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Effect of Microbial Ratio, Storage Temperature, and Packaging on the Viability and Stability of Co-Inoculated *Rhizobium tropici* and *Azospirillum* spp.in Nitrogen-Fixing Biofertilizer

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ABSTRACT

Access to reliable microbial biofertilizers in the Philippines remains limited, underscoring the need for locally produced, stable, and farmer-ready alternatives. This study evaluated the 12-month viability patterns of a solid co-inoculated biofertilizer containing *Rhizobium tropici* (isolated from *Saccharum spontaneum*) and *Azospirillum* spp. formulated at five microbial ratios and stored under different packaging and temperature conditions. The research employed a descriptive experimental approach to document colony-forming unit (CFU/g) trajectories, identify stability trends, and determine whether each formulation maintained viability above the regulatory threshold of ≥1 × 10^6 CFU/g. Solid formulations were packaged in polyethylene (PPE) or aluminum foil and stored under ambient (28–32 °C) or air-conditioned (20–25 °C) conditions. Monthly viability was assessed using serial dilution and spread plating on Dobereiner's medium. Results showed a general pattern of early stabilization (Months 1–3) followed by gradual CFU decline. The balanced 50:50 mixture (1:1 co-culture) exhibited the highest stability, particularly when stored in aluminum foil at 20–25 °C, maintaining 2.6 × 10^8 CFU/g at Month 12. *Azospirillum* spp.-dominant

mixtures showed strong resilience, while *R. tropici*-dominant and single-strain formulations declined more rapidly. Packaging and temperature were major determinants of shelf-life performance, with aluminum foil and cool storage markedly improving microbial survival. The descriptive study provides foundational stability data essential for local product development and quality assurance. Future research should validate agronomic performance through field trials and explore alternative carrier systems and cost-efficient storage strategies to further optimize biofertilizer formulations.

Keywords: *biofertilizer stability, Rhizobium tropici, Azospirillum* spp., c*o-inoculation, microbial viability*

Introduction

Microbial biofertilizers are increasingly recognized as sustainable alternatives to chemical fertilizers due to their capacity to enhance plant growth, improve nutrient use efficiency, and support soil health. However, access to affordable and locally produced biofertilizers in the Philippines remains limited. The privatization of Bio-N, a widely used microbial-based biofertilizer developed by UPLB–BIOTECH, has posed significant challenges to sustainable agricultural production in the Philippines by limiting its availability and increasing procurement costs for farmers. This shift has created an urgent need to develop alternative biofertilizer solutions that are both accessible and cost-effective (Peña, 2024).

Recent industry initiatives, such as ROMARC Enterprises' production of liquid *Azospirillum* spp. concentrates represent important steps toward expanding microbial input availability. *Azospirillum* spp., a well-studied nitrogen-fixing bacterium, was recognized for its ability to promote plant growth and enhance soil fertility (Cassán et al., 2020; Pedraza et al., 2020). Building upon this, the Department of Agriculture–Ilagan Soil Laboratory (DA-ISL), with its established product baseline for legume inoculants and Agri-N Plus biofertilizer, launched a research initiative to evaluate and optimize nitrogen-fixing biofertilizers through co-inoculation strategies. Co-inoculation, which involves the combination of complementary microbial species within a single formulation, has emerged as a promising strategy to enhance microbial survival, improve nitrogen fixation efficiency, and maintain long-term stability (Garcia et al., 2021). By harnessing synergistic physiological traits, co-inoculated systems often outperform single-strain inoculants in both functionality and resilience (Liu et al., 2023).

As part of this initiative, the Department of Agriculture–Ilagan Soil Laboratory (DA-ISL) successfully isolated *Rhizobium tropici* from the roots of tallgrass (*Saccharum spontaneum*), a hardy grass species that thrives in marginal soils (Pandey & Singh, 2020). This discovery is notable because *R. tropici* is traditionally associated with leguminous plants, making its isolation from a non-leguminous species (Maximiano et al., 2020). However, limited studies in the Philippines have examined the storage stability, microbial interactions, and long-term viability of multi-strain solid biofertilizer formulations (Garcia et al., 2021). To harness its potential, the *Azospirillum* spp.-based

concentrate developed by ROMARC Enterprises was co-inoculated with the *R. tropici* strain into solid carrier systems, resulting in a dual-strain nitrogen-fixing biofertilizer formulation. This progression from strain isolation to formulation and finally to stability assessment provides a complete workflow for developing locally adapted biofertilizer products. Subsequent stability and viability assessments were performed to evaluate microbial survival, product quality, and packaging performance under different storage conditions, with the overarching goal of providing farmers with a reliable and cost-effective biofertilizer alternative (Bahuguna et al., 2025).

Specifically, this study aimed to (1) document 12-month CFU/g trajectories of co-inoculated *Rhizobium tropici* and *Azospirillum* spp. formulations under different microbial ratios, (2) assess the effects of packaging material and storage temperature on microbial viability relative to the regulatory threshold of $\geq 1 \times 10^6$ CFU/g, and (3) identify formulation–storage combinations that provide the most stable and farmer-ready nitrogen-fixing biofertilizer.

To address these objectives, the study employed a descriptive research design to evaluate the 12-month viability patterns of the dual-strain formulations across packaging types, microbial ratios, and storage temperatures. Instead of performing inferential comparisons, the study focused on describing CFU/g trajectories, identifying stability trends, and determining which conditions maintain viable microbial populations above regulatory requirements. This approach provides essential baseline data for local production protocols, quality assurance standards, and decision-making for farmer-ready distribution of microbial inoculants.

Methods

Research Design

The study employed an experimental research design to evaluate the stability and viability of co-inoculated *Rhizobium tropici and Azospirillum* spp. formulated with a soil-charcoal solid carrier. Controlled laboratory experiments were conducted to monitor microbial viability over a 12-month storage period under varying environmental conditions. This design enabled a systematic assessment of microbial load, carrier stability, shelf life, and packaging performance.

