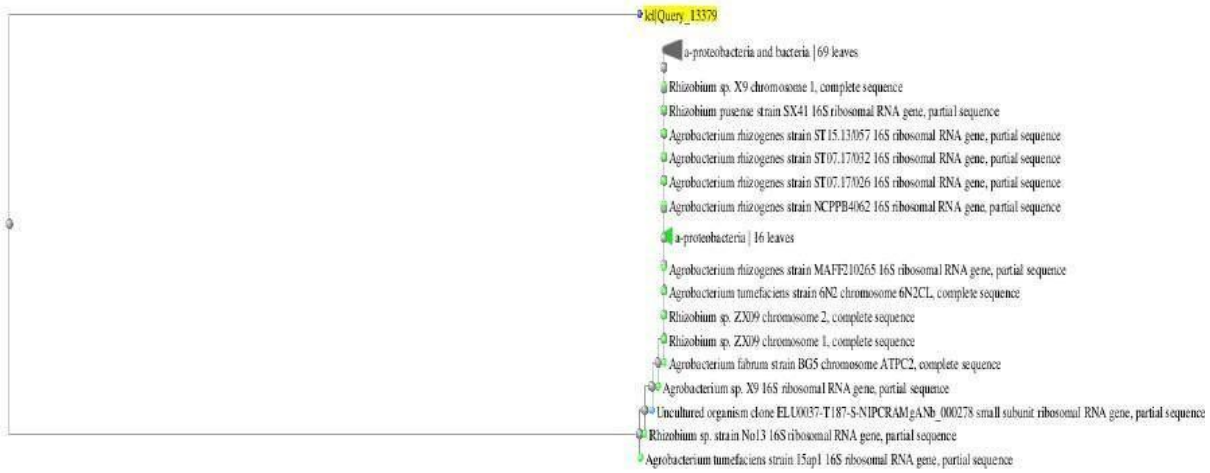

Figure 1: Pedigree analysis of *Rhizobium* species

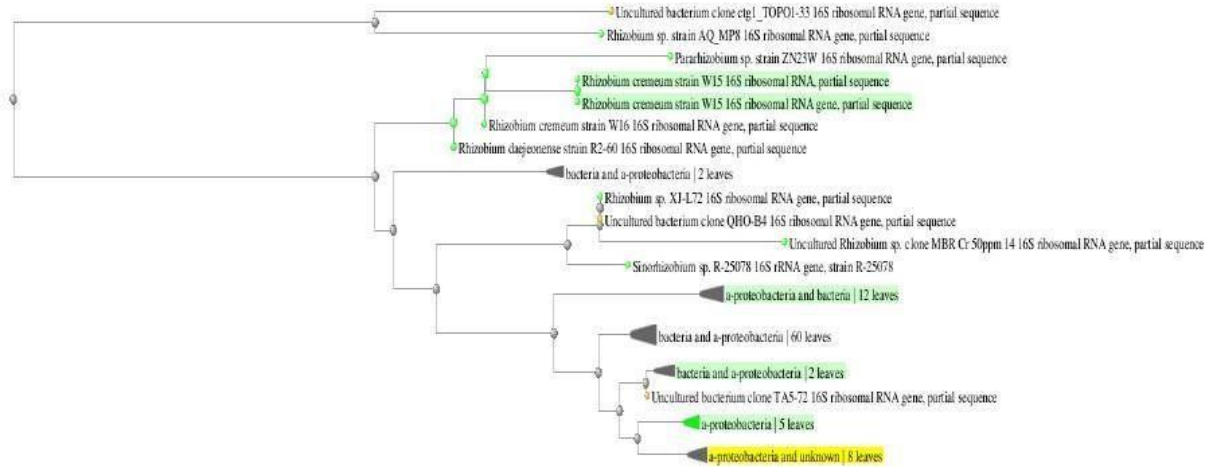

Figure 2: Pedigree analysis of *Ciceribacter* species

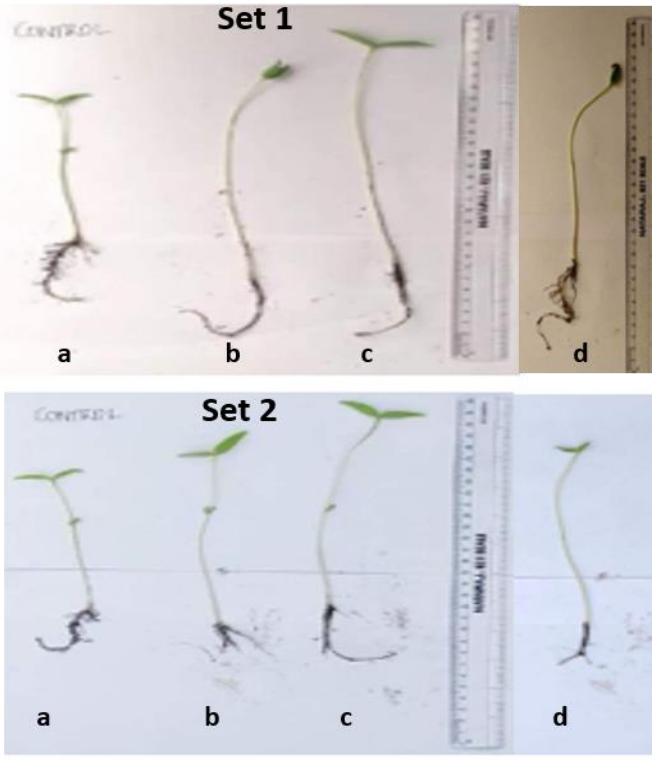

Figure 3: Set 1: Cocopeat with vermicompost (5g:5g); Set 2: Cocopeat with vermiwash (5g: 5ml)
a: Control; b: *R. pusense;* c: *C. thiooxidans*; d: Consortium (1:1)

These results also highlight that vermiwash performance was much lower than vermicompost, however vermiwash offers certain advantages such as easy application and the presence of soluble hormones and enzymes, its nutrient-holding capacity and structural benefits are limited compared to vermicompost[3]. Vermicompost improves soil texture, water retention and microbial colonisation zones, creating a more stable environment for root development. These findings align with prior studies emphasising the superior plant growth response with solid organic amendments over liquid extracts, though both play a role in sustainable cultivation systems[13,14,23]. Overall, vermicompost proved to be more effective as a base carrier and was used for pilot-scale study.

Pilot Study: The application of vermicompost and microbial inoculants markedly enhanced the growth parameters of *Vigna radiata* compared to the untreated control (Table 4). While vermicompost alone (PT 2) modestly increased shoot length by 9.5% and total length by 7.1%, the addition of *R. pusense* (PT 3–5) and *C. thiooxidans* (PT 6–8) led to substantial improvements, particularly in root development. Among single-strain treatments, PT 7 (2.0 mL *C. thiooxidans*) achieved the highest total plant length (42.5 ± 4.1 cm; +51.7%) and root length (15.0 ± 0.9 cm; +150.0%), indicating a strong role of sulfur-oxidizing bacteria in stimulating belowground growth (Figure 4). Similarly, increasing concentrations of *R. pusense* (PT 3 to PT 5) correlated with steady improvements in overall plant size highlighted its nitrogen-fixing potential.

