Research Article

SURVEY, ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF RHIZOBIUM ISOLATES FROM COWPEA (*VIGNA UNGUICULATA* L.)IN FARMERS' FIELDSAT THIRUVALLUR, TAMIL NADU AND ITS EFFECT ON GROWTH AND YIELD OF COWPEA

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Abstract

The present investigation was undertaken to assess the diversity, biochemical traits, and field efficacy of *Rhizobium* isolates associated with cowpea (*Vigna unguiculata* L.) root nodules in Thiruvallur district, Tamil Nadu. A survey across four locations (Thiruvalangadu, Chakaramanallur, Pallipattu, and Kanagamachathiram) revealed significant variation in *Rhizobium* populations, with densities ranging from 18.0 × 10^3 to 25.5 × 10^3 CFU/g of soil, and Kanagamachathiram recording the highest population. All isolates exhibited typical Gram-negative, rod-shaped, mucoid colonies and were positive for starch and gelatin hydrolysis but negative for the Voges–Proskauer test, with variability noted in citrate utilization. Field evaluation demonstrated that Rhizobium inoculation significantly improved plant growth, nodulation, dry matter production, and yield attributes over uninoculated and fertilizer controls. Among the treatments, 75% recommended nitrogen + Rhizobium inoculation (T_2) consistently outperformed others, recording the highest pod yield per plant (37.57 g), followed by 50% nitrogen + Rhizobium (T_3). The control (C_1) recorded the lowest values across all traits, while chemical fertilizer control (C_2) showed intermediate responses. The results underscore the potential of native Rhizobium isolates as effective biofertilizers for cowpea, enabling partial substitution of chemical nitrogen inputs while maintaining high productivity. This study highlights the dual importance of Rhizobium in improving cowpea yield and promoting sustainable soil fertility management in Tamil Nadu.

Keywords: Vigna unguiculata, Rhizobium, biological nitrogen fixation, biofertilizer, nodulation, cowpea yield, sustainable agriculture.

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Introduction

Cowpea (*Vigna unguiculata* L.) is one of the most ancient human food sources which has been used as a crop from Neolithic period (Summerfield *et al*., 1976). The global area of cultivation of cowpea is 151.9 lakh hectares (1,51,90,896 ha) with production of 97.75 lakh tonnes (97,74,865.72 t) and productivity of 943.5 kilogram per hectare (643.5 kg/ha), as of 2023 (FAO STAT, 2025).). It is not only a low-cost source of plant-derived proteins but also sources of plant-based amino acids and nutrients globally. The protein of cowpea grain is comparable to that of soybean with 250 mg/g of grain. In addition to its dietary importance, cowpea improves soil fertility by fixing atmospheric nitrogen through a symbiotic association with Rhizobium bacteria, thereby reducing dependence on costly chemical fertilizers (Vinay *et al*., 2022).

Nitrogen is one the essential elements for all organisms. Even though, the nitrogen concentration is abundant in the atmosphere (~78%), its amount in soil, crust rocks and sea water are comparatively low. Terrestrial plants primarily absorb nitrogen from the soil through their roots, although foliar uptake is also possible when compounds such as urea are applied as sprays. Since atmospheric nitrogen (N_2) cannot be directly utilized by plants, its availability largely depends on specific microbial associations. Leguminous plants, in particular, form symbiotic relationships with rhizobia, while some non-leguminous plants associate with diazotrophic endophytes to access biologically fixed nitrogen. Despite the fact that soil organic matter represents the largest reservoir of nitrogen, plants are unable to directly assimilate it. Instead, inorganic nitrogen forms become available through the mineralization of organic matter, decomposition of organic residues, and the addition of chemical fertilizers (Ohyama*et. al*., 2010). The heavy reliance on chemical fertilizers to achieve higher yields is costly and poses serious risks to environmental sustainability, while also contributing to global warming. Biological nitrogen fixation (BNF) is an economical and eco-friendly process that supplies plants with nitrogen by converting atmospheric N_2 into forms they can utilize. Rhizobial strains establish symbiotic relationships with legumes and facilitate the conversion of atmospheric nitrogen into ammonium (NH_4^+) within specialized structures known as root or stem nodules. This symbiotic association not only enhances the growth and productivity of the host legume but also improves soil fertility, benefiting subsequent crops cultivated in the same field. (Nagalingam*et al*., 2020).