The primary aim of the study was descriptive in nature to document the viability patterns of *R. tropici* and *Azospirillum* spp. across different microbial ratios, packaging types, and storage temperatures over time. Thus, the objective was not to establish statistical differences among treatments, but rather to describe stability trends, determine whether each formulation maintains CFU levels above regulatory thresholds, and generate baseline data essential for local biofertilizer development.

Participants/Respondents

No human or animal subjects were involved. The subjects of investigation were microbial cultures (*R. tropici* and *Azospirillum* spp.) and solid carrier formulations composed of sterilized garden soil and charcoal. All experimental procedures were conducted by trained laboratory personnel with experience in microbiological techniques.

Locale of the Study

The study was conducted at the Department of Agriculture – Regional Field Office 02 (DA-RFO 02), Ilagan Soil Laboratory, located at Purok 02, DA-CVRC Compound, San

Felipe, City of Ilagan, Isabela, Philippines. Root samples of *Saccharum spontaneum* were collected onsite to isolate the *Rhizobium tropici* used in this study.

Figure 1. *Geographical Location of the Saccharum Spontaneum Sampling Site for Rhizobium Tropici Isolation*

Data Collection Procedure

A sterilized soil–charcoal substrate (3:1 w/w) was utilized as the solid carrier for all formulations. Each biofertilizer packet contained 180 g of carrier that was sterilized by autoclaving at 121 °C for 30 minutes over three consecutive days to ensure effective decontamination. Five inoculum mixtures were prepared using a standardized total culture volume of 40 mL: (a) Mixture A consisting of 20 mL *Rhizobium tropici* and 20 mL *Azospirillum* spp.; (b) Mixture B with 28 mL *R. tropici* and 12 mL *Azospirillum* spp.; (c) Mixture C with 12 mL *R. tropici* and 28 mL *Azospirillum* spp.; (d) Mixture D containing 40 mL *Azospirillum* spp. only; and (e) Mixture E containing 40 mL *R. tropici* only. Each inoculum preparation was aseptically incorporated into the cooled, sterilized carrier and mixed thoroughly until a homogenous distribution was achieved.

Formulated biofertilizers were portioned into 200 g units and sealed either in polypropylene (PPE) plastic or aluminum foil packaging. Packets were subsequently stored for 12 months under two temperature regimes representing common storage environments: ambient laboratory conditions (28–32 °C) and controlled air-conditioned conditions (20–25 °C). This factorial arrangement of microbial composition, packaging material, and storage temperature enabled systematic assessment of the shelf-life performance of each formulation.

Microbial viability was evaluated monthly throughout the 12-month storage period. Colony-forming units per gram (CFU/g) were quantified via serial dilution and spread plating on Dobereiner's agar. Colony morphology served as the basis for descriptive differentiation between the co-inoculated organisms, with *R. tropici* forming small, smooth, mucoid, white colonies, and *Azospirillum* spp., producing larger, smooth, green–blue colonies. Carrier moisture content was also monitored using the oven-drying method at 105 °C for 24 h to determine carrier stability and support interpretation of long-term viability trends.

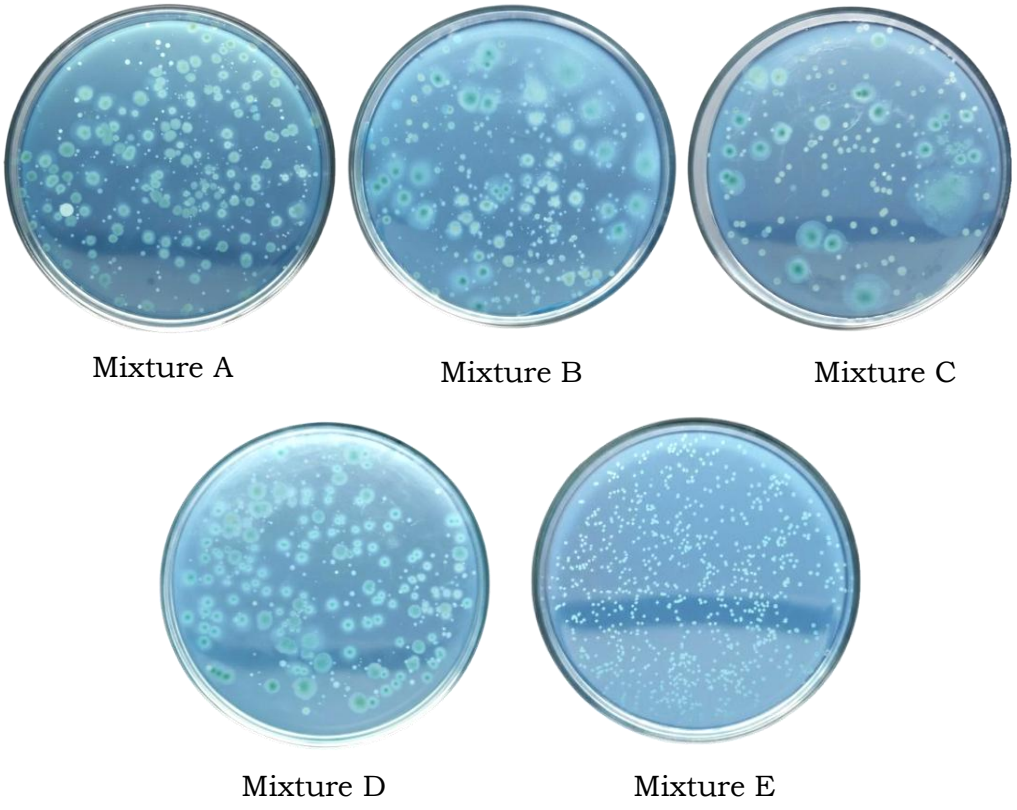

Figure 2. *Quality Control Assessment of Co-Inoculated Soil–Charcoal Biofertilizer on Döbereiner's Agar Medium Showing Distinct Colony Growth of Rhizobium tropici and Azospirillum spp.*

Analysis of Data

Colony-forming unit (CFU/g) values were systematically tabulated and graphically plotted to visualize viability trajectories across the 12-month storage period. The results were interpreted descriptively with reference to the regulatory viability requirement of $\geq 1 \times 10^6$ CFU/g, the comparative stability patterns observed among the different formulations, and the apparent influences of microbial ratio, packaging material, and storage temperature on overall shelf-life performance. No inferential statistical tests were applied in this evaluation.

