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Article

Advancing Sustainable Wheat Production in the Andes Through Biofertilization with *Azospirillum*, *Trichoderma* and Fermented Anchovy-Based Under Rainfed Conditions

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Abstract

Wheat (*Triticum aestivum* L.) sustains global caloric intake, but its productivity in Andean highlands is constrained by soil fertility and input reliance. This study represents one of the first field-based evaluations of biofertilizers under high-altitude, rainfed Andean conditions, addressing a major knowledge gap in low-input mountain agroecosystems. This study evaluated three seed-applied biofertilizers—*Azospirillum brasilense*, *Trichoderma viride* (Trichomax), and an anchovy (*Engraulis ringens*) based liquid biofertilizer, compared with an untreated control and a soil-test mineral fertilization benchmark in rainfed wheat (*Triticum aestivum* L.) cv. INIA 405 in the central Andes of Peru. A 5 × 5 Latin square design (25 plots) was established under farmer-realistic conditions. At physiological maturity (Zadoks 9.5), plant height, spike length, grains per spike, thousand-grain weight, test weight, root dry mass, and grain yield were recorded. Mineral fertilization achieved the highest yield (1.20 ± 0.79 t ha^{-1}), nearly doubling the control (0.60 ± 0.47 t ha^{-1}). Notably, *A. brasilense* delivered an intermediate yield of 0.90 ± 0.64 t ha^{-1}, representing a 50% increase over the control—accompanied by a marked rise in root dry mass. *T. viride* and the anchovy-based input yielded 0.85 ± 0.59 and 0.81 ± 0.59 t ha^{-1}, respectively. Grain physical quality remained stable across treatments (thousand-grain weight $\approx$ 42 g; test weight 68–75 kg hL^{-1}). Trait responses were complementary: root dry mass increased with mineral fertilization and *A. brasilense*, whereas spike length increased with mineral fertilization and the anchovy-based input. Overall, the evidence supports biofertilizers, particularly *A. brasilense*, as effective complements that enable partial fertilizer substitution within integrated nutrient-management strategies for sustainable wheat production in Andean rainfed systems.

Keywords: wheat; biofertilizer; *Azospirillum brasilense*; *Trichoderma viride*; root biomass; grain yield; sustainable agriculture

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1. Introduction

Common wheat (*Triticum aestivum* L.), originating from the Fertile Crescent in Western Asia [1], is the most important cereal worldwide, representing approximately 95% of total

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global wheat production and cultivated on more than 215 million hectares [2,3]. Along with rice and maize, wheat is among the three major cereals that provide more than 20% of global dietary energy [4,5]. This cereal is notable not only for its high protein content, exceeding 12%, and its elevated gluten levels, but also for contributing nearly 20% of daily caloric intake in the human diet [6–8]. Furthermore, wheat is a significant source of minerals, B vitamins, and dietary fiber, reinforcing its role as an essential component of a healthy diet [4,9,10]. In 2020, global wheat production surpassed 755 million tons, with average yields close to 3.5 t ha^{-1} [11]. The leading producers, China, India and Russia, account for nearly 40% of the global production volume [12].

National wheat production in Peru experienced a slight variation during the last decade. Until 2015, it remained above 200,000 tonnes, reaching approximately 215,000 tonnes, and then began a decline that culminated in about 186,800 tonnes in 2020 [13]. According to the most recent records from MIDAGRI, production was 188,469 t in 2019 and decreased marginally to 187,694 t in 2020, representing a drop of 0.41%. Efforts to expand production have been driven by policies aimed at reducing dependence on imports and strengthening food security in highland areas [14]. In the northern Andes, particularly in the department of Cajamarca, the *Avance Económico Departamental* bulletin from the National Institute of Statistics and Informatics (INEI, January 2025) reported that the area planted with wheat reached 9281 hectares during the 2023/2024 growing season [15].

However, this expansion has often been accompanied by the intensive and, in many cases, indiscriminate use of chemical fertilizers, leading to soil degradation, reduced fertility, and contamination of water resources [16,17].

Wheat cultivation in the Andean highlands occurs within a highly constrained agroecological environment where both soil and water limitations interact to restrict crop performance. Andean soils are typically shallow, acidic, and poor in organic matter, reflecting long-term degradation driven by steep terrain, intensive land-use, and limited nutrient replenishment [18]. These properties significantly reduce nutrient availability and hinder root exploration. Water-driven soil erosion is an additional and pervasive challenge: throughout the Peruvian and Ecuadorian Andes, the combination of rugged topography, sparse vegetation, and short but intense rainfall events accelerates topsoil loss and reduces soil structural stability [19,20]. In parallel, hydrometeorological assessments in regions such as the Peruvian Andes reveal a pronounced rainfall seasonality [21,22]. Land-use intensification—including the conversion of native grasslands to annual cropping systems and the degradation of temporary pastures—further exacerbates these trends by decreasing soil organic carbon, altering nutrient cycling, and increasing erosion susceptibility [23]. Together, these soil physical constraints and hydrological pressures create a fragile production environment in which wheat experiences limited nutrient uptake, reduced biomass accumulation, and heightened vulnerability to climatic variability, underscoring the need for management strategies that enhance nutrient-use efficiency and support the long-term resilience of high-Andean agroecosystems.