The *rhizobia* represent the most significant group of nitrogen-fixing soil bacteria, forming mutualistic symbiotic associations with legume roots through nodule development. By enhancing the organic nitrogen pool, *Rhizobium* contributes greatly to sustainable crop production. The success of biological nitrogen fixation and subsequent yield improvement in legumes largely depends on the compatibility between the Rhizobium strain and the host plant genotype, as well as the influence of soil conditions and agronomic practices. In cowpea (*Vigna unguiculata* L.), this interaction is particularly critical, since the crop is often cultivated in low-input systems where chemical fertilizers are limited, making *Rhizobium* symbiosis a key contributor to sustainable productivity. Moreover, *Rhizobium* inoculation has been consistently reported to improve cowpea growth, biomass accumulation, pod yield, and protein content by enhancing nodulation and nitrogen fixation. The integration of *Rhizobium* with other biofertilizers such as cyanobacteria has also shown positive effects on legume yield, offering promising avenues for improving cowpea productivity while reducing reliance on synthetic nitrogen fertilizers (Shahrajabian*et. al.*,.2021).

The root nodules of legumes are predominantly inhabited by bacterial species such as *Rhizobium*, *BradyRhizobium*, *AzoRhizobium*, *MesoRhizobium*, *AlloRhizobium*, and *SinoRhizobium*. In addition, certain rhizobacteria, known as plant growth-promoting (PGP) bacteria, enhance plant development through multiple mechanisms, including the production of growth regulators, improved nutrient availability, stimulation of root exudation, and suppression of phytopathogens. These PGP bacteria actively colonize plant roots, ultimately contributing to increased plant growth and yield. Rhizobia are capable of promoting the growth of non-leguminous plants both directly and indirectly, particularly by suppressing or eliminating harmful microorganisms through the production of antibiotics and hydrogen cyanide. Owing to these traits, rhizobia hold significant potential as biofertilizers and as biological control agents against plant pathogens. In recent decades, the emphasis on eco-friendly and sustainable agricultural practices has intensified, thereby highlighting the importance of biofertilizers like rhizobia, which can reduce dependency on chemical fertilizers while minimizing their negative environmental impacts (Nagalingam*et al*., 2020).

Despite the known benefits of rhizobial inoculation, variability in strain performance remains a major challenge, as not all isolates confer the same level of nitrogen fixation or growth promotion. For this reason, screening and evaluating different Rhizobium isolates for cowpea production is essential to identify the most promising strains under local agroecological conditions (Kumari *et. al*., 2025). Such efforts not only improve yield but also contribute to sustainable agriculture by minimizing synthetic nitrogen fertilizer use and maintaining soil health.

The present study the diversity, population density, and biochemical traits of *Rhizobium* isolates from cowpea root nodules across four locations in Thiruvallur district, Tamil Nadu, and its effect on the growth and yield of cowpea, with the aim of identifying potential strains that could serve as effective biofertilizers to improve cowpeayield, to recommended for biofertilizer development and support sustainable agricultural practices.

Materials and Methods

A survey was carried out in four locations in Thiruvallur district, Tamil Nadu (Figure 1). The locations are Thiruvalangadu (13.13129226318784°N, 79.77391071421451°E), Chakaramanallur (12.86056025684469°N, 79.4121104680451°E), Pallipattu (13.337290157247661°N, 79.4396838345508°E), and Kanagamachathiram (13.206680959229423°N, 79.74866779377301°E) during April, 2023. From each selected village, a cowpea farmer's field was selected. From each farmer's fields, five healthy cowpea plants were uprooted at flowering stage, and intact root nodules were carefully collected. Nodules were stored in sterile polythene bags, labelled, and transported to the laboratory under refrigerated conditions.