Inferential analysis was intentionally excluded due to the inherent variability and often non-linear decay patterns characteristic of CFU dynamics in biofertilizer storage studies. Factors such as carrier moisture content, oxygen diffusion across packaging materials, and microenvironmental heterogeneity within the carrier matrix can interact in complex ways, making parametric statistical assumptions difficult to satisfy. The primary aim of this investigation was to establish baseline shelf-life profiles and identify formulation–storage combinations capable of sustaining microbial populations above the mandated viability threshold, rather than to test formal hypotheses regarding treatment effects. The descriptive approach used in this study aligns with current local biofertilizer quality assurance protocols and is consistent with published literature that emphasizes trend characterization and stability profiling in long-term storage assessments.

Figure 3. *Sample of a Soil-Charcoal-Based Solid Biofertilizer Formulation Packaged in Aluminum Foil and PPE Plastic and Stored Under Ambient and Air-Conditioned Rooms*

Ethical Considerations

This study did not involve human or animal testing; however, ethical standards for laboratory research were strictly followed. Biosafety measures were implemented, including the use of autoclaves for decontamination, personal protective equipment, and sterilization of laboratory materials to prevent contamination. Laboratory protocols were based on the Bureau of Soils and Water Management Testing Method Manual (2021) and ASTM standards. Waste management procedures included safe handling, sterilization, and disposal of biohazard materials to ensure environmental safety and regulatory compliance.

Results and Discussion

Results

This study monitored the 12-month viability of *Rhizobium tropici* and *Azospirillum* spp. in five co-inoculated and single-strain mixtures stored under different packaging materials and temperature conditions. Results are presented descriptively to illustrate CFU/g trajectories, identify stability patterns, and determine whether each formulation maintained viability above the regulatory threshold of $\geq 1 \times 10^6$ CFU/g.

General Viability Trends Across Treatments

All biofertilizer formulations displayed a consistent overall pattern characterized by an initial stabilization or slight increase in CFU counts during the first two to three months, followed by progressive declines throughout the remainder of the storage period. This trend aligns with established microbial adaptation behaviors in solid carriers, in which early metabolic activity is succeeded by gradual moisture loss and nutrient depletion (Elita et al., 2025). Across all treatments, formulations stored at 20–25 °C and packaged in aluminum foil exhibited markedly slower reductions in CFU/g compared with those stored in PPE plastic under ambient conditions, highlighting the

combined importance of temperature control and barrier-protective packaging for sustaining long-term microbial viability (Indratmi et al., 2021).

Effect of Microbial Ratio

Balanced 1:1 Co-Culture (Mixture A)

Mixture A consistently exhibited the highest stability among all formulations. When packaged in aluminum foil and stored under air-conditioned conditions, CFU/g values remained within the 10^8–10^9 range throughout the 12 months, reaching 2.6 × 10^8 CFU/g at month 12, well above the regulatory threshold of ≥1 × 10^6 CFU/g.

In contrast, PPE-packaged samples stored under ambient temperatures experienced accelerated decline and eventually reached TFTC (too few to count) by month 12. Aluminum foil provided a moderate stabilizing effect even under ambient conditions, although CFU/g values showed gradual reductions beginning around Month 9, as shown in Table 1.

Table 1. Monthly Viability of Mixture A (50% *Rhizobium tropici* + 50% *Azospirillum* spp.) Under Different Storage and Packaging Conditions

Mixture	Packing	Storage	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
A	PPE	Ambient	6.4×10^7	6.0×10^7	5.3×10^7	5.8×10^7	5.3×10^7	4.5×10^7
A	PPE	Air-cond	7.4×10^7	6.0×10^7	5.7×10^7	5.6×10^7	1.5×10^8	2.4×10^8
A	Foil	Ambient	1.7×10^8	6.3×10^7	5.5×10^7	4.8×10^7	1.6×10^8	2.3×10^8
A	Foil	Air-cond	2.0×10^8	3.6×10^8	1.9×10^8	5.0×10^7	2.0×10^8	3.4×10^8
Mixture	**Packing**	**Storage**	**Month 7**	**Month 8**	**Month 9**	**Month 10**	**Month 11**	**Month 12**
A	PPE	Ambient	4.0×10^7	3.1×10^7	2.5×10^7	4.9×10^6	1.0×10^6	TFTC
A	PPE	Air-cond	3.0×10^8	3.1×10^8	3.4×10^8	2.5×10^8	2.1×10^8	1.9×10^8
A	Foil	Ambient	2.7×10^8	2.8×10^8	3.2×10^8	2.7×10^8	1.0×10^8	4.7×10^7
A	Foil	Air-cond	4.9×10^8	5.2×10^8	5.9×10^8	3.3×10^8	2.9×10^8	2.6×10^8

