



RESEARCH ARTICLE

Improved lentil production and revitalised soil health using liquid formulation of *Rhizobium leguminosarum* and *Bacillus tequilensis* in the Indo-Gangetic Plain

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Abstract

The use of bacterial inoculants as biofertilisers has gained considerable attention in agriculture due to their ability to enhance crop productivity and improve soil fertility. However, the limited shelf life of liquid-based bacterial formulations remains a major constraint to their commercialisation and widespread adoption. To address this challenge, the present study evaluated the effect of different additives on the shelf life of newly developed liquid bacterial formulations and assessed their impact on lentil growth, microbial activity and soil health. *In vitro* experiments were conducted to determine the influence of various additives on the survival of two bacterial strains, *Rhizobium leguminosarum* bv. *viciae* DPR2 and *Bacillus tequilensis* DPB4. Among the tested combinations, the formulation containing 1.5–2 % polyvinyl pyrrolidone (PVP) supplemented with 1.5 % cassava starch proved to be optimal, significantly enhancing bacterial viability during storage. A field experiment was subsequently carried out to evaluate the agronomic performance of co-inoculation using both bacterial strains in the optimised liquid formulation and to assess its influence on soil microbial activity. The application of liquid biofertiliser treatment (T6), comprising both bacterial strains in the optimised formulation (1.5–2 % PVP + 1.5 % cassava starch), significantly improved plant height, nodule number and other growth attributes of lentil. Furthermore, key soil health parameters including available nitrogen (N), phosphorus (P) and potassium (K), soil microbial biomass carbon (SMBC), CO₂ evolution and dehydrogenase activity were significantly enhanced compared to charcoal-based inoculation and the uninoculated control, indicating increased soil microbial activity. Overall, the findings demonstrate that the optimised liquid formulation (1.5–2 % PVP + 1.5 % cassava starch) effectively extends shelf life and improves the performance of co-inoculated bacterial strains, leading to enhanced lentil growth, soil nutrient availability and microbial activity. These results highlight the potential of this formulation for sustainable agricultural applications.

Keywords: *Bacillus*; cassava starch; co-inoculation; lentil; liquid biofertilisers; microbial activity; polyvinyl pyrrolidone; *Rhizobium*; shelf life

Introduction

In the modern world, the application of chemical-based fertilisers are essential to enhance crop production and ensure a stable supply of food for the rapidly growing human population. Chemical fertilisers undoubtedly have the potential to boost productivity levels. However, their excessive use can cause significant harm to the soil biodiversity and the delicate balance of nutrient levels. This, in turn, can lead to emerging nutrient deficiencies and exacerbate soil physical, chemical and biological processes, ultimately impacting

the health of the soil and its ability to sustain plant growth. Therefore, the application of biofertiliser along with organics, rejuvenates the ecological diversity in most soils (1). However, the effectiveness of biofertilisers is often limited by poor cell viability and persistence in soil and inoculant media. Therefore, there is an urgent need for suitable media for cell stability. Traditionally, pulses are the second most important group of crops after cereals. Pulses are widely recognised for their essential characteristics, including being an excellent source of protein and their ability to fix atmospheric nitrogen (N), a process that significantly enhances soil fertility (2).

In India, lentil (*Lens culinaris* Medik.) is the second most important legume crop after chickpea, grown on 5.21 % of total pulse area (29.03 million ha) and contributing 6.67 % of total pulse production (23.40 MT) in 2018–19. It is a winter-season crop and is especially valued for its high-quality dietary proteins. However, the use of high-yielding cultivars significantly increased the yield of pulse crops (3). In addition, the application of fertilisers can significantly augment the growth and productivity of food crops, but their extensive use poses serious environmental problems and adversely affects soil biodiversity due to their long persistence in soil (4). Therefore, employing microorganisms as potential bioinoculants offers a feasible solution to synthetic fertilisers for the growth of leguminous crops (5). *Rhizobium* is an essential symbiont for lentils and provides biologically fixed N (6). However, other bacteria like *Pseudomonas* (7) and *Bacillus* (8) also played a noteworthy contribution in lentil growth. By combining the symbiotic N₂ fixer *Rhizobium leguminosarum* with two *Pseudomonas* species increased the growth and nutrient uptake (iron (Fe), phosphorous (P) and N) of *L. culinaris*. *Rhizobium* with other bacterial strains, particularly *Bacillus* sp. and *Pseudomonas* sp. which exhibit plant growth promoting traits, has been shown to enhance the agronomic characteristics of lentils (9, 10). In the field, the use of *Rhizobium* as a bioinoculant is expected to address the N requirements for improved lentil yield. Moreover, using *Bacillus* having numerous plant growth enhancing activities, including a prominent P solubilisation trait, promotes plant growth because it forms endospores, which foster bacterial survival in harsh environments.

Plant probiotics can be used to develop biofertilisers that provide an eco-friendly and cost-effective strategy for enhancing crop growth (11). In India, most biofertilisers are developed using solid carriers with a shelf life of no more than six months (12). However, carrier-based formulations are often limited by a shorter shelf life, high risk of contamination, poor survival rates under unfavourable environmental conditions and variable performance in the field. Additionally, the manufacturing process of these formulations, which involves mining, drying, milling and neutralising, as well as sterilisation, formulation and transportation can be expensive. Therefore, there is a need for an alternative to carrier-based inoculants that can extend the viability of microbial cells. One potential approach to address these challenges is the use of liquid formulations, which not only provide the intended microorganisms and nutrients but also enable the inclusion of cell protectants or other additives that can enhance stability and resilience under unfavourable conditions (13). Several additives are frequently used to improve the shelf life of liquid biofertilisers. These include natural polymers, such as alginate, arabic gum, xanthan gum, carrageenan and gelatin, as well as synthetic polymers like polyvinyl pyrrolidone (PVP) and polyvinyl alcohol. Additionally, glycerol, horticultural oil and various types of saccharides, such as monosaccharides (e.g., glucose) and disaccharides (e.g., lactose), can also be used as additives to improve the viability and resilience of microbial cells in biofertiliser formulations (14, 15). *Rhizobium* strains can be used as liquid biofertilisers for pulse crops, as shown by studies conducted over the past few decades (16). However, research conducted on inoculants production and formulation technologies is still limited. There is a need for advancements in biofertiliser technology to enhance the field efficacy and shelf life of biofertilisers. Additionally, it is crucial to make them economically feasible and suitable for farmers.

Based on the aforementioned facts, the present study was carried out with two objectives: (i) to develop liquid biofertiliser formulations of two plant probiotic strains (*Rhizobium leguminosarum* and *Bacillus tequilensis*) using suitable additives and (ii) to evaluate the effect of these liquid formulations on growth, yield attributes of lentil and soil quality in calcareous soils of the Indo-Gangetic Plain region of Bihar.

Materials and Methods

Bacterial strains

Two bacterial strains i.e., *Rhizobium leguminosarum* bv. *viciae* DPR2 and *Bacillus tequilensis* DPB4, used in the present study, were acquired from the Department of Microbiology, College of Basic Sciences and Humanities, Dr. Rajendra Prasad Central Agricultural University, Pusa, Samastipur, Bihar. These bacterial strains have already been annotated for functional plant growth promoting properties.

Preparation of liquid bioformulation

Growth medium

The standard media used were:

Yeast extract mannitol medium (YEM): for *Rhizobium leguminosarum* bv. *viciae* DPR2 (10.0 g/L mannitol; 0.5 g/L K₂HPO₄; 0.2 g/L MgSO₄·7H₂O; 0.1 g/L NaCl; 0.5 g/L yeast extract)

Nutrient yeast salt medium (NYSM): for *Bacillus tequilensis* DPB4 (10.0 g/L glucose; 5.0 g/L peptone; 5.0 g/L NaCl; 3.0 g/L beef extract; 0.5 g/L yeast extract; 0.203 g/L MgCl₂; 0.102 g/L CaCl₂; 0.01 g/L MnCl₂)

Liquid inoculant formulation

Liquid inoculant formulations were modified to assess shelf-life persistence. The experiment was conducted in 125 mL Erlenmeyer flasks containing 50 mL of YEM or NYSM amended with different concentrations of additives as follows:

Glycerol: YEM: 0, 2.0, 4.0 and 6.0 mL

NYSM: 0, 4.0, 8.0 and 12.0 mL

Trehalose: 0, 2.5, 5.0 and 10.0 mM (w/v) for both YEM and NYSM

Polyvinyl pyrrolidone (PVP): YEM: 0, 1.5, 2.0 and 2.5 % (w/v)

NYSM: 0, 1.0, 1.5 and 2.0 % (w/v)

Polyethylene glycol (PEG): 0, 0.25, 0.5 and 1.0 % (w/v) for both media

Late log-phase cultures (approximately 10⁸–10⁹ colony forming unit (CFU) mL⁻¹) were inoculated (1 % v/v) into respective media supplemented with additives. Flasks were incubated at 28 ± 2 °C on a rotary shaker (200 rpm) for 6 days. Viable cell populations were determined by serial dilution and plate count method on respective agar media and expressed as CFU per millilitre.