The Rhizobia was isolated from the nodules with procedure given by Beck *et. al.*, (1993) in three replicates. Ten pink nodules of cowpea plants from each of the sampling site, were sterilized with 95% ethyl alcohol and washed with sterile distilled water several times to remove the sterilization residue. Sterilized nodules were transferred by sterile forceps to sterile test tubes with two drops of sterile distilled water, and then nodules were crushed at the bottom of the tube by end of a sterile glass rod until the slurry was formed. A small amount of slurry was taken with a sterile inoculation loop and streaked on surface of culture medium of Yeast Extract Mannitol Agar (YEMA) (Rao and Sinha, 1963) (Table 1) containing Congo red stain and the plates were incubated inverted at 30℃ for 3-7 days (Figure 2). The emergence of mucous white colonies on surface of culture media is an indication of the presence of the presence of *Rhizobium* colonies. *Rhizobium* colonies were purified by re-cultivation on the same medium (YEMA), then colonies are purified on slant nutrient medium and were preserved at 40℃ for subsequent studies.

Figure 1: Survey locations in Thiruvallur district, Tamil Nadu.

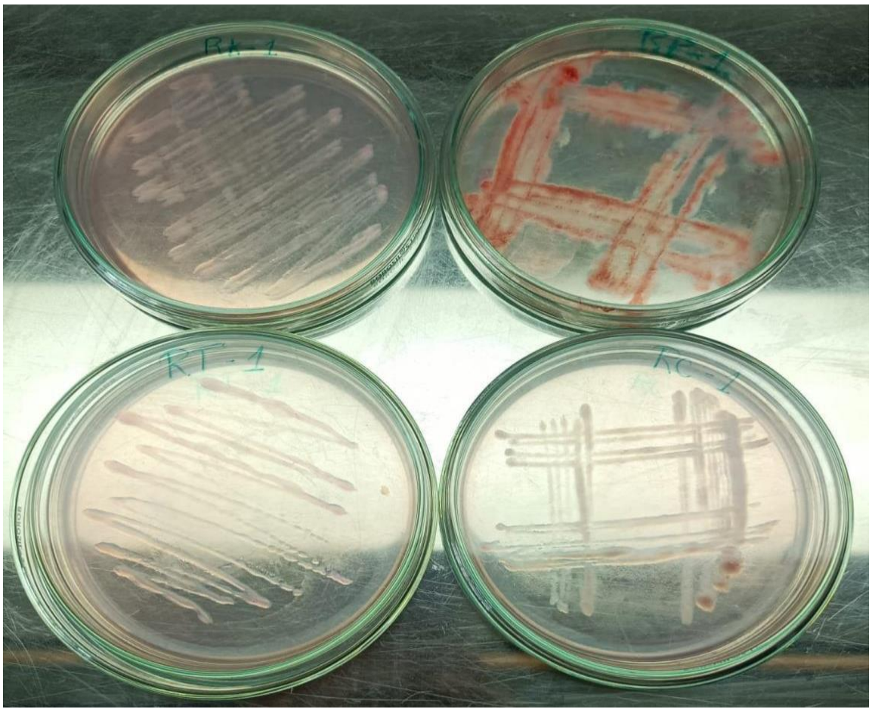

Figure 2: *Rhizobium* culture plates from four different locations.

Table 1: Yeast Extract Agar Mannitol medium.

Ingredient	Quantity
Yeast Extract	1g
Mannitol	10g
Dipotassium phosphate	0.5g
Sodium Chloride	0.1g
Magnesium Chloride	0.2g
Agar	20g
Congo Red	0.5g
Distilled water	1000ml
pH	6.8

Rhizobium populations from cowpea root nodules were estimated using the serial dilution plate method described by Sperber (1958). Soil samples were serially diluted up to 10^{-4}, and 1 ml of the final dilution was spread onto Yeast Extract Mannitol Agar (YEMA) medium. The inoculated plates were incubated at 28 ± 2°C for up to two weeks, after which the resulting bacterial colonies were counted and expressed as colony-forming units (cfu) per gram of moisture free soil, based on the formula,

$$\textbf{Microbial population} = \frac{(\textbf{No.of colonies counted})\ \textbf{x}\ (\textbf{Dilution factor})}{\textbf{Volume plated}\ (\textbf{ml})}\ \textbf{cfu/g}$$

The *Rhizobium*isolates were subjected to a series of standard biochemical tests (Table 2) to confirm their identity and metabolic traits.