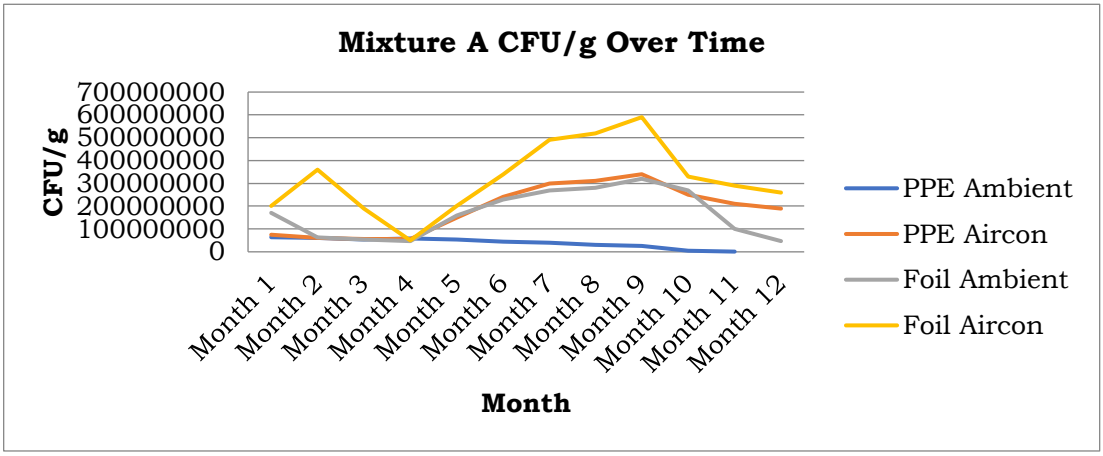

Figure 4. *Microbial Stability Trends of Mixture A (50% R. tropici + 50% Azospirillum spp.) Stored for 12 Months Under Different Packaging and Temperature Conditions*

The 1:1 co-culture (Mixture A) demonstrated the strongest and most consistent long-term stability, reinforcing the advantage of balanced co-inoculation and the superior barrier properties of aluminum foil. PPE packaging and ambient temperatures accelerated microbial decline and reduced viability toward the end of storage.

Azospirillum spp.-Dominant Formulations (Mixtures C and D)

Mixtures with higher proportions of *Azospirillum* spp. (30:70 and 100%) demonstrated strong resilience across storage conditions. *Azospirillum* spp.-rich formulations generally retained viable populations longer than *Rhizobium*-dominant mixtures, reflecting the inherent stress tolerance of the genus. Contrastingly, Mixture D (100% *Azospirillum* spp.), stored in aluminum foil under air-conditioned conditions, achieved the highest stability among the single-strain formulations, maintaining counts above 10^8 CFU/g and peaking at 4.1×10^8 CFU/g during the storage period. Conversely, PPE-packaged samples, especially under ambient temperatures, showed faster declines and often reached TFTC by months 10-12 as presented in Table 2.

Table 2. Monthly Viability of Mixture D (100% *Azospirillum* spp.) Under Different Storage and Packaging Conditions

Mixture	Packing	Storage	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
D	PPE	Ambient	3.5×10^7	1.6×10^8	3.6×10^7	2.3×10^7	2.0×10^7	1.7×10^7
D	PPE	Air-cond	5.1×10^7	2.7×10^8	7.0×10^7	4.5×10^7	1.9×10^7	1.5×10^7
D	Foil	Ambient	1.9×10^7	5.3×10^7	3.1×10^7	4.0×10^7	1.3×10^8	3.8×10^7
D	Foil	Air-cond	3.4×10^7	6.1×10^7	6.2×10^7	5.7×10^7	7.6×10^7	2.2×10^8
Mixture	**Packing**	**Storage**	**Month 7**	**Month 8**	**Month 9**	**Month 10**	**Month 11**	**Month 12**
D	PPE	Ambient	1.4×10^7	1.3×10^7	1.1×10^7	6.6×10^6	1.1×10^6	TFTC
D	PPE	Air-cond	1.4×10^7	1.3×10^7	1.2×10^7	5.5×10^6	1.4×10^6	TFTC
D	Foil	Ambient	1.9×10^7	Too Few	Too Few	Too Few	Too Few	TFTC
D	Foil	Air-cond	3.1×10^8	3.7×10^8	4.1×10^8	3.1×10^8	2.9×10^8	1.0×10^8

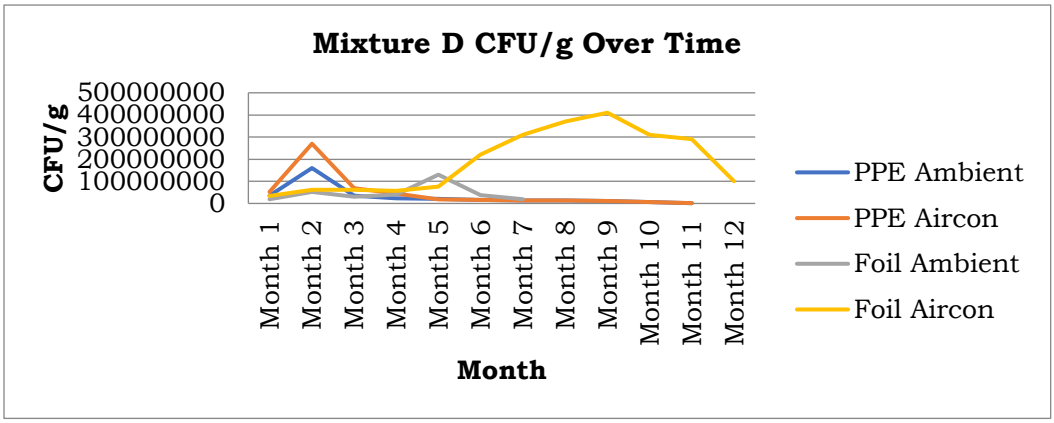

Figure 5. *Viability Trends of Mixture D (100% Azospirillum* spp.*) Under Varying Packaging and Storage Temperatures*

Azospirillum spp. remained highly resilient, particularly in foil packaging and cooler temperatures, while PPE–ambient conditions consistently produced the earliest declines across all *Azospirillum* spp.-dominant treatments.

***Rhizobium*-Dominant Formulations (Mixtures B and E)**

Formulations dominated by *Rhizobium tropici* (70:30 and 100%) demonstrated the weakest overall stability. Both mixtures exhibited sharp CFU reductions by months 6–8, with many treatments declining to TFTC by months 9–12. This pattern reflects R. tropici's lower tolerance to desiccation, oxygen exposure, and fluctuating storage conditions.

Moderate improvement was observed in aluminum foil combined with air-conditioning; however, this was insufficient to maintain month 12 viability above the regulatory threshold, particularly for Mixture B.