Shelf life studies of liquid inoculants

Liquid inoculants containing the optimal concentration of each additive (based on highest viable cell counts) were stored at room temperature (25 ± 2 °C). Viable cell counts were evaluated at monthly intervals up to 12 months using the plate count technique.

Selected best performing formulations were further evaluated with natural, low-cost biopolymer stabilising agents:

Cassava starch: 0, 0.5, 1.0 and 1.5 % (w/v)

Potato starch: 0, 0.25, 0.5 and 1.0 % (w/v)

Field experiment

Site description and experimental design

The field trial was conducted during the rabi season of 2019–2020 at Tirhut College of Agriculture, Dholi, Dr. Rajendra Prasad Central Agricultural University (RPCAU), Pusa, Bihar, India.

Date of sowing: 20 November 2019

Date of harvest: 28 February 2020 (100 days after sowing)

Details of the treatments employed during the field trial are presented in Table 1. The experiment was laid out in a randomized block design (RBD) with 4 replications and 6 treatments. Each plot measured 4 × 3 m (12 m²), resulting in 24 total plots.

Fertiliser application

The recommended dose of fertilisers (RDF) for lentil was applied as:

Nitrogen (N): 20 kg ha⁻¹

Phosphorus (P₂O₅): 40 kg ha⁻¹

Potassium (K₂O): 20 kg ha⁻¹

Nitrogen was applied through urea, P through single superphosphate (SSP) and K through muriate of potash (MOP). The entire dose was applied as basal before sowing.

Method of liquid inoculant application

One-week-old liquid inoculant cultures having a cell density of approximately 10⁸ CFU mL⁻¹ were used for seed treatment.

Seeds were coated at the rate of 10 mL liquid inoculant per kg of seed.

A 10 % (w/v) jaggery solution was used as an adhesive.

The required quantity of inoculant was mixed thoroughly with seeds to ensure uniform coating.

Treated seeds were shade-dried for 30 min prior to sowing.

For dual inoculation treatments, equal volumes of each bacterial inoculant were mixed before application.

Preparation of charcoal-based inoculant

Finely powdered, sterilised charcoal (particle size < 212 µm) was used as carrier material.

Table 1. Treatments employed for field trial

Labels	Treatment descriptions
T1	N ₀ PK (Nitrogen omitted control)
T2	Recommended NPK fertiliser
T3	NPK + <i>Rhizobium leguminosarum</i> DPR2 (charcoal-based formulation)
T4	NPK + <i>Rhizobium leguminosarum</i> DPR2 + <i>Bacillus tequilensis</i> DPB4 (charcoal-based formulation)
T5	NPK + <i>Rhizobium leguminosarum</i> DPR2 (liquid formulation)
T6	NPK + <i>Rhizobium leguminosarum</i> DPR2 + <i>Bacillus tequilensis</i> DPB4 (liquid formulation)

Note: N, P and K denote nitrogen, phosphorus and potassium, respectively. N₀PK indicates omission of nitrogen fertilizer while phosphorus and potassium were applied at recommended doses. Recommended NPK fertiliser was applied as per the recommended dose of the crop. CR and CB indicate charcoal-based formulation of *Rhizobium leguminosarum* DPR2 and *Bacillus tequilensis* DPB4, respectively; LR and LB indicate liquid-based formulation of *Rhizobium leguminosarum* DPR2 and *Bacillus tequilensis* DPB4 respectively. The treatments were structured to compare the effects of fertilisation, single inoculation and dual inoculation while simultaneously evaluating the performance of charcoal-based and liquid formulations of microbial inoculants.

Charcoal was sterilised by autoclaving at 121 °C for 20 min on two consecutive days.

The sterilised carrier was mixed with broth culture containing ~10⁹ CFU mL⁻¹ to achieve a final moisture content of 35–40 %.

The final population density was maintained at ≥ 10⁸ CFU g⁻¹ of carrier material.

The mixture was cured under sterile conditions for 24 hr and packed in low-density polyethylene bags.

Charcoal-based inoculant was applied at the rate of 20 g per kg of seed using 10 % jaggery as adhesive.

Biometric observation and yield assessment

At harvest (100 DAS), ten plants were randomly selected from each plot to record:

Number of nodules per plant

Grain yield per plot

Grain N content was estimated using the standard Kjeldahl method illustrated by Bremner and Mulvaney (17).

Soil parameters

Post harvest soil samples were collected from each plot to assess a range of physicochemical characteristics including pH, electrical conductivity (EC), soil organic carbon (SOC) content, N, P, K as well as microbial biomass carbon. The initial physico-chemical and biological properties of the experimental field soil are presented in Table 2. The pH was determined following the procedure given by Bower and Wilcox (18), while EC was measured according to the method described by Jackson (19). The OC content was estimated using the protocol of Walkley and Black (20) and the available of N, P and K was assessed using the protocols of Hanway and Heide (21), Olsen et al. (22) and Subbiah and Asija (23) respectively. To determine the microbial biomass carbon, the chloroform fumigation extraction technique was applied, as per the methods illustrated by Vance et al. (24).

Statistical analysis

Data were analysed using SPSS and R software. One-way ANOVA was performed for RBD with 4 replications. Treatment means were compared using least significant difference (LSD) at $p \leq 0.05$.

Principal component analysis (PCA) was performed using mean-centred data based on eigenvalues to evaluate relationships among treatments and variables. Graphs were generated using R and GraphPad Prism.

Table 2. Physiochemical properties of experimental field soil

Soil characteristics	
Soil Taxonomy	Haplusterts
Texture	Sandy loam
pH	7.92
EC (ds/m)	0.43ds/m
Organic carbon (%)	0.62
N (kg/ha)	216
P(kg/ha)	18.9
K(kg/ha)	146
SMBC (mg/ka)	124
Dehydrogenase activity (µgTPF/g soil/day)	8.7

Values represent the initial physico-chemical and biological properties of the experimental field soil before sowing. EC : Electrical conductivity; N, P and K denote available nitrogen, phosphorus and potassium, respectively; SMBC : Soil microbial biomass carbon.

Table 3. Effect of different chemical additives on the population of *Rhizobium leguminosarum* DPR2 at monthly intervals

Chemical additives	Months				
	1	3	6	9	12
4 mL Gly	1.58×10 ⁸	0.6×10 ⁸	5.7×10 ⁶	0.4×10 ⁵	0.33×10 ³
8 mL Gly	1.66×10 ⁸	0.5×10 ⁸	1.5×10 ⁷	0.61×10 ⁶	2.5×10 ⁴
12 mL Gly	1.72×10 ⁸	1.24×10 ⁸	1.51×10 ⁸	3.51×10 ⁶	1.36×10 ⁴
2.5 mM Treh.	0.15×10 ⁹	1.15×10 ⁷	1.45×10 ⁶	0.45×10 ⁵	0.21×10 ⁴
5 mM Treh.	0.41×10 ⁹	0.98×10 ⁸	0.19×10 ⁷	0.32×10 ⁵	0.55×10 ⁴
10 mM Treh.	0.55×10 ⁹	1.15×10 ⁹	0.71×10 ⁷	0.21×10 ⁵	0.83×10 ⁵
1 % PVP	1.52×10 ⁹	1.50×10 ⁹	0.75×10 ⁸	0.19×10 ⁶	0.55×10 ⁵
1.5 % PVP	1.30×10 ⁹	1.70×10 ⁹	1.70×10 ⁸	2.58×10 ⁷	0.95×10 ⁵
2 % PVP	0.11×10 ¹⁰	0.20×10 ⁹	0.8×10 ⁷	1.10×10 ⁶	0.80×10 ⁵
0.25 % PEG	0.75×10 ⁹	1.51×10 ⁸	0.07×10 ⁸	1.42×10 ⁵	1.11×10 ⁴
0.5 % PEG	0.15×10 ¹⁰	0.9×10 ⁹	1.09×10 ⁷	1.15×10 ⁵	0.5×10 ⁵
1 % PEG	1.06×10 ⁹	1.2×10 ⁸	0.63×10 ⁷	1.31×10 ⁵	1.09×10 ⁴

Each value represents a mean of 3 replications.