Table 2: Biochemical tests for characterization of *Rhizobium*

S. No.	Biochemical test	Reference
1.	Gram reaction – Hucker's modification	Rangaswami (1975)
2.	Hydrolysis of starch	Stolpe and Godkeri (1981)
3.	Hydrolysis of gelatin	Stolpe and Godkeri (1981)
4.	Voges-Proskauer test	Aneja (1993)
5.	Citrate utilization test	Aneja (1993)

To investigate the effect of isolated *Rhizobium*on growth and yield of cowpea, Ankur Hybrid cowpea was used, whose seeds were obtained from a local authenticated input store. The experimental was laid out in Jaya Agricultural College field at Vyasapuram in Thiruvallur, Tamil Nadu. The field is located at an altitude of 84 meters above the mean sea level with a geographical bearing at 13.07°N latitude and 79.65°E longitude. The average rainfall of the district is 1104.4 mm of which the North East monsoon contributes to the tune of 690 mm. The average temperature of the district ranges from 18.5 °C to 37.9°C. The experiment was laid out in Randomized Complete Block Design (RCBD) with six treatments (Table 3) and three blocks. The surface sterilized seeds were soaked in 48 hours old *Rhizobium* cultures for 30 minutes, shade-dried and then sown in plots of size 2.5 x 2 m^2.

Table 3: Description of treatments.

S. No.	Treatment	Description
1.	C_1 (Control 1)	No biofertilizers and chemical fertilizers
2.	C_2 (Control 2)	Recommended doses of chemical fertilizers
3.	T_1	*Rhizobium* only
4.	T_2	75% N + *Rhizobium*
5.	T_3	50% N + *Rhizobium*
6.	T_4	25% N + *Rhizobium*
7.	T_5	10% N + *Rhizobium*

Note: *All the treatments except C_1 (Control) were given recommended doses of P and K.*

The traits recorded for the study were plant height (cm) at 30 and 60 DAS (PH -30 and PH – 60), dry matter production (t/ha) (DMP), number of nodules per plant at 30 and 60 DAS (NP – 30 and NP – 60), number of pods per plant (PP) and pod yield per plant (g/plant) (PYP). From each plot, five plants were randomly selected for data collection and mean value was used in the analysis. The mean sum of squares from ANOVA was calculated by Agricolae package (de Mendiburu et. al., 2023) in R-studio v4.3.2 (R Core Team, 2023).

A clustered heat map was generated to visualize the relationships among the cowpea growth and yield traits across treatments using Pheatmap package (Kolde, 2025) in R-studio v4.3.2 (R Core Team, 2023).

Results and Discussion

The Mean Sum of Squares (MSS) from ANOVA (Table 4) revealed that the *Rhizobium* population had significant difference based on the location of survey. The critical difference (CD) at 5% level of significance was calculated to be 0.471.

Table 4: Mean Sum of Squares (MSS) from ANOVA.

Sources of Variation	Degrees of freedom	Mean Sum of Squares (MSS)
Location	3	29.797***
Error	8	0.062
Critical Difference (CD) at 5%		0.471

'***' – Significant at 0.1%.

The mean *Rhizobium* population recorded in the four different locations of Thiruvallur district were tabulated (Table 5 and Figure 3). The mean population is estimated to be 21.563 cfu x 10^3/g of moisture free soil. Among the four locations, the *Rhizobium*from Kanagamachathiram had the highest population (25.5 cfu x 10^3/g), while the *Rhizobium* from Pallipattu had the lowest population (18 cfu x 10^3/g).

Table 5: Mean *Rhizobium* population recorded at the survey locations**.**

S. No.	Location	*Rhizobium* population (cfu X 10^3/g of moisture free soil)
1.	Thiruvalangadu[b]	22.25
2.	Chakaramanallur[c]	20.50
3.	Pallipattu[d]	18.00
4.	Kanagamachathiram[a]	25.50
Mean		**21.563**

Locations with same letter are not significantly different.