Table 3. Monthly Viability of Mixture B (70% *Rhizobium tropici* + 30% *Azospirillum* spp.) Under Different Storage and Packaging Conditions

Mixture	Packing	Storage	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
B	PPE	Ambient	1.3×10^{7}	3.7×10^{7}	3.0×10^{7}	9.4×10^{6}	5.2×10^{6}	2.7×10^{6}
B	PPE	Air-cond	2.5×10^{7}	4.1×10^{7}	4.4×10^{7}	1.4×10^{7}	9.3×10^{6}	9.3×10^{6}
B	Foil	Ambient	3.8×10^{7}	4.0×10^{7}	4.0×10^{7}	2.5×10^{7}	1.9×10^{7}	1.2×10^{7}
B	Foil	Air-cond	5.8×10^{7}	6.6×10^{7}	4.7×10^{7}	4.5×10^{7}	3.4×10^{7}	2.3×10^{7}
Mixture	**Packing**	**Storage**	**Month 7**	**Month 8**	**Month 9**	**Month 10**	**Month 11**	**Month 12**
B	PPE	Ambient	1.1×10^{6}	1.1×10^{6}	TFTC	TFTC	TFTC	TFTC
B	PPE	Air-cond	9.2×10^{6}	4.0×10^{6}	TFTC	TFTC	TFTC	TFTC
B	Foil	Ambient	4.0×10^{6}	1.3×10^{6}	TFTC	TFTC	TFTC	TFTC
B	Foil	Air-cond	1.1×10^{7}	8.1×10^{6}	5.9×10^{6}	5.2×10^{6}	5.2×10^{6}	4.9×10^{6}

Table 4. Monthly Viability of Mixture E (100% *Rhizobium tropici*) Under Different Storage and Packaging Conditions

Mixture	Packing	Storage	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
E	PPE	Ambient	8.0×10^{6}	4.3×10^{7}	5.2×10^{7}	3.3×10^{7}	2.7×10^{7}	8.5×10^{6}
E	PPE	Air-cond	7.4×10^{6}	3.3×10^{7}	3.6×10^{7}	3.9×10^{7}	2.1×10^{7}	1.9×10^{7}
E	Foil	Ambient	7.5×10^{7}	2.1×10^{8}	7.0×10^{7}	1.7×10^{8}	1.3×10^{8}	8.9×10^{7}
E	Foil	Air-cond	8.0×10^{7}	3.5×10^{8}	2.3×10^{8}	2.3×10^{8}	1.9×10^{8}	1.4×10^{8}
Mixture	**Packing**	**Storage**	**Month 7**	**Month 8**	**Month 9**	**Month 10**	**Month 11**	**Month 12**
E	PPE	Ambient	3.2×10^{6}	TFTC	TFTC	TFTC	TFTC	TFTC
E	PPE	Air-cond	1.4×10^{7}	8.2×10^{6}	6.0×10^{6}	TFTC	TFTC	TFTC
E	Foil	Ambient	4.6×10^{7}	9.9×10^{6}	8.3×10^{6}	5.5×10^{6}	1.9×10^{6}	TFTC
E	Foil	Air-cond	9.6×10^{7}	5.8×10^{7}	3.9×10^{7}	3.1×10^{7}	2.9×10^{7}	2.5×10^{7}

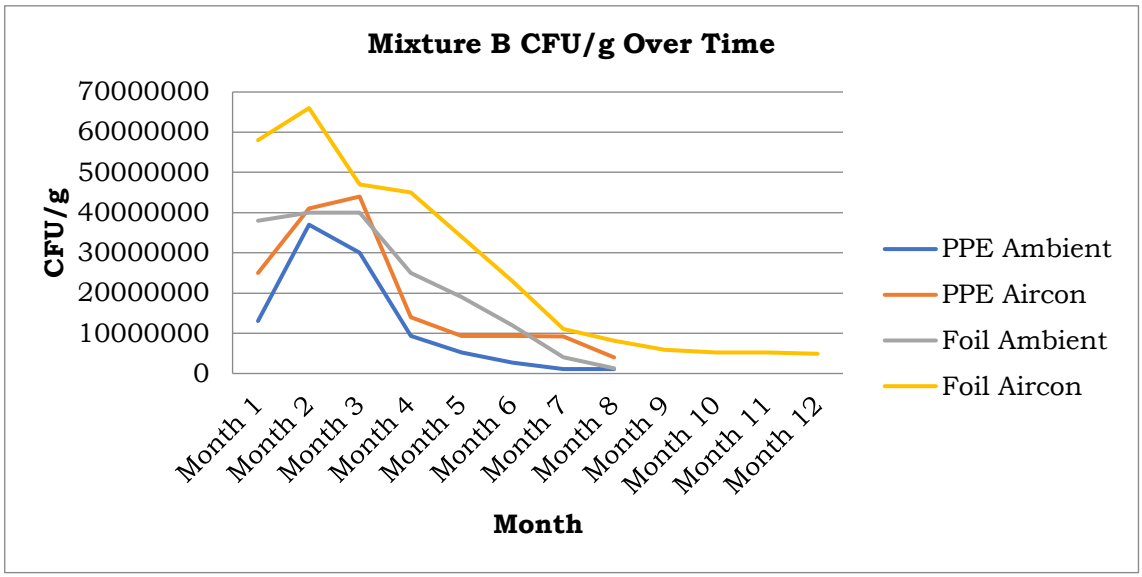

Figure 6. *CFU/g Trajectories of Mixture B (70% R. tropici + 30% Azospirillum* spp.*) over a 12-Month Storage Period*

Rhizobium-dominant mixtures showed sharp declines under all conditions, often reaching "too few to count" by months 9–12. Improved stability was observed only in foil-packaged samples stored at 20–25 °C.