Results and Discussion

The effects of different concentrations of four additives to YEM on the final cell concentration of *Rhizobium leguminosarum* DPR2 are shown in Table 3. After 12 months of storage, the viable count of all liquid rhizobia inoculants that were prepared in amended media was found to be higher than that of inoculants prepared in the control medium YEMB. The population of *Rhizobium leguminosarum* bv. *viciae* DPR2 was highest in 1.5 % PVP when compared to other additives. The highest population of *Rhizobium leguminosarum* (1.11×10⁸CFU mL⁻¹) was maintained in the case of PVP (1.5 %) for extended period of time (up to 8 months) and after that, the population declined to 10⁵ CFU mL⁻¹ when determined after 12 months. Similarly, data representing the effect of four additives to NYSM on the final cell population of *Bacillus tequilensis* DPB4 is illustrated in Table 4. The population of *Bacillus tequilensis* DPB4 was highest in 2 % PVP supplementation when compared with others. The highest population of this bacterial strain (1.19 × 10⁸CFU mL⁻¹) was maintained in the case of PVP (2 %) up to 10 months and after that, the population declined to 10⁷ CFU mL⁻¹ when determined at 12 months. Hence, PVP with concentrations of 1.5 % and 2 % was the most suitable supplement for enhancing the shelf life of *Rhizobium leguminosarum* DPR2 and *Bacillus tequilensis* DPB4 with 10⁸ CFU mL⁻¹ which is also the quality standard for liquid formulation of bacterial inoculants. The enhanced survival of bacterial cells in PVP-supplemented media may be attributed to the protective polymeric matrix formed by PVP, which helps maintain cellular hydration and stabilises bacterial metabolism during prolonged storage. (13, 18–26).

The log₁₀ reduction in viable population of *R. leguminosarum* DPR2 after 12 months of storage ranged from approximately 3.8 to 5.7 across treatments, reflecting differential formulation stability (Fig. 1). Although a gradual decline in viable counts was observed in all

treatments, variations in reduction magnitude suggest that certain additives may enhance survival by improving osmotic balance, moisture retention and membrane stabilization during prolonged storage. These observations are consistent with the protective role of osmoprotectants and polymer-based additives in reducing desiccation stress. The graphical summary complements the detailed monthly trends presented in Table 3 and provides a consolidated descriptive comparison of long-term storage performance.

In addition, Tables 5 and 6 illustrate the effect of PVP supplemented with stabilising agent (potato starch at 0, 0.25, 0.5 and 1.0 % (w/v) and cassava starch at 0, 0.5, 1.0 and 1.5 % (w/v)) added to NYSM and YEMB on the final cell population of *R. leguminosarum* DPR2 and *B. tequilensis* DPB4. YEMB supplemented with 1.5 % PVP + 1.5 % cassava starch maintained the highest population of *Rhizobium* inoculant (1.71 × 10⁸CFU mL⁻¹) for 11 months. On the contrary, NYSM supplemented with 2 % PVP+ 1.5 % cassava starch maintained the highest cellular population of *B.s tequilensis* DPB4 (1.50 × 10⁸CFU mL⁻¹) for 12 months. Result clearly indicates that blend of PVP as supplement (1.5 % for *Rhizobium* and 2 % for *Bacillus*) and cassava starch (1.5 %) depicted higher cellular population of bacterial strains. In addition to PVP, the improved viable cell counts may also be attributed to the inclusion of cassava starch, which provides favorable rheological properties, enhances water retention and creates a protective microenvironment that supports microbial stability during storage. Moreover, cassava starch possesses a high molecular weight biopolymer complex that provides stabilising properties. Likewise, cassava starch also contains high water binding ability, which might retain water surrounding the cells for their metabolism (27). Cassava starch also offers important economic and practical advantages for large-scale adoption of liquid biofertilisers. Being an inexpensive and widely

Table 4. Effect of different chemical additives on the population of *Bacillus tequilensis* DPB4 at monthly interval

Chemical additives	Months				
	1	3	6	9	12
4 mL Gly	1.7×10 ⁸	0.8×10 ⁸	6.7×10 ⁶	0.6×10 ⁵	MAP
8 mL Gly	1.76×10 ⁸	0.7×10 ⁸	2.9×10 ⁷	0.86×10 ⁶	3.8×10 ³
12 mL Gly	8.1×10 ⁸	8.7×10 ⁸	6.3×10 ⁸	6.3×10 ⁶	4.6×10 ⁴
2.5 mM Treh.	0.24×10 ⁹	1.64×10 ⁷	1.54×10 ⁶	0.60×10 ⁵	0.33×10 ⁴
5 mM Treh.	0.52×10 ⁹	1.04×10 ⁸	0.29×10 ⁷	0.45×10 ⁵	0.86×10 ⁴
10 mM Treh.	0.68×10 ⁹	1.24×10 ⁹	0.82×10 ⁷	0.33×10 ⁵	0.14×10 ⁵
1 % PVP	1.44×10 ⁹	1.0×10 ⁹	0.73×10 ⁷	0.71×10 ⁶	0.26×10 ⁵
1.5 % PVP	1.64×10 ⁹	1.12×10 ⁹	1.89×10 ⁸	1.230×10 ⁶	0.48×10 ⁵
2 % PVP	0.28×10 ¹⁰	0.4×10 ⁹	3.1×10 ⁸	1.46×10 ⁸	1.24×10 ⁷
0.25 % PEG	0.88×10 ⁹	1.72×10 ⁸	0.13×10 ⁸	1.51×10 ⁵	1.17×10 ⁴
0.5 % PEG	0.22×10 ¹⁰	1.2×10 ⁹	1.12×10 ⁷	1.24×10 ⁵	0.11×10 ⁵
1 % PEG	1.22×10 ⁹	1.2×10 ⁸	0.85×10 ⁷	1.44×10 ⁵	1.11×10 ⁴

Each value represents a mean of 3 replications.

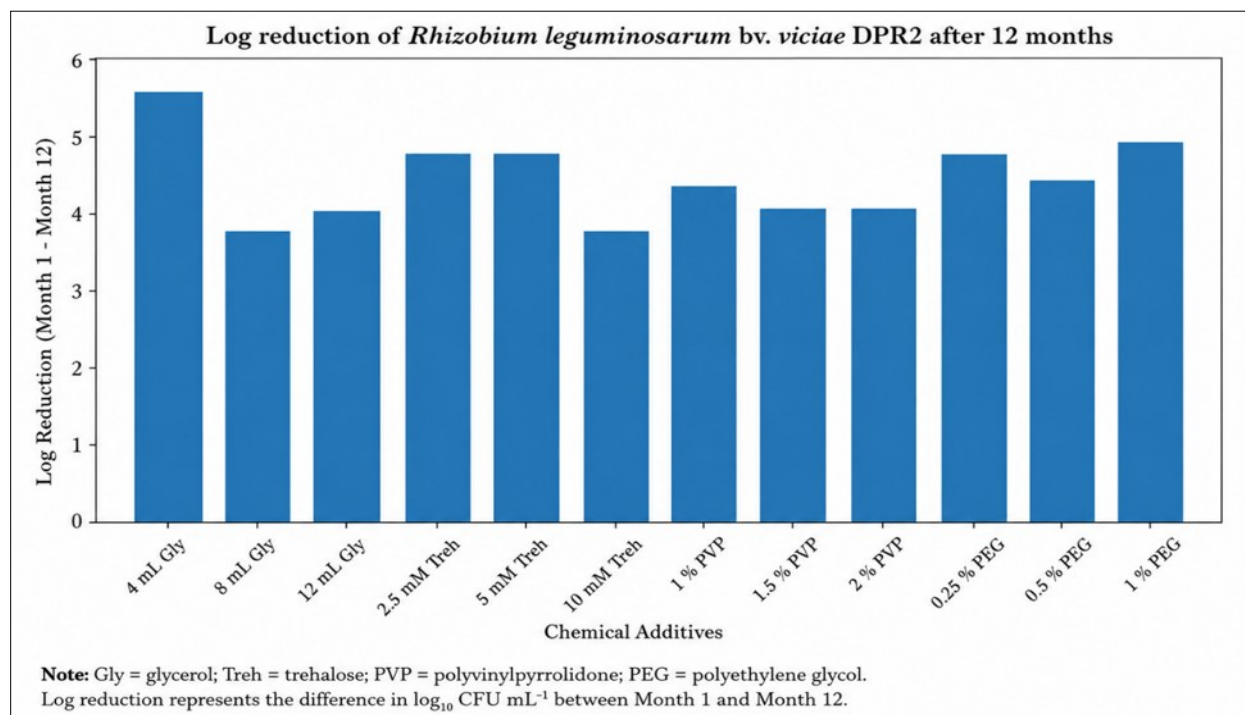


Fig. 1. $\log_{10}$ reduction in viable population of *Rhizobium leguminosarum* DPR2 after 12 months of storage under different chemical additives.

available agricultural product, cassava starch can substantially reduce the formulation cost compared with synthetic stabilisers or commercially refined polymers (Fig. 2 and 3). This makes technology more affordable and accessible for small and marginal farmers, particularly in developing regions where input costs often limit the adoption of microbial inoculants. In addition to its stabilising function, cassava starch improves the viscosity and adhesive properties of liquid formulations, facilitating better seed coating and uniform distribution of microbial cells during seed treatment. The improved shelf life achieved through cassava starch supplementation also enhances the reliability of biofertiliser products during storage and transportation, thereby minimising economic losses associated with reduced microbial viability. Consequently, the incorporation of cassava starch not only improves formulation stability but also contributes to the economic feasibility and practical applicability of liquid biofertiliser technology for sustainable pulse production systems. In addition, it is utilised as a thickener and a low-cost starch ingredient, making it easy for farmers to adopt (Fig. 4).