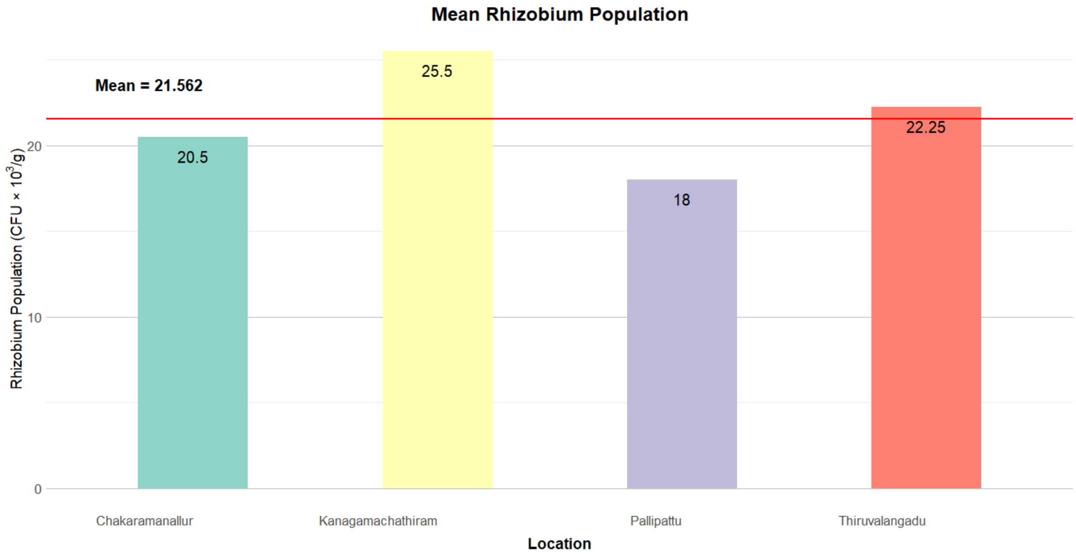

Figure 3: Rhizobium population in different locations**.**

The cowpea root nodule isolates from four different locations were authenticated as *Rhizobium* sps. through confirmative tests – gram reaction (Figure 4), hydrolysis of starch (Figure 4), hydrolysis of gelatin (Figure 4), Voges-Proskauer test and Citrate utilization test and the results are tabulated (Table 6). The hydrolysis of starch test resulted in formation of colourless halo around the growth and presence of blue colour in the remaining area of the plates showed utilization of starch. The hydrolysis of gelatin test resulted in development of yellow halo around the growth indicated utilization of gelatin.

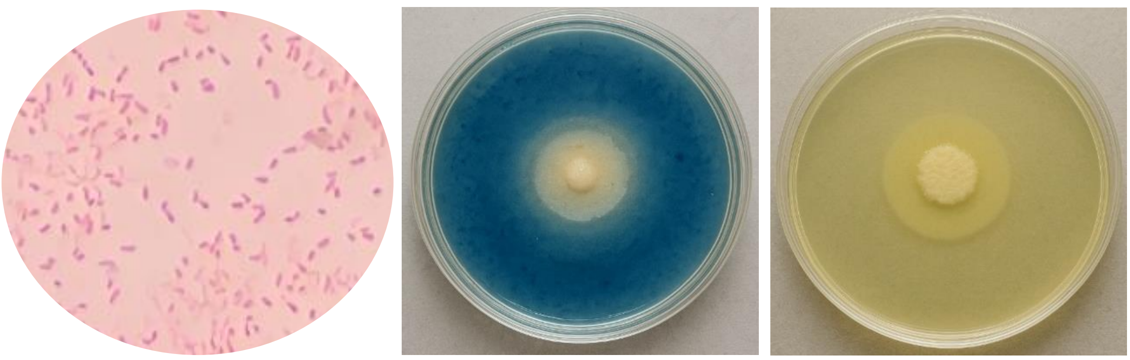

Figure 4: Test results of gram staining, hydrolysis of starch and gelatin tests.

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Table 6: Morphological and biochemical characterization of the isolated.