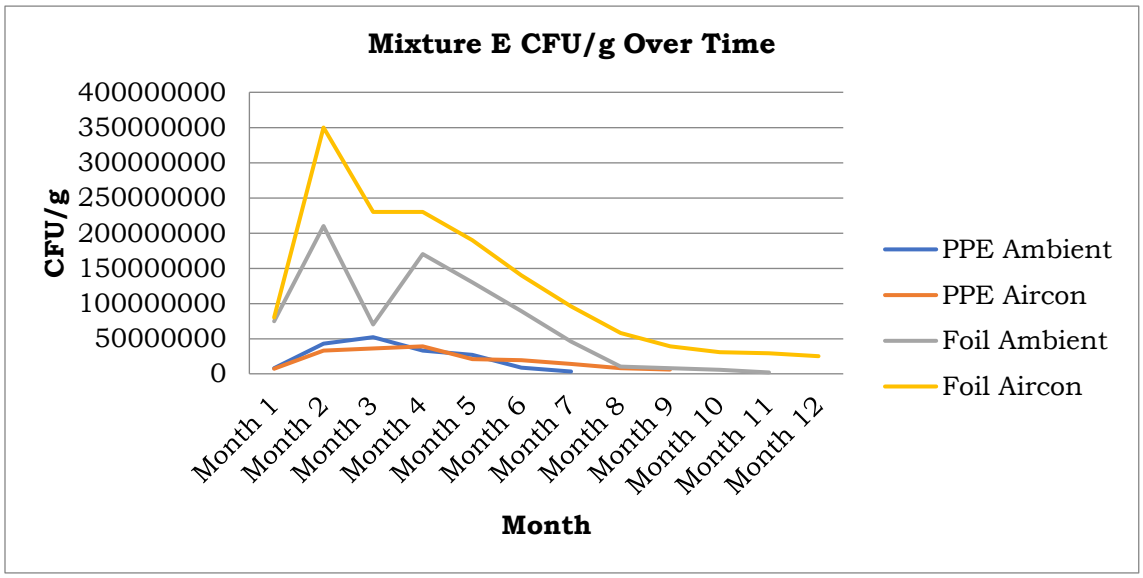

Figure 7. *Microbial Viability Trends of Mixture E (100% R. tropici) Across Packaging and Temperature Conditions*

Across all treatments, pure *R. tropici* (Mixture E) exhibited the lowest long-term stability, with most conditions reaching TFTC before month 12. Foil packaging and cooler storage delayed—but did not prevent—viability loss.

Effect of Storage Temperature

Storage temperature exerted a pronounced effect on microbial survival throughout the 12-month evaluation period. Formulations stored under air-conditioned conditions (20–25 °C) consistently maintained higher CFU counts, whereas those kept under ambient temperatures (28–32 °C) exhibited accelerated declines in viability across all treatments.

For instance, Mixture A packaged in aluminum foil retained a population of approximately 2.6×10^8 CFU/g under air-conditioned storage but showed a markedly sharper reduction in viability when stored at ambient temperatures. The superior performance at lower temperatures is attributed to reduced metabolic stress, slower desiccation rates, and overall stabilization of microbial activity, collectively contributing to prolonged survival within the carrier matrix.

Effect of Packaging Material

Packaging material demonstrated a substantial influence on long-term microbial stability, with aluminum foil consistently outperforming PPE plastic across all mixture formulations and storage temperatures. The superior performance of foil packaging is attributed to its enhanced moisture-retention capacity, lower oxygen permeability, and effective protection against light exposure—factors that collectively support microbial persistence within the carrier matrix. In contrast, PPE plastic was more susceptible to moisture loss, exhibited greater sensitivity to temperature fluctuations, and provided a comparatively weaker barrier against environmental stressors.

As an example, Mixture D stored in aluminum foil maintained CFU levels exceeding 10^8 CFU/g at month 12 under cooled storage conditions, whereas the corresponding PPE-packaged formulation stored under ambient conditions declined below detectable limits before month 11. These results underscore the critical importance of packaging material in maintaining microbial viability and ensuring the long-term stability of solid biofertilizer products.

Comparative Stability Among Formulations

Quantitative assessment of microbial viability at month 12 indicated that co-inoculated and *Azospirillum* spp.-enriched formulations exhibited superior stability. Mixture A (50:50 *Rhizobium* tropici: *Azospirillum* spp.) demonstrated the highest long-term viability (1.9×10^8 – 4.7×10^7 CFU/g) when stored in aluminum foil under controlled (20–25 °C) conditions, followed by Mixture D (100% *Azospirillum* spp.; 1.0×10^8 CFU/g), which maintained high resilience. Mixture C (30:70 *Rhizobium*: *Azospirillum* spp.) showed moderate stability (1.0×10^7 – 8.1×10^6 CFU/g), whereas Mixture B (70:30 *Rhizobium*-dominant; 4.9×10^6 CFU/g) and Mixture E (100% *Rhizobium*; 2.5×10^7 – too few to count) exhibited weak and lowest stability, respectively.

Overall, balanced co-inoculation or *Azospirillum*-dominant formulations maintained superior shelf-life performance, while ambient temperature and porous packaging correlated with reduced microbial viability.

Table 5. Comparative Summary of the Most Stable Formulations Across All Treatments

Mixture	Composition	Best Storage	Month 12 CFU/g	Stability Rank
A	50:50 *Rhizobium* : *Azospirillum*	Aluminum foil / AC room	1.9×10^{8} – 4.7×10^{7}	Highest
D	100% *Azospirillum* spp.	AC room / Aluminum foil	1.0×10^{8} – 1.0×10^{8}	Highly resilient
C	30:70 *Rhizobium* : *Azospirillum*	AC room / Aluminum foil	1.0×10^{7} – 8.1×10^{6}	Moderately stable
B	70:30 *Rhizobium* : *Azospirillum*	AC room	4.9×10^{6}	Weak stability
E	100% *Rhizobium*	AC room / Aluminum foil	2.5×10^{7} – too few to count	Lowest

Discussion

The descriptive results demonstrate that microbial ratio, packaging material, and storage temperature exert interactive effects on the long-term stability of co-inoculated solid biofertilizers. These patterns are consistent with established physiological and ecological principles governing microbial persistence in solid carrier systems. Collectively, the findings highlight how strain compatibility, environmental buffering, and physical protection provided by the carrier–packaging matrix determine the survival trajectories of nitrogen-fixing microbes during storage.