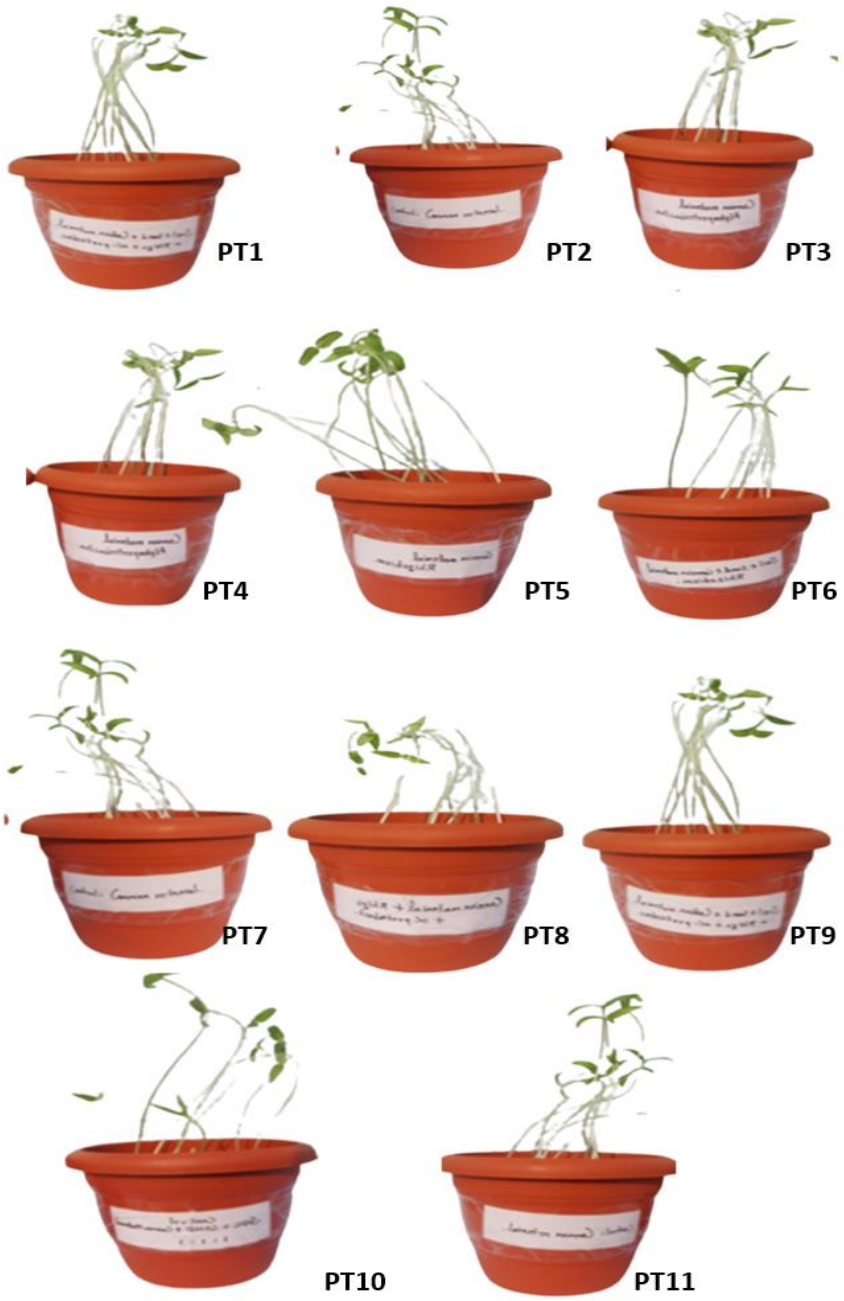

Figure 4: Plant growth observed after 20 days of sowing seed

Table 3
Plant height and growth rates observed after 5 days of growth- Small Scale Experiment

Plant Type	Shoot Length (cm)	Root Length (cm)	Total Length (cm)
ST 1 (Control)	10.0 ± 1.4	4.0 ± 0.6	14.0 ± 1.9
ST 2	11.0 ± 1.1 (+10%)	4.5 ± 0.3 (+12.5%)	15.5 ± 1.0 (+10.7%)
ST 3	13.0 ± 1.4 (+30.0%)	5.0 ± 0.7 (+25.0%)	18.0 ± 2.0 (+28.6%)
ST 4	15.0 ± 2.2 (+50.0%)	6.0 ± 0.8 (+50.0%)	21.0 ± 1.9 (+50.0%)
ST 5	14.0 ± 0.8 (+40.0%)	5.5 ± 0.5 (+37.5%)	19.5 ± 2.2 (+39.3%)
ST 6	9.0 ± 1.6 (−10.0%)	3.0 ± 0.4 (−25.0%)	12.0 ± 2.3 (−14.3%)
ST 7	11.0 ± 1.2 (+10.0%)	3.5 ± 0.9 (−12.5%)	14.5 ± 1.8 (+3.6%)
ST 8	12.0 ± 1.3 (+20.0%)	4.5 ± 0.7 (+12.5%)	16.5 ± 1.5 (+17.9%)
ST9	11.0 ± 1.8 (+10.0%)	4.0 ± 1.0 (0%)	15.0 ± 1.2 (+7.1%)