S. No.	Isolate location	Morphology	Shape	Gram reaction	Voges-Proskauer test	Citrus Utilisation test
1.	Thiruvalangadu	Translucent and mucoid	Rod	-	-	+
2.	Chakaramanallur	Translucent and mucoid	Rod	-	-	-
3.	Pallipattu	Translucent and mucoid	Rod	-	-	+
4.	Kanagamachathiram	Translucent and mucoid	Rod	-	-	+

The MSS from ANOVA (Table 7) showed that the treatments were significantly different for all the traits at 0.1% level of significance, while the replications and errors were non-significant for all the traits.

Table 7: Mean Sum of Squares (MSS) from ANOVA for growth and yield traits of cowpea.

Source of Variation	Degrees of freedom (df)	PH – 30 DAS	PH – 60 DAS	DMP (t/ha)	NP – 30 DAS	NP – 60 DAS	PP	PYP (g/plant)
Replication	2	4.08	1.61	0.08	13.76	0.62	0.571	2.23
Treatment	6	43.73***	50.03***	32.56***	202.19***	184.54***	26.27***	283.49***
Error	12	1.55	0.69	0.20	19.10	0.23	1.46	0.42
Mean Significant Difference (MSD) at 5%		3.563	2.371	1.270	12.487	1.371	3.453	1.847

'***' – Significant at 0.1%.

As the ANOVA revealed significant differences among the treatments, Tukey's HSD test (Tukey, 1949) was used to estimate the Mean Significant Difference (MSD) to separate the treatment means (Table 8).

Significant differences were observed among treatments for all measured traits (Table 3). At 30 DAS, plant height ranged from 36.5 cm in C_1 to 48.4 cm in T_2. Treatments T_2 and T_3 recorded the tallest plants (48.4 cm and 45.9 cm, respectively), significantly higher than the control C_1, while C_2 showed an intermediate response. A similar trend was evident at 60 DAS, with T_2 (66.9 cm) and T_3 (65.0 cm) maintaining superiority, whereas C_1remained lowest (54.2 cm). Dry matter production followed the same pattern, where T_2 (22.1 t/ha) was markedly superior to all treatments, while C_1 and T_4 recorded the lowest biomass values. For nodules production, both at 30 and 60 DAS, T_2 produced the highest number of nodules (45.0 and 44.7, respectively), followed closely by T_3, while C_1 produced the fewest. Pod production and pod yield per plant were also maximized in T_2 (25.7 pods and 37.6 g), statistically comparable with T_3, but significantly higher than both controls. The control C_1 consistently exhibited the lowest performance across all traits, while C_2 remained moderate and statistically comparable with intermediate treatments (T_1, T_4, and T_5). Overall, T_2 proved to be the most effective treatment, consistently outperforming both controls and other treatments across growth, biomass, and yield attributes, while T_3 ranked second in performance.

Table 8: Treatment means for growth and yield traits of cowpea.

Treatment	PH – 30 DAS	PH – 60 DAS	DMP (t/ha)	NP – 30 DAS	NP – 60 DAS	PP	PYP (g/plant)
C_1	36.5^{c}	54.23^{d}	12.63^{c}	21.33^{c}	21.67^{g}	17.33^{d}	26.03^{f}
C_2	45.07^{ab}	62.50^{b}	16.90^{b}	31.00^{bc}	30.67^{f}	23.67^{ab}	33.93^{bc}
T_1	41.63^{b}	59.33^{c}	17.03^{b}	31.00^{bc}	36.67^{d}	20.00^{cd}	28.53^{e}
T_2	48.37^{a}	66.90^{a}	22.10^{a}	45.00^{a}	44.67^{a}	25.67^{a}	37.57^{a}
T_3	45.9^{a}	64.97^{a}	17.63^{b}	42.67^{ab}	43.00^{b}	24.00^{ab}	35.23^{b}
T_4	42.27^{b}	62.10^{b}	12.37^{c}	39.67^{ab}	38.33^{c}	22.00^{bc}	33.10^{c}
T_5	41.83^{b}	60.57^{bc}	16.57^{b}	32.67^{abc}	33.33^{e}	19.33^{cd}	31.10^{d}

Treatments with same letters are not statistically significant.