Agronomic parameters

The data illustrate various agronomical parameters such as number of nodules per plant, weight of nodules per plant, grain yield and N content (%) in grain under the influence of various treatments are presented in Table 7. Inoculation of *Rhizobium* and *Bacillus* based liquid biofertiliser (T6) as well as charcoal-based biofertiliser (T4) showed higher number of nodules (20.8 and 20.6) followed by treatments T3 and T5 over uninoculated control. However, the increased nodule weight was recorded significantly for the treatments containing liquid and charcoal-based biofertilisers over uninoculated control. In terms of biological N fixation and root growth development, two different bacterial inoculants could have synergistic effects on nodule number and nodule weight. A similar type of study conducted by early researchers showed improved patterns in plant growth and yield, significant increment in nodulation and also enhanced nutrient uptake in 11 grain legumes under co-inoculation of rhizobia and *Bacillus* (28). Moreover, similar trends in results were observed in previous studies in chickpea and

Table 5. Effect of 1.5 % polyvinyl pyrrolidone (PVP) supplemented with different concentrations of potato starch (PS) and cassava starch (CS) on the population of *Rhizobium leguminosarum* DPR2 at monthly intervals

Concentration	Months											
	1	2	3	4	5	6	7	8	9	10	11	12
0.25 % PS	1.62×10^9	1.16×10^8	1.12×10^8	0.80×10^8	1.25×10^7	1.27×10^7	0.43×10^7	1.18×10^6	0.54×10^6	1.62×10^5	1.51×10^5	1.31×10^5
0.5 % PS	1.82×10^9	1.08×10^9	0.96×10^9	1.68×10^8	1.80×10^8	1.89×10^8	1.56×10^8	1.62×10^7	1.68×10^7	1.76×10^7	1.70×10^6	1.64×10^5
1 % PS	1.16×10^9	1.12×10^9	1.86×10^9	1.70×10^8	1.56×10^9	1.77×10^8	0.61×10^8	0.32×10^8	1.26×10^7	1.40×10^6	1.44×10^6	1.41×10^5
0.5 % CS	1.84×10^8	1.16×10^9	1.12×10^9	0.88×10^9	1.82×10^8	1.89×10^8	1.56×10^8	1.62×10^8	1.67×10^8	1.68×10^7	1.57×10^6	0.38×10^7
1 % CS	1.68×10^9	2.60×10^9	2.52×10^9	1.08×10^8	1.76×10^9	1.79×10^8	1.67×10^8	0.78×10^8	1.76×10^7	1.48×10^7	1.15×10^7	1.39×10^6
1.5 % CS	1.56×10^9	2.52×10^9	2.60×10^9	1.84×10^9	1.72×10^9	1.80×10^9	2.18×10^8	2.1×10^8	1.93×10^8	1.85×10^8	1.71×10^8	1.34×10^7

Each value represents a mean of 3 replication.

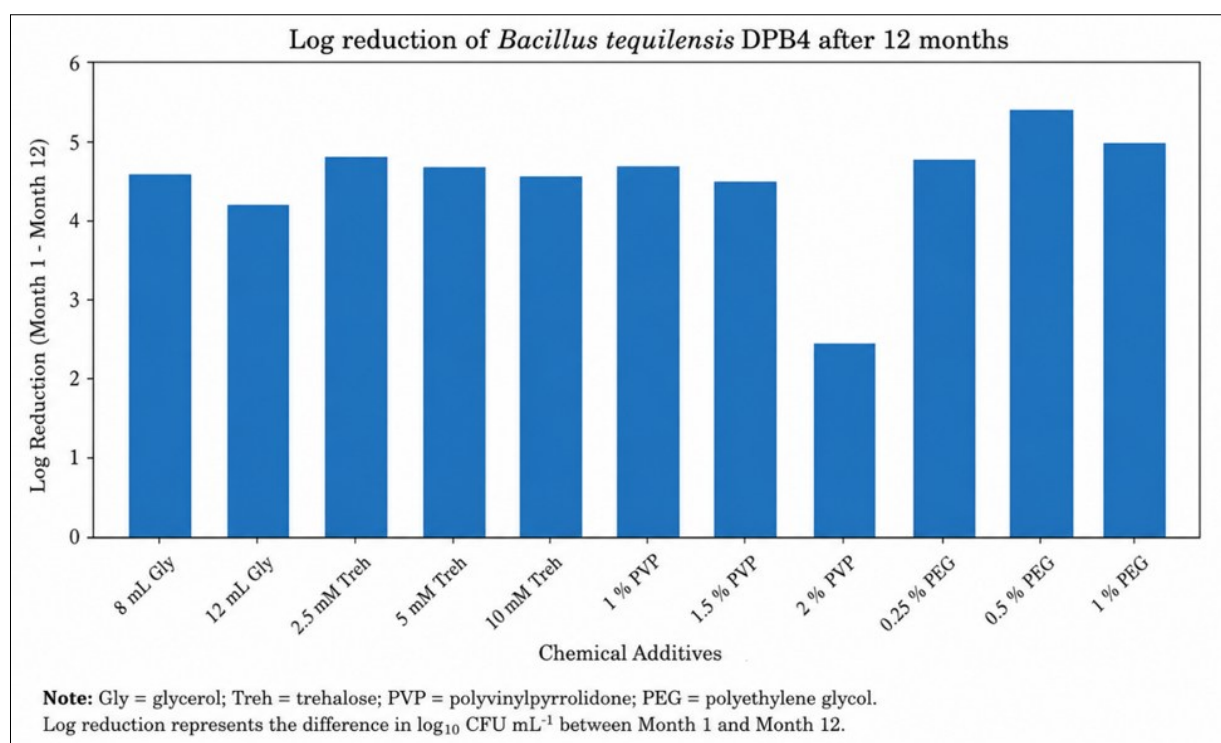
Values represent viable cell population (CFU mL^{-1}) of *Rhizobium leguminosarum* bv. *viciae* DPR2 recorded at monthly intervals during storage. PVP : polyvinyl pyrrolidone; PS : potato starch; CS : cassava starch. CFU : colony forming units. Liquid biofertilizer formulations were stored under laboratory conditions during the study period.

Table 6. Effect of 2 % polyvinyl pyrrolidone (PVP) supplemented with different concentrations of potato starch (PS) and cassava starch (CS) on the population of *Bacillus tequilensis* DPB4 at monthly intervals

Concentration	Months											
	1	2	3	4	5	6	7	8	9	10	11	12
0.25 % PS	1.41×10 ⁹	2.11×10 ⁹	1.05×10 ⁸	1.68×10 ⁸	1.18×10 ⁸	1.51×10 ⁷	1.30×10 ⁷	1.11×10 ⁷	2.43×10 ⁶	2.51×10 ⁶	2.39×10 ⁶	1.69×10 ⁵
0.5 % PS	2.09×10 ⁹	1.04×10 ⁹	2.78×10 ⁹	1.55×10 ⁹	1.67×10 ⁸	1.76×10 ⁸	1.42×10 ⁸	1.46×10 ⁷	1.53×10 ⁷	1.62×10 ⁷	1.59×10 ⁶	1.51×10 ⁵
1 % PS	4.12×10 ⁹	1.08×10 ⁹	1.65×10 ⁹	1.52×10 ⁸	1.42×10 ⁸	1.45×10 ⁸	1.54×10 ⁷	1.28×10 ⁷	1.17×10 ⁶	2.30×10 ⁶	2.08×10 ⁶	1.33×10 ⁵
0.5 % CS	1.67×10 ⁹	2.09×10 ⁹	1.04×10 ⁹	1.72×10 ⁸	1.68×10 ⁸	1.75×10 ⁸	1.41×10 ⁷	1.50×10 ⁷	1.55×10 ⁶	1.53×10 ⁶	1.41×10 ⁵	1.30×10 ⁵
1 % CS	2.55×10 ⁹	1.47×10 ⁹	3.39×10 ⁸	3.01×10 ⁸	2.63×10 ⁹	2.21×10 ⁹	2.93×10 ⁸	2.67×10 ⁸	1.84×10 ⁸	1.67×10 ⁸	1.43×10 ⁸	1.20×10 ⁸
1.5 % CS	1.42×10 ¹⁰	1.39×10 ⁹	2.49×10 ⁸	2.65×10 ⁹	2.58×10 ⁹	1.69×10 ⁹	1.13×10 ⁹	2.8×10 ⁸	1.81×10 ⁸	1.69×10 ⁸	1.60×10 ⁸	1.50×10 ⁸

*Each value represents a mean of three replication.