Co-Inoculation Synergy and Microbial Interaction

The superior performance of Mixture A, formulated at a 50:50 ratio of *Rhizobium tropici* and *Azospirillum* spp., highlights the synergistic advantages of co-inoculating complementary nitrogen-fixing bacteria within a single biofertilizer. Several ecological and physiological mechanisms likely contribute to its enhanced long-term stability. First, resource partitioning between the two species minimizes competitive exclusion; *Azospirillum* spp. can metabolize a wider range of carbon substrates, whereas *R. tropici* exhibits more substrate specificity, thereby reducing niche overlap and supporting sustained coexistence (Machado et al., 2020). Second, *Azospirillum*'s inherent resilience to abiotic stressors—such as desiccation, osmotic fluctuations, and temperature variation—combined with its ability to produce exopolysaccharides (EPS), creates micro-moisture zones within solid carriers that may protect the more moisture-sensitive *R. tropici* (Gureeva et al., 2023). Third, differences in early adaptation dynamics further stabilize the consortium; although both species establish rapidly after inoculation, *Azospirillum* tends to maintain viability longer, potentially delaying the overall decline in microbial populations typically observed during storage (Fernández et al., 2022). Collectively, these complementary interactions explain why the balanced co-culture of Mixture A sustained higher CFU/g values across storage periods compared to single-strain and *Rhizobium*-dominant formulations.

Physiological Basis for Temperature-Driven Stability Differences

Storage temperature emerged as a major determinant of microbial survival in solid biofertilizer formulations, with cooler conditions (20–25 °C) consistently maintaining higher viable counts than ambient temperatures (28–32 °C) (Aloo et al., 2022). This pattern aligns with well-established temperature-dependent physiological responses. Lower temperatures suppress metabolic activity, thereby slowing nutrient depletion and reducing the accumulation of inhibitory metabolic by-products (Consiglio et al., 2022). Cooler storage also decreases moisture loss, mitigating desiccation stress that disproportionately impacts *Rhizobium* spp., which is more sensitive to drying than other plant growth–promoting bacteria (Rizvi et al., 2021). Furthermore, reduced oxidative and thermal stress at lower temperatures contributes to the stabilization of cellular enzymes and membrane structures, enhancing overall survival (Somero et al., 2020). Given that many solid biofertilizers deteriorate during storage due to heat accumulation and rapid carrier desiccation, these findings underscore the critical importance of temperature regulation to preserve microbial viability over extended storage periods.

Packaging Material as a Determinant of Shelf Life

Packaging material exerted a significant influence on microbial viability trajectories during storage, with aluminum foil consistently outperforming PPE plastic due to its superior barrier properties. Aluminum foil's lower oxygen transmission rate (OTR) reduces microbial exposure to oxidative stress, thereby stabilizing sensitive nitrogen-fixing bacteria (Ge et al., 2020). Its high moisture barrier capacity also limits desiccation—a primary cause of CFU decline in *Rhizobium* spp.—by preventing excessive water loss from the solid carrier matrix (Nguyen et al., 2021). Additionally, aluminum packaging provides complete light impermeability, protecting the inoculants from UV-induced damage and temperature fluctuations (Shamim et al., 2020). In contrast, PPE plastic allows greater moisture diffusion and possesses weaker thermal and oxygen barrier properties, resulting in accelerated viability losses, particularly under ambient temperature conditions (Li et al., 2024). These results collectively reinforce the critical role of packaging selection in ensuring biofertilizer stability and maintaining product quality throughout storage.

Differential Performance of *Azospirillum* spp. and *Rhizobium* Strains

The pronounced performance differences between *Azospirillum*-dominant and *Rhizobium*-dominant formulations can be attributed to fundamental physiological distinctions between the two genera. *Azospirillum* spp. exhibited strong tolerance to environmental stress, supported by efficient colonization traits, exopolysaccharide (EPS) production, and adaptive mechanisms that enable sustained viability under fluctuating storage conditions (Nievas et al., 2023). In contrast, *Rhizobium tropici*, while highly effective in symbiotic nitrogen fixation, possesses narrower environmental tolerances and is particularly sensitive to moisture depletion, often showing rapid population decline once carrier hydration falls below critical thresholds. These contrasting characteristics explain why *Azospirillum*-rich mixtures (Mixtures C and D) maintained high viability throughout storage, whereas *Rhizobium*-dominant mixtures (Mixtures B and E) exhibited pronounced reductions in CFU counts. Collectively, the findings suggest that *Azospirillum* functions as a stabilizing component in multi-strain solid biofertilizer formulations, enhancing overall resilience and long-term microbial persistence.

Integrative Interpretation of Key Findings

Across all formulations and storage conditions, three overarching patterns emerged that collectively explain the long-term stability behavior of the co-inoculated solid biofertilizer. First, co-inoculation markedly enhances microbial persistence, with balanced consortia—such as the 50:50 formulations in Mixture A—demonstrating superior stability due to synergistic functional interactions and complementary metabolic capabilities between *Rhizobium* and *Azospirillum* species (Cassán et al., 2020; Cunha et al., 2025).

Second, cooler storage environments (20–25 °C) significantly improve microbial viability. Reduced temperatures slow metabolic degradation, limit moisture loss, and mitigate cumulative desiccation stress, establishing temperature control as a primary determinant of long-term shelf-life performance (Aloo et al., 2022; Greffe et al., 2020).