The clustered heatmap (Figure 2) provided a clear visualization of treatment effects on cowpea growth and yield traits. C_1, which received neither biofertilizers nor chemical fertilizers, clustered at the lower end of the scale, with predominantly green shades across traits. In contrast, C_2, which received the recommended dose of chemical fertilizers, formed a separate cluster characterized by red shades, representing consistently higher values. Among the biofertilizer-based treatments, T_1 (*Rhizobium* only) grouped closer to C_1, showing only modest improvements. However, as nitrogen supplementation increased in T_2–T_5, a gradient emerged: T_2 (75% N + *Rhizobium*) performed closest to C_2, T_3 was intermediate, while T_4 and T_5 shifted closer to T_1 with relatively lower trait values. Overall, the heatmap separated treatments into a high-performance cluster (C_2 and T_2) and a low-performance cluster (C_1, T_1, T_4 and T_5), with T_3 occupying an intermediate position.

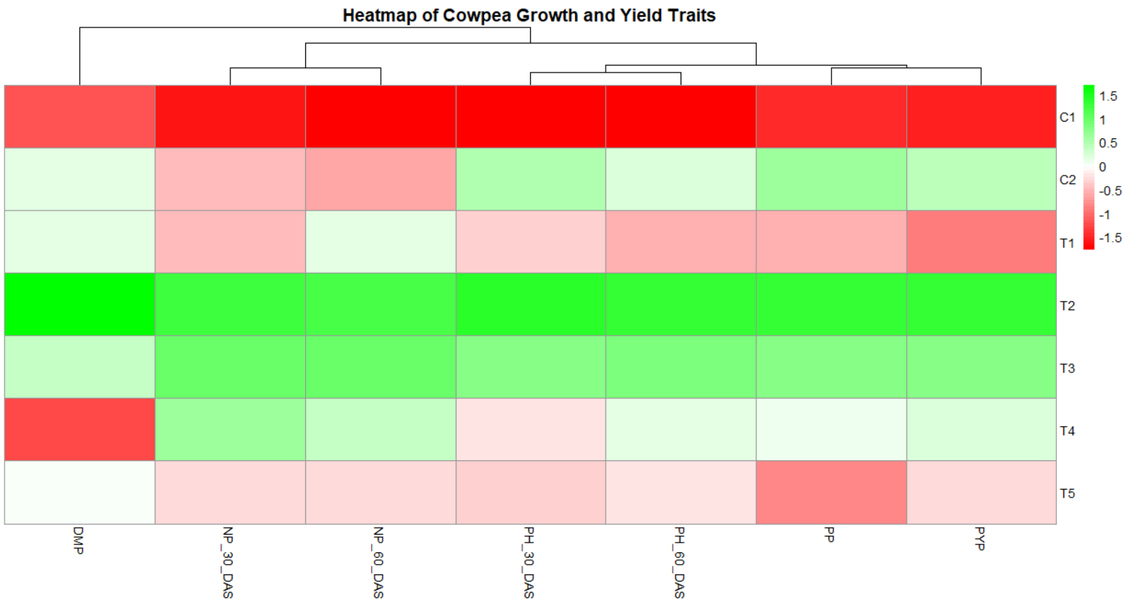

Figure 5: Clustered heatmap of growth and yield traits of cowpea.

Conclusion

The present study revealed considerable variation in the native *Rhizobium* populations associated with cowpea root nodules across four locations in Thiruvallur district, Tamil Nadu, with Kanagamachathiram recording the highest density and Pallipattu the lowest. Morphological and biochemical characterization confirmed all isolates as *Rhizobium*, exhibiting typical Gram-negative, rod-shaped, mucoid colonies with consistent enzymatic profiles, though variation was observed in citrate utilization. Field evaluation further demonstrated that *Rhizobium* inoculation significantly enhanced cowpea growth, nodulation, biomass, and yield performance compared to uninoculated and fertilizer controls. Among the treatments, 75% recommended nitrogen + Rhizobium inoculation proved most effective, followed by 50% nitrogen + Rhizobium, underscoring that partial substitution of chemical nitrogen with bioinoculants not only sustains productivity but also reduces fertilizer dependence. Overall, the findings highlight the diversity and effectiveness of native Rhizobium strains as promising biofertilizers, offering a sustainable and environmentally friendly approach to enhance cowpea production and soil fertility in Tamil Nadu.

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