Note: Per cent values in parentheses are growth rate; p=<0.05 for all treatments

Table 4
Plant height and growth rates observed after 20 days of growth-Pilot Study

Plant ID	Shoot Length (cm)	Root Length (cm)	Total Length (cm)
PT 1	22.0 ± 2.4	6.0 ± 0.7	28.0 ± 2.3
PT 2	24.1 ± 1.3 (+9.5%)	6.0 ± 0.6 (0%)	30.0 ± 3.5 (+7.1%)
PT 3	25.0 ± 3.1 (+13.6%)	7.0 ± 0.9 (+16.7%)	32 ± 2.1 (+14.3%)
PT 4	26.5 ± 2.6 (+20.5%)	9.0 ± 0.8 (+50.0%)	35.5 ± 4.4 (+23.2%)
PT 5	27.0 ± 4.2 (+22.7%)	12.0 ± 1.2 (+100.0%)	39.0 ± 2.6 (+39.3%)
PT 6	26.0 ± 1.8 (+18.2%)	12.0 ± 0.4 (+100.0%)	38.0 ± 3.7 (+35.7.0%)
PT 7	27.5 ± 3.7 (+25.0%)	15.0 ± 0.9 (+150.0%)	42.5.0 ± 4.1 (+51.7%)
PT 8	25.5 ± 2.1 (+15.9%)	13.0 ± 1.1 (+116.7%)	38.5 ± 2.5 (+37.5%)
PT 9	25.5 ± 4.6 (+15.9%)	14.0 ± 0.5 (+133.3%)	39.5 ± 4.0 (+41.0%)
PT 10	26.5 ± 3.3 (+20.5%)	20.0 ± 1.2 (+233.3%)	46.5 ± 3.8 (+66.0%)
PT 11	26.0 ± 2.7 (+18.2%)	16.0 ± 0.6 (+166.7%)	42.0 ± 2.9 (+50.0%)

Note: Per cent values in parentheses are growth rate; p=<0.05 for all treatments

The dual inoculation treatments (PT 9–11) demonstrated the most pronounced growth responses. Notably, PT 10 (1.0 mL each of *R. pusense* and *C. thiooxidans*) recorded the greatest total plant length (46.5 ± 3.8 cm; +66.0%) and a 233.3% increase in root length, underscoring the synergistic effect of co-inoculation. This suggests a complementary mechanism where nitrogen fixation, sulfur mobilization and enhanced phytohormone production collectively optimize nutrient uptake and stimulate vigorous growth[21,23]. These findings affirm the potential of tailored biofertilizer blends to significantly improve legume productivity sustainably.

Physico-chemical characteristics of biofertilizer formulation: Bioformulation PT10 physicochemical characteristics were evaluated and it was found that they broadly align well with the Fertilizer Control Order (FCO) guidelines[11] and standards for kitchen compost and organic manure. The pH of 7.55 is within the acceptable range for compost and organic manure (6.0–8.0 and 6.5–7.5 respectively), indicating a neutral to slightly alkaline environment favourable for microbial activity and plant growth[15]. The moisture content (14%) is slightly below ideal levels (15–25%), which might affect microbial viability during storage and suggests a need for moisture adjustment. Low electrical conductivity (0.45 dS/m) suggests minimal salinity risk, promoting safer soil application (Table 5). The particle size passing through a 4 mm sieve with >90% efficiency and bulk density of 0.69 g/cm³ is within the recommended ranges, which supports good aeration and handling properties[21].