Third, aluminum foil packaging facilitates superior microbial preservation compared with plastic-based materials due to its enhanced moisture barrier, low oxygen transmission properties, and protective physical structure (Indratmi et al., 2021). The stabilizing influence of foil packaging was especially evident in *Azospirillum*-containing mixtures, which maintained high viability even at month 12 when stored under foil and cool conditions. Collectively, these converging results identify the optimal stability strategy as a combination of balanced co-inoculation, aluminum foil packaging, and cool storage (20–25 °C), which together provide the most effective conditions for sustaining high CFU counts of nitrogen-fixing bacteria over extended storage periods.

Implications for Biofertilizer Development

The findings of this study provide several scientific, operational, and policy-relevant insights essential for advancing local biofertilizer production. The findings of this study offer several scientific insights that are crucial for advancing local biofertilizer development. First, co-inoculation demonstrably enhances microbial resilience, as the synergistic interactions between *Rhizobium tropici* and *Azospirillum* spp. significantly improved long-term viability. This supports existing evidence that multi-strain formulations can outperform single-strain inoculants in terms of shelf-life stability and functional persistence (Patil et al., 2025; Thomloudi et al., 2019). Second, *Azospirillum* functions as a stabilizing component within dual-strain nitrogen-fixing formulations. Its strong tolerance to desiccation, temperature fluctuations, and oxygen exposure positions it as a reliable anchor species that enhances the robustness of co-inoculated biofertilizers (Al-Tawaha et al., 2021). Third, descriptive viability profiling under actual storage conditions proves valuable for strain selection. Identifying long-term stability trajectories provides an evidence-based foundation for optimizing microbial ratios in future formulations and ensuring that selected strains can withstand real-world production and distribution environments.

From a production and operational perspective, aluminum foil should be adopted as the standard packaging material for regional biofertilizer manufacturers due to its superior barrier properties, which effectively protect microbial inoculants against moisture loss and oxidative stress (Indratmi et al., 2021). Temperature-controlled storage at 20–25 °C markedly extends shelf life and is practically achievable for Department of Agriculture (DA), Local Government Unit (LGU), and cooperative-level production facilities through the use of small-scale cooling rooms or insulated storage spaces. In addition, implementing standardized viability monitoring protocols based on CFU/g trajectories can strengthen quality assurance systems and ensure regulatory

compliance during production and distribution, providing consistent product performance and safety for end-users (Bahuguna et al., 2025).

With regard to policy and farmer adoption implications, locally produced co-inoculated formulations of *Azospirillum* spp. and *Rhizobium tropici* demonstrated microbial counts equal to or exceeding 1×10^8 CFU/g, surpassing the viability of commercially available Bio-N products, which are reported at approximately 1×10^7 CFU/g on a soil-charcoal carrier, based on Department of Agriculture, Fertilizer and Pesticide Authority's (FPA) List of Active Registered Fertilizers (Finished Products/Raw Materials) as of February 28, 2025**.** These results indicate that locally developed formulations have the potential to reduce dependence on imported or commercially supplied Bio-N inoculants, addressing supply limitations that have emerged following privatization. The viability and stability data generated in this study provide essential baseline information to support product registration, accreditation processes, and future scaling of technology within government-led biofertilizer programs. Furthermore, the development of stable, farmer-ready inoculants is expected to enhance field adoption of microbial alternatives, promoting more sustainable nutrient management practices and reducing reliance on synthetic nitrogen fertilizers. Hence, these findings highlight a clear pathway for strengthening the Philippine biofertilizer sector through the use of locally isolated strains, optimized carrier systems, and context-appropriate storage and handling technologies.

Conclusion and Future Works

This study demonstrated that the co-inoculated formulation of *Rhizobium tropici* and *Azospirillum* spp. possessed strong potential as a practical and sustainable nitrogen-fixing biofertilizer for local agricultural systems. Among all treatments, the balanced 50:50 mixture stored in aluminum foil under air-conditioned conditions showed the highest stability, maintaining 2.6×10^8 CFU/g at month 12—well above the regulatory threshold of $\geq 1 \times 10^6$ CFU/g. These findings underscore the importance of balanced co-inoculation, high-barrier foil packaging, and cool storage (20–25 °C) in extending shelf life and maintaining microbial viability during distribution.

Scientifically, the results confirm that co-inoculated systems benefit from complementary physiological traits, with *Azospirillum* spp. providing greater resilience and contributing to the overall stability of dual-strain formulations. Operationally, the study offers practical recommendations for DA-led and LGU-level production units to adopt improved packaging and storage strategies.

However, the study was conducted under controlled laboratory conditions using a soil–charcoal carrier, and real-world factors such as transport stress, sunlight exposure, handling variability, and fluctuating field temperatures were not evaluated. Additionally, the descriptive design does not support inferential comparisons among treatments.

To advance the implications of this work, future research should prioritize multi-location field trials to confirm the agronomic performance and nitrogen-fixation efficiency of the most stable formulations identified in the study. Further evaluation of the scalability and cost-effectiveness of aluminum foil packaging and temperature-controlled storage systems is also needed, particularly for production units operated by the Department of Agriculture and local government partners. In addition, exploring alternative or nanostructured carrier materials—such as rice hull biochar, corn cob fibers, and nanocellulose—may offer opportunities to further enhance moisture retention and microbial survival. The development of predictive models describing CFU

decline under varying storage scenarios would also support evidence-based storage recommendations and improve quality assurance standards. Collectively, these research directions will help translate laboratory findings into scalable, farmer-ready biofertilizer technologies that strengthen sustainable nutrient management across Philippine agricultural systems.

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Conflict of Interest

The authors declare the absence of any potential conflicts of interest that could have influenced the conduct, results, or interpretation of this study and its publication.

Artificial Intelligence (AI) Declaration Statement

The authors acknowledge the use of OpenAI's ChatGPT in formatting suggestions during manuscript preparation, and Grammarly AI tool for assistance in language refinement, and Scribbr for proper APA citation formatting. The said tools were not used for data analysis, interpretation of results, or the writing of original scientific content. All AI-assisted content was thoroughly reviewed and edited by the authors to ensure accuracy and integrity of the manuscript.