Note: PS: potato starch; CS: cassava starch; PVP: polyvinyl pyrrolidone; Population expressed as CFU mL⁻¹.

**Fig. 2.** The log₁₀ reduction in viable population of *Bacillus tequilensis* DPB4 after 12 months of storage.

The log₁₀ reduction in viable population of *B. tequilensis* DPB4 after 12 months of storage is presented in Fig. 2. Log reductions varied among treatments, indicating differential formulation stability during storage. While most additives exhibited moderate population decline, certain polymer- and osmoprotectant-based treatments demonstrated comparatively lower reductions, suggesting improved survival likely associated with moisture retention and cellular protection mechanisms. The graphical summary complements the detailed monthly data presented in Table 4.

lentil, where the combination of nitrogen fixer, a classical endosymbiont such as *Rhizobium* and other plant-growth promoting bacterial strain exhibited improvement in the nodulation (29, 30).

Biofertilisers based on liquid and charcoal significantly increased lentil grain total N content (4.37 % and 4.38 % for T4-NPK+R+B and T6-NPK+LR+B, respectively) over non-biofertiliser-based treatment and control. The outcome of the study recorded 37.7 % N content under response to integrated use of *R. leguminosarum* and *Pseudomonas* spp. The significant results related to grain yield were recorded due to the influence of liquid and charcoal-based biofertilisers containing both bacterial strains (Table 7). The highest grain yield was measured for T6-NPK+R+B (10.88 q/ha) followed by T4-NPK+R+B (10.85 q/ha). Indeed, all treatments containing bacterial inoculants in the form of liquid and carbon-based biofertilisers showed maximum values for grain yield compared to treatments without biofertiliser (T1-N0PK and T2-NPK).

Our study shows that the application of liquid biofertilisers supplemented with 2 % PVP and 1.5 % cassava starch results in a higher grain yield, possibly due to the better survival of bacterial inoculants under field conditions. Previous studies have also reported the beneficial effects of liquid and carrier-based biofertilisers on leguminous crops. The liquid *Rhizobium* inoculants had a greater impact on yield than carrier-based formulations (31). The liquid biofertilisers containing *Rhizobium* showed higher yields in chickpea compared to charcoal-based biofertilisers (32). Bacterial inoculants play a prolific role in nutrient uptake by plants, leading to increased crop biomass and grain yield (33).

Although the PVP + cassava starch formulation markedly enhanced *in vitro* shelf-life stability, the field advantage over charcoal-based inoculants was comparatively modest. The relatively modest difference in field performance, despite improved shelf-life stability, likely reflects the complexity of soil ecosystems where microbial competition, environmental stress and rhizospheric interactions

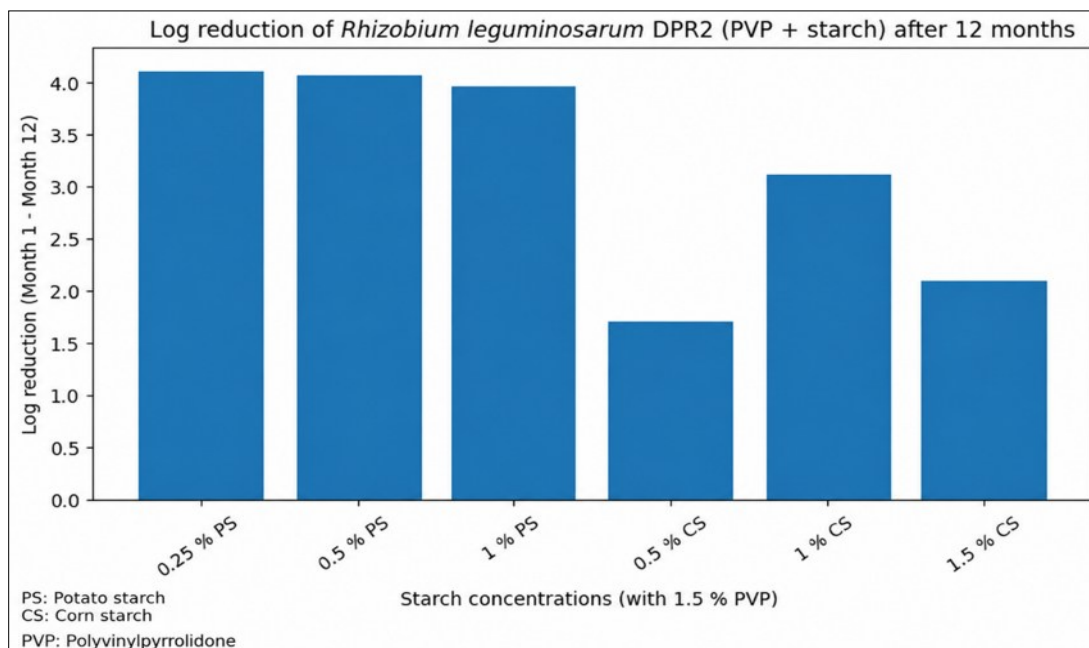


Fig. 3. Log reduction bar graph for *Rhizobium leguminosarum* bv. *viciae* DPR2 (1.5 % PVP + starch combinations).

The $\log_{10}$ reduction in viable population of *R. leguminosarum* DPR2 supplemented with 1.5 % PVP and different starch concentrations is presented in Fig. 3. Reductions varied among treatments, indicating differential survival stability during storage. Cassava starch at higher concentration exhibited comparatively lower reduction, suggesting improved long-term viability, possibly due to enhanced moisture retention and protective matrix formation. The figure complements the detailed monthly data presented in Table 5.

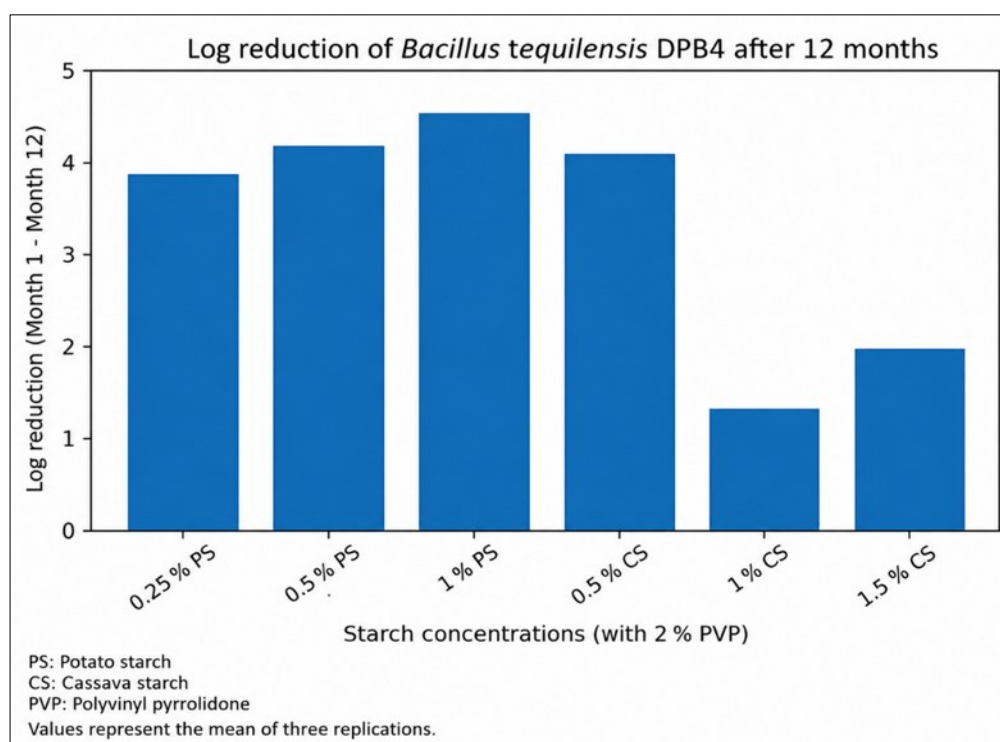


Fig. 4. Log reduction bar graph for *Bacillus tequilensis* DPB4 (2 % PVP + starch combinations).

The $\log_{10}$ reduction in viable population of *B. tequilensis* DPB4 supplemented with 2 % PVP and different starch concentrations is illustrated in Fig. 4. The degree of population decline varied among treatments. Potato starch treatments exhibited relatively higher log reductions compared with cassava starch treatments, whereas cassava starch at higher concentrations showed comparatively lower reductions, indicating improved survival stability during storage. The graphical representation complements the detailed monthly population data presented in Table 6.

regulate effective colonisation and plant growth promotion. Both formulations exceeded the biological threshold required for nodulation and nutrient mobilisation, leading to functional convergence under field conditions. Therefore, while improved shelf life ensures product reliability and commercial viability, field performance is ultimately governed by ecological interactions rather than initial inoculum density alone.