Nutrient analysis shows that PT10 formulation met kitchen compost standards but is lower than vermicompost and organic manure standards for essential nutrients, micronutrients and macronutrients (Table 6). Primary macronutrient content (NPK total 1.30%) meets minimal kitchen compost requirements but is insufficient compared to vermicompost and organic manure benchmarks, suggesting that the formulation might be more suited as a basic soil amendment rather than a high-nutrient biofertilizer. Secondary nutrients like calcium and sulfur are present in adequate but modest amounts[15]. Heavy metals were all well below FCO limits, reflecting product safety. Elevated iron and manganese, while unregulated, could be beneficial in trace amounts for plant nutrition[18].

Hence, the formulated biofertilizer (PT10) meets the quality criteria for kitchen compost under FCO[11] particularly for physical parameters and basic nutrient content. However, it does not fulfil the stricter nutrient and moisture requirements for vermicompost or organic manure categories[4]. Nonetheless, the absence of pathogens and toxic heavy metals indicates that it is safe and beneficial for agricultural application, especially for organic farming[22].

Table 5
Physical and Chemical parameters of formulated biofertilizer with FCO[11] Standard Values

Parameters	Tested Values	FCO Standard Values for Kitchen Compost	FCO Standard Values for Vermicompost	FCO Standard Values for Organic Manure
pH	7.55	6-8	-	6.5- 7.5
Color	Light Red	Red to Black	-	-
Odour	None	There should be no odour	There should be no odour	There should be no odour
Conductivity (dsm $^{-1}$)	0.45	<6	-	<5
Particle size after passing through 4mm sieve	Passes	Minimum 90% material should pass through the pores of the seive	Minimum 90% material should pass through the pores of the sieve	Minimum 90% material should pass through the pores of the sieve
Bulk Density (g/cm^3)	0.69	<1.20	0.7 – 0.9	<1
Pathogens Present	None	No pathogens to be present	No pathogens to be present	No pathogens to be present

Table 6
Percentage of Important Nutrients in Biofertilizer with FCO (1989) Standard Values

Parameters (%)	Tested Value (%)	FCO Standard Values (%) for Kitchen Compost	FCO Standard Values (%) for Vermicompost	FCO Standard Values (%) for Organic Manure
Essential Nutrients				
C	13.39	> 12	>18	>14
Moisture (H, O_2)	14	15 – 25	15-25	25
Primary Macro Nutrients				
N	1.09	A sum total of N,P,K should not be less than 1.0%	A sum total of N,P,K should not be less than 2.5%	A sum total of N,P,K should not be less than 2.0%
P	0.03			
K	0.18			
Secondary Macro Nutrients				
Ca	0.26	-	-	-
S	0.04	-	-	-
Micro Nutrients				
Ni	22.93	<50	<50	<50
Cd	ND	<5	<5	<5
Cr	3.86	<50	<50	<50
Cu	12.36	<300	-	<300
Zn	-	<1000	-	<1000
Fe	12457.96	-	-	-
Mn	77.66	-	-	-
Heavy Metals				
Pb	14.25	<100	<100	-

Conclusion

This study successfully isolated and characterised *Rhizobium pusense* and *Ciceribacter thiooxidans* from legume rhizospheres and demonstrated their potential as biofertilizer formulation for *V. radiata*. Both microorganisms significantly enhanced plant growth with co-inoculation showing superior effects compared to single-strain treatments. The dual consortium promoted root elongation, shoot growth and overall biomass, owing to complementary mechanisms of nitrogen fixation and sulfur oxidation. Physicochemical analysis confirmed that the formulated biofertilizer (PT10) met essential quality and safety standards, making it suitable for organic farming as kitchen compost. The findings suggest that microbial consortium-based biofertilizers can reduce dependency on chemical fertilizers, can improve soil fertility and can support sustainable crop production. Further research on field-scale trials, storage stability and long-term soil impact is recommended to validate commercial applicability.

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(Received 18th August 2025, accepted 11th October 2025)