Soil parameters

Post-harvest soil samples were collected for the determination of available NPK, SMBC, CO_2 evolution and dehydrogenase activity (Fig. 5–8). A significant increase related to soil available N, P and K was observed in the soil of the plots given treatments with biofertilisers (Fig. 5). The maximum available N content was observed for the T6-NPK+LR+LB (238.2 kg/ ha) followed by T4-

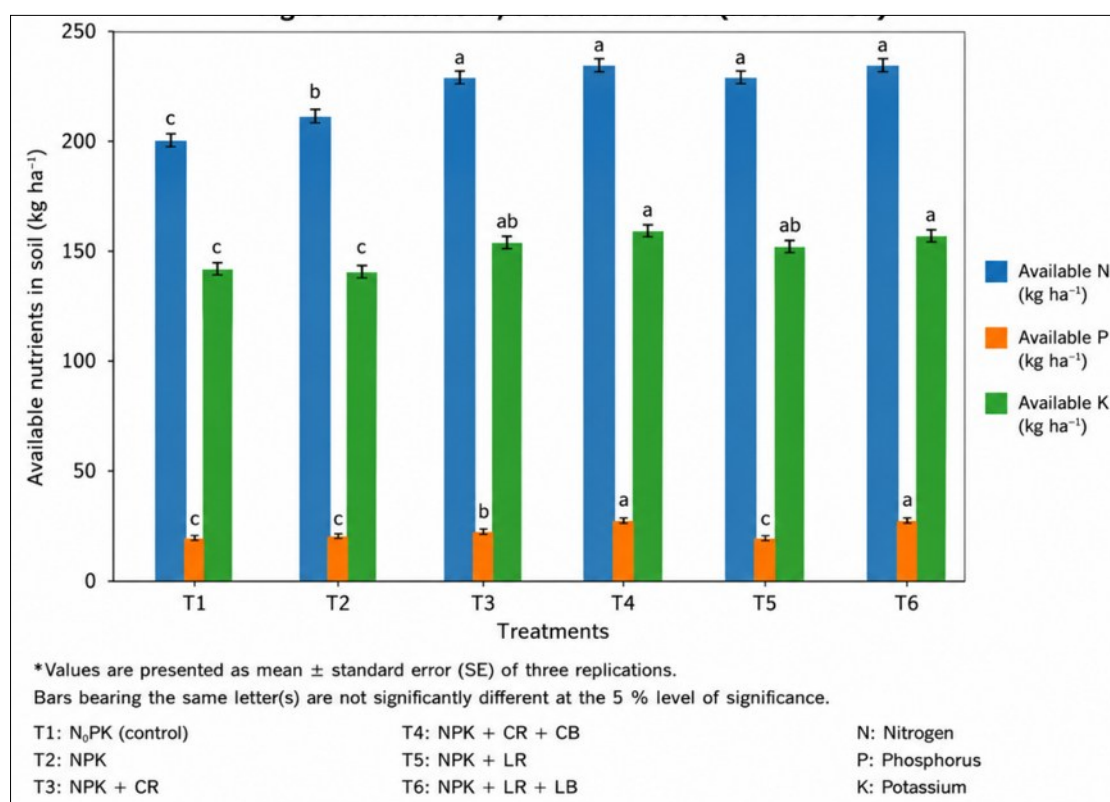
Table 7. Effect of liquid and charcoal based biofertilisers amended with additives on number of nodules, weight of nodules, nitrogen content in lentil grain and grain yield

Labels	Treatment	No. of nodules/ plant (mg)	Wt. of nodules /plant (mg)	Grain yield (q/ha)	N content (%) in lentil grain
T1	N0PK	13.4 ± 0.81 ^b	9.6 ± 0.80 ^c	6.05 ± 0.43 ^a	3.19 ± 0.06 ^c
T2	NPK	16.2 ± 0.74 ^b	12.2 ± 0.90 ^b	8.96 ± 0.38 ^a	3.64 ± 0.06 ^b
T3	NPK+ CR	20.4 ± 0.82 ^a	15.7 ± 0.68 ^a	10.72 ± 0.34 ^a	4.36 ± 0.04 ^a
T4	NPK+ CR+ CB	20.6 ± 0.80 ^a	15.5 ± 0.79 ^a	10.85 ± 0.68 ^a	4.37 ± 0.07 ^a
T5	NPK+ LR	19.9 ± 1.50 ^a	15.3 ± 0.71 ^a	10.64 ± 0.24 ^a	4.30 ± 0.04 ^a
T6	NPK+ LR+ LB	20.8 ± 1.39 ^a	15.9 ± 0.76 ^a	10.88 ± 0.10 ^a	4.38 ± 0.04 ^a
	LSD ($p \leq 0.05$) [*]	1.15	1.93	0.95	0.17

Data were analysed statistically at the 5 % ($p < 0.05$) level of significance.

Treatments with same alphabet are at par.

LSD: Least significant difference, NPK: Recommended dose of nitrogen, phosphorus and potassium, CR: Charcoal-based *Rhizobium* inoculant, CB: Charcoal-based *Bacillus* inoculant, LR: Liquid *Rhizobium* inoculant, LB: Liquid *Bacillus* inoculant

**Fig. 5.** Available nitrogen (N), phosphorus (P) and potassium (K) in soil under different treatments. Bars represent Mean ± SE (n=3). Means followed by different letters differ significantly at $p = 0.05$ according to the CD test.

NPK+CR+CB (237.5 kg/ha). A greater proliferation of soil bacteria that convert organically bound N to inorganic form is possible due to the direct input of N through fertilisers and biofertilisers under the treatment. It was found that the study results corroborated with early research (34). Similarly, the organic manures could reduce N losses and preserve soil N through mineralisation, maintaining a continuous supply of N throughout legume plants' lives. The highest value for the available soil P was recorded for the biofertilisers based treatment T6-NPK+LR+LB (27.8 kg/ha) in comparison of non-biofertilisers treatments. i.e., T1-N₀PK (18.5 kg/ha) and T2-NPK (19.4 kg/ha). The increased soil phosphorus availability could be due to the phosphorus solubilisation potential of bacterial inoculants because of the secretion of wide arrays of organic acids and mineralisation of the organic form of P via the action of phosphatases. Organic materials and biofertilisers form a protective covering over sesquioxide, decreasing the soil's phosphate-fixing capacity and thus increasing the available P-status in the soil. Similarly, the highest value for the available K, i.e., 162.4 kg/ha, was determined for the treatment T6-NPK+LR+LB followed by T4-

NPK+CR+CB (160.0 kg/ha). The higher K availability in the soil indicates that *Rhizobium* and *Bacillus* co-inoculation may have a better ability to maintain K in an accessible form and reduce K fixation. The results similar to the findings of early researchers where effective microbial strains including *Bacillus megaterium* and *Bacillus mucilaginosus* increased available form of K in the rhizospheric soil by 28.1 % (35–37).

Application of biofertiliser with chemical fertiliser maintains the soil fertility status. This may be due to application of higher quantity of nutrients and their complementary effects. Therefore, it can be ideal to enhance available nutrient content in soil and soil quality by application of plant growth stimulating bacterial strains (38–40).

The evolution of CO₂ in soil is a measure of microbial activity and rate of decomposition. The results showed that soil treated with liquid biofertilisers evolved significantly more CO₂ and microbial biomass carbon i.e. 17.6 mg/100 g soil of CO₂ (Fig. 6) and 163.7 mg/kg of SMBC (Fig. 7) for T6-NPK+LR+LB followed by charcoal-based

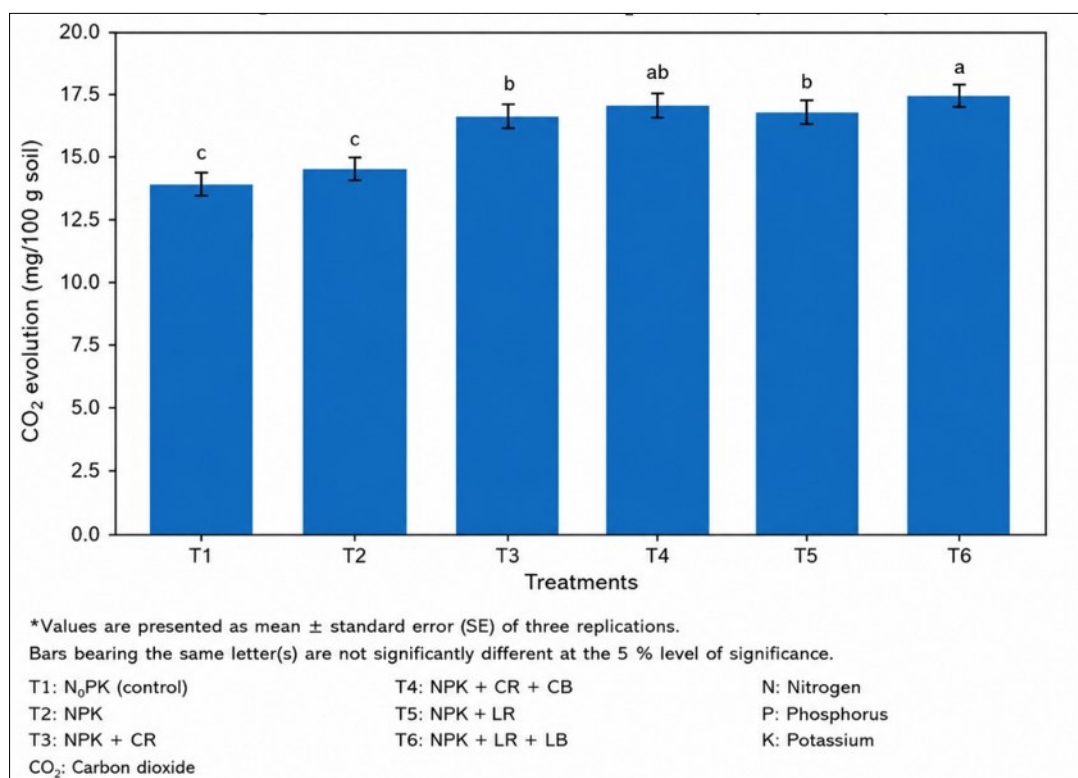


Fig. 6. Effect of liquid and charcoal-based biofertilisers amended with additives on CO₂ evolution of soil. Bars represent mean $\pm$ SE (n=3). Means followed by different letters differ significantly at $p = 0.05$ (CD test).

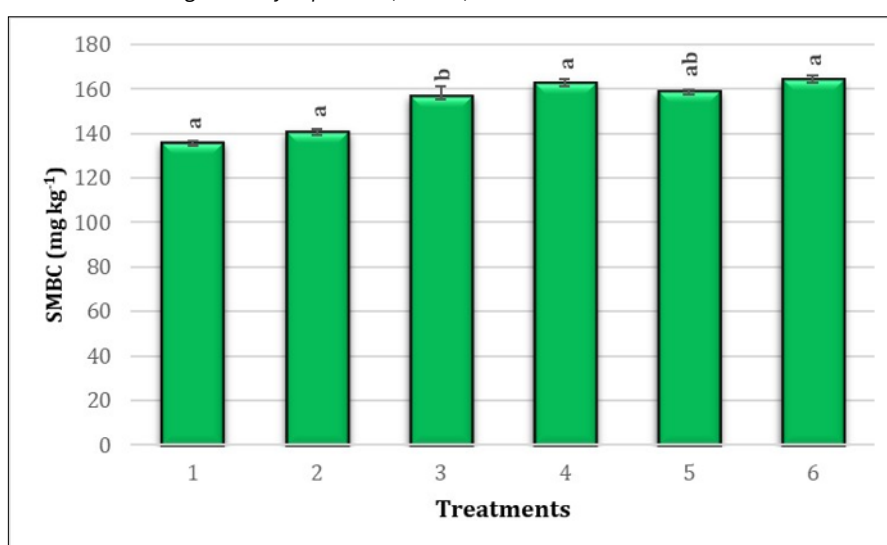


Fig. 7. Effect of liquid and charcoal based biofertilisers amended with additives on soil microbial biomass carbon (SMBC) of soil.

biofertilisers i.e. 17.2 mg/100 g soil of CO₂ (Fig. 6) and 162.4 mg/kg of SMBC (Fig. 7) for T4-NPK+CR+CB over uninoculated control. Compared with inorganic fertilisation and control, liquid biofertilisers led to an increase in microbial biomass carbon (MBC). At different crop ages, similar results reported with a liquid formulation containing *Azotobacter* and PSB (41). The use of inorganic fertilisers may have suppressed microbial growth, resulting in lower microbial respiration (42). However, the supplementation of energy sources in the form of liquid-based *Rhizobium* and *Bacillus* inoculants may have increased microbial activity, leading to higher CO₂ evolution (17.6 mg/100 g soil) compared to the corresponding control plot (13.9 mg/100 g soil). The increased CO₂ evolution in plots receiving liquid biofertilisers indicate enhanced microbial respiration and activity, suggesting that inoculation with *Rhizobium* and *Bacillus* stimulated soil microbial metabolism. By utilising substrates in the form of root exudates metabolic activity increased (43).

Significantly high dehydrogenase activity was recorded for T6-NPK+LR+LB (15.96 μ g TPF/g soil/day) followed by charcoal-based biofertiliser (15.86 μ g TPF/g soil/day for T4-NPK+CR+CB) over uninoculated control (Fig. 8). The impact of dual liquid inoculants of *Mesorhizobium ciceri* and *Pseudomonas diminuta* on chickpea growth and revealed that the soil dehydrogenase activity was significantly higher compared to single inoculants in liquid and charcoal-based biofertilisers (44). The use of a blend of PVP and cassava starch as additives in liquid biofertilisers containing *Rhizobium* and *Bacillus* might have increased the overall microbial activity under field conditions. Polymeric additives such as PVP and cassava starch may also improve seed adhesion of microbial inoculants, thereby enhancing bacterial survival and early rhizospheric colonisation after sowing.

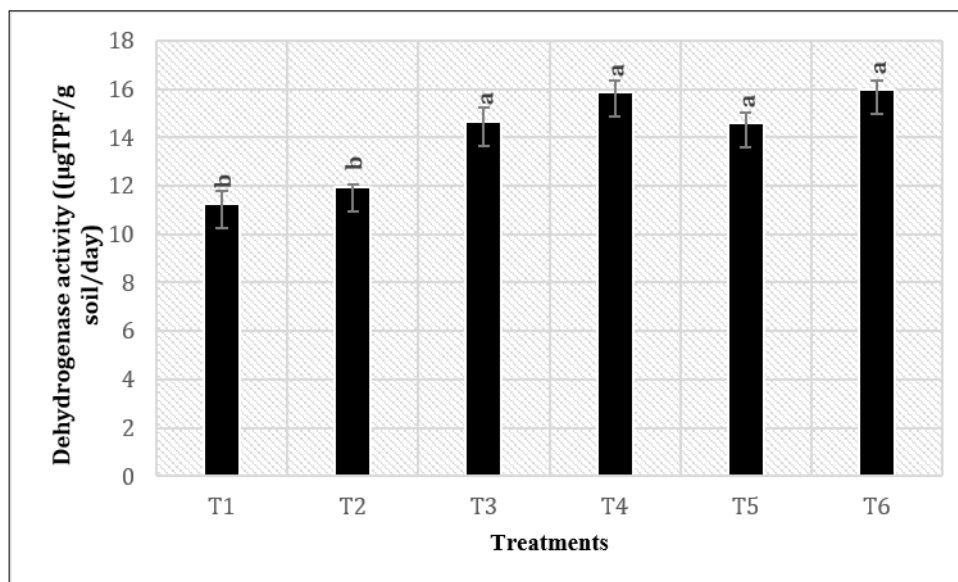


Fig. 8. Effect of liquid and charcoal based biofertilisers amended with additives on soil dehydrogenase activity.

Correlation matrix was performed to study relation among the plant soil properties and plant morphological parameters (Fig. 9 and 10). All ten parameters observed are positively related to each other, no parameter shows any negative relation. From the above figure it can be noticed that available P content is positively correlated to all soil as well as plant parameters, however this parameter establishes a positive plus highly significant correlation with dehydrogenase activity.

The integrated PCA and correlation analyses revealed that soil biological activity parameters, particularly microbial biomass

carbon and dehydrogenase activity, were the dominant drivers of treatment differentiation, explaining 93 % of total variance. Dual inoculation treatments (T4 and T6) were primarily characterised by enhanced microbial functioning and P availability, which were strongly and positively associated with nodulation and grain yield, demonstrating that improved rhizospheric microbial activity was the central mechanism underlying enhanced lentil productivity. The PCA revealed that soil biological activity parameters explained 93 % of treatment variance, indicating that enhanced microbial functioning not merely nodulation was the primary driver of yield improvement. Furthermore, the integration of *Rhizobium* and

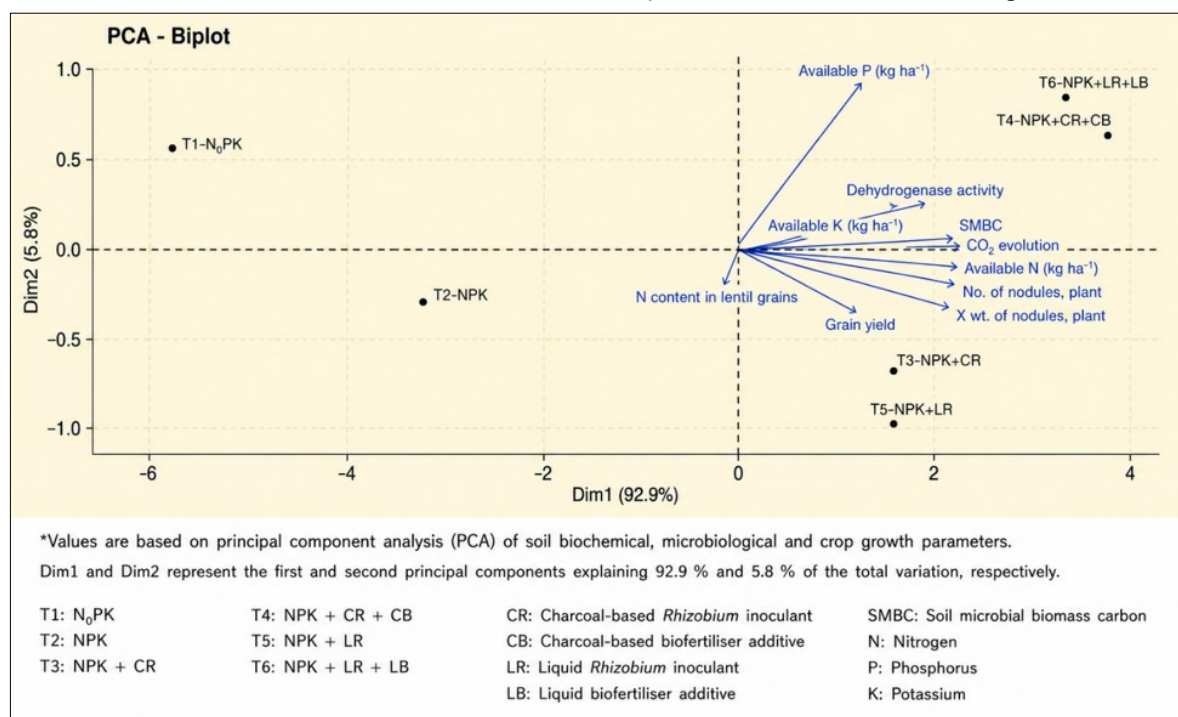


Fig. 9. Treatments for lentil plant in the principal component analysis (PCA).

The first two principal components (PCs) were associated with eigen values higher than one and explained 99 % of the cumulative variance. PC1 accounted for 93 % of the variance and PC2 for 6 %. PC1 was positively and strongly correlated with available K, dehydrogenase activity and SMBC and negatively correlated with other parameters. PC2 was positively correlated with available P. The loading plots illustrate the relationships among parameters where two vectors with an angle < 90° are positively correlated and two vectors with an angle > 90° are not correlated. The vector length shows how well-represented the variables are in the biplot. The variations in all the parameters except available P were closely aligned to each other. The score plot of PCA also revealed information on parameters in relation to different treatments. The treatments from the lower right quadrant i.e., T3 and T5 were characterised by high grain yield, no. of nodules, weight of nodules and available N. The treatments from upper right quadrant T4 and T6 were characterised by high available P only (Fig. 9).

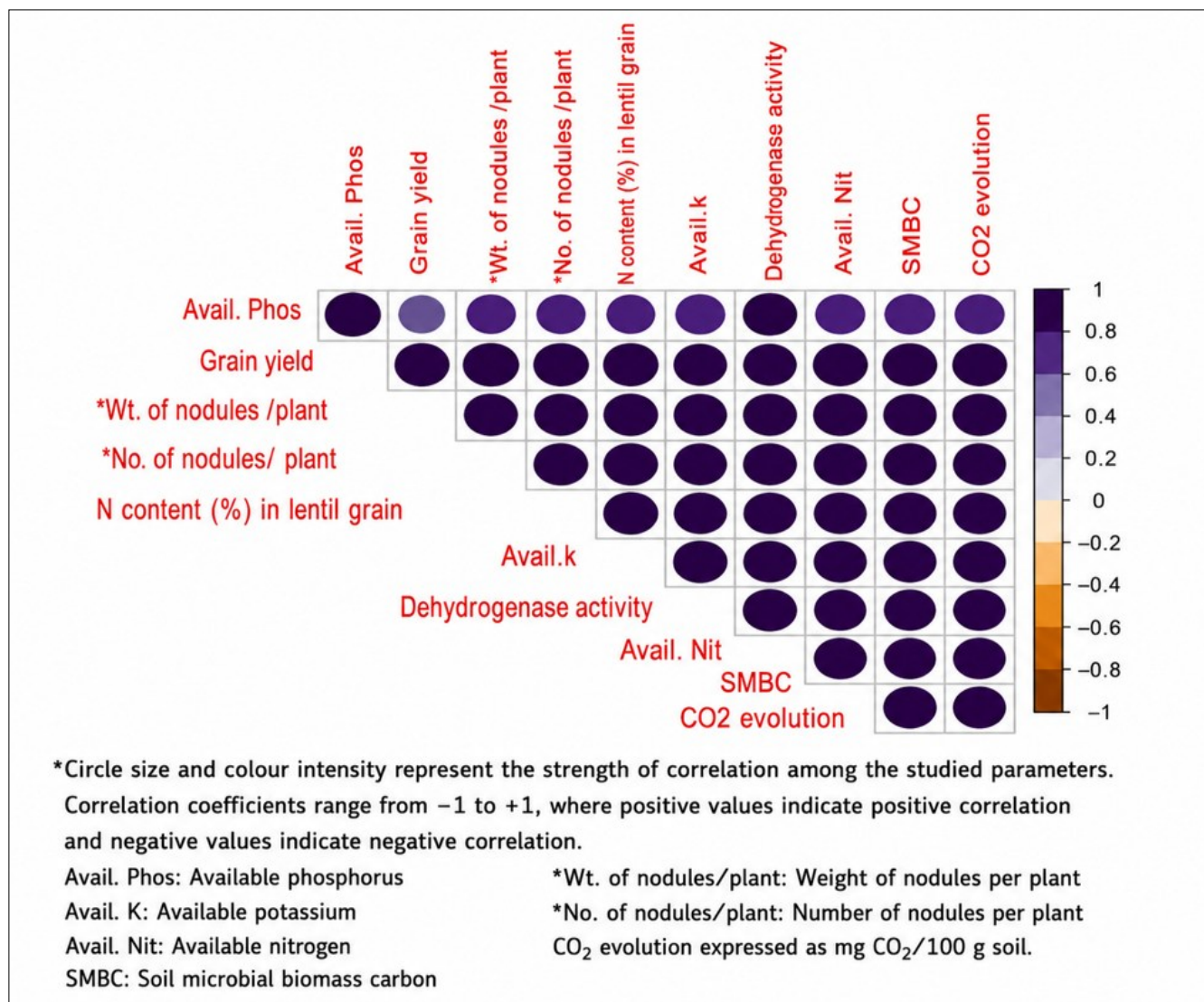


Fig. 10. The correlation matrix for soil variables and plant performance parameters.

Bacillus within a biodegradable carbon matrix enabled synergistic N fixation and P solubilisation under field conditions, representing a functional advancement in liquid biofertiliser technology.

Previous studies have demonstrated the advantages of liquid biofertiliser formulations in improving the survival and field performance of rhizobial inoculants. For instance, previous researchers reported that liquid formulations of *Rhizobium* significantly enhanced shelf life and nodulation efficiency in legume crops compared with conventional carrier-based inoculants (45). In agreement with these findings, the present study also observed improved microbial survival and agronomic performance using liquid formulations. However, our study extends these findings by incorporating a PVP cassava starch blend as a stabilising matrix, which further enhanced the long-term viability of both *R. leguminosarum* and *B. tequilensis*. Unlike earlier studies that primarily focused on single-strain inoculants, the present work demonstrates the synergistic benefits of dual inoculation combined with a biodegradable stabilising system. Moreover, the integration of soil biological indicators and multivariate analyses revealed that improved microbial activity in the rhizosphere was a key driver of enhanced lentil productivity. These findings highlight the functional advancement of the PVP-cassava starch formulation beyond conventional liquid inoculant technologies.

Conclusion

The present study demonstrated that supplementation of liquid biofertiliser formulations with polyvinyl pyrrolidone (PVP) and cassava starch significantly enhanced the survival and stability of *Rhizobium leguminosarum* and *Bacillus tequilensis* during prolonged storage. Among the tested formulations, the combination of 2 % PVP with 1.5 % cassava starch maintained higher viable cell populations and improved the shelf life of the inoculants. Field evaluation further confirmed that the optimised formulation enhanced nodulation, plant growth, soil biological activity and nutrient availability, resulting in increased lentil yield compared with conventional carrier-based inoculants. These findings highlight the potential of a biodegradable PVP-cassava starch matrix as an effective stabilising system for liquid biofertilisers, offering a cost-effective and sustainable strategy to improve microbial inoculant performance and support environmentally friendly pulse production.

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Authors' contributions

GK, SSP, and AJ, developed and conceptualised the idea behind the proposed work. VKU, JP and SK have performed the essential experiment and validations. DP reviewed and compiled the manuscript. K, H and N edited and performed statistical analysis of the available data. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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