High-Andean soils such as those of Cajamarca are further constrained by the combined effects of low soil pH and only moderate soil organic matter (SOM), conditions that sharply limit nutrient availability and root functioning in wheat systems. Acidic pH enhances the solubility of Al^{3+} and Mn^{2+}, leading to root toxicity, reduced elongation, and impaired water and nutrient uptake (N, P, Mg, and Mo) [24–27]. Likewise, moderate SOM contents limit nitrogen mineralization, cation exchange capacity, and microbial activity, thereby constraining the soil's ability to supply nutrients in synchrony with crop demand [28]. These constraints occur within a broader Andean context characterized by slow SOM turnover, heterogeneous SOC distributions, and strong climatic controls imposed by cold temperatures and steep terrain [29]. Land-use studies across the Central Andes further show that cultivation accelerates soil acidification, increases exchangeable Al, reduces microbial biomass, and disrupts nutrient cycling [30], while erosion-driven nutrient losses dominate N, P, and K budgets in sloping fields [29,31]. Collectively, these soil constraints create a nutrient-limited environment in which wheat productivity depends heavily on practices that enhance nutrient-use efficiency and mitigate the chemical limitations imposed by soil acidity and low-to-moderate SOM.

Against this backdrop, biofertilizers have emerged as a sustainable alternative to improve plant nutrition, increase tolerance to stress factors, and reduce reliance on synthetic fertilizers [32,33]. Within this group, species of the genus *Trichoderma*, particularly *T. viride*, stand out for their ability to promote plant growth, enhance soil fertility, and biologically control soil pathogens, making them key tools for developing more resilient and sustainable agricultural systems [34–36]. In addition, other plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) have shown high potential for improving crop growth and yield, including in wheat [37–40]. PGPR, such as those from the genus *Azospirillum*, stimulate root and shoot development through the production of phytohormones like indole-3-acetic acid and gibberellins, while AMF, primarily from the genus *Glomus*, enhance water and phosphorus uptake and increase tolerance to abiotic stress [41,42]. Recent studies have reported that the co-inoculation of PGPR and AMF significantly increases root length, surface area, and dry biomass [43,44].

Empirical evidence supports the potential of these biofertilizers in wheat and other grasses, showing yield and grain quality improvements. Grageda-Cabrera et al. [45] demonstrated that AMF inoculation in wheat increased yields by up to 1.2 t ha^{-1} and improved nitrogen use efficiency by 11%. Similarly, Cisse et al. [46] found that combining biofertilizers with manure reduced the need for chemical fertilizers by up to 50% without compromising yields and, in some seasons, even increased yields by 6.8% to 12.4%. Likewise, Bhawana et al. [47], working with pearl millet (*Pennisetum glaucum*) in a pearl millet–wheat cropping system, reported significant improvements in yield and morphophysiological traits with the combined application of liquid biofertilizers and standard fertilization—results that can be extrapolated to wheat systems Similarly, Zaheer et al. [48] demonstrated that the cytokinin-producing strain *A. brasilense* RA-17 enhanced the net assimilation rate, leaf area, and grain yield of wheat, evidencing the positive physiological influence of biofertilizers on C3 crops.

In parallel, fish-derived liquid inputs (hydrolysates/ferments) provide readily available amino acids, peptides, micronutrients, and bioactive compounds that can stimulate early seedling vigor, microbial activity, and nutrient cycling [49,50]. Furthermore, research on wheat crop has confirmed significant gains in growth and yield through the application of liquid biofertilizers containing *Azospirillum* and *Azotobacter* [51,52]. In Egypt, El-Sorady et al. [53] reported a 14% increase in grain yield with *Azotobacter* inoculation, while Kaur et al. [54] observed the maximum yield with a combined inoculation with *Streptomyces* sp.

Despite this promise, evidence from high-Andean rainfed systems remains sparse, particularly for wheat. Many studies have been conducted in irrigated or lowland environments, have focused on a single biofertilizer, or have emphasized pot experiments that poorly represent field heterogeneity. Moreover, few trials around the world have evaluated seed-applied *Azospirillum*, *Trichoderma*, and fish-based liquid biofertilizers side-by-side in the same field context, with standardized measurements of root biomass, spike traits, grain yield, and grain quality. Addressing these gaps is critical because the Andean agroecological matrix—characterized by steep topographic gradients, acidic clay-loam soils, and cool night temperatures—may condition both the magnitude and stability of biofertilizer responses. Within this regional framework, the present work provides one of the first field-based evaluations conducted in Cajamarca-Perú, where rainfed conditions and soil constraints pose major challenges for nutrient management. The findings emphasize the potential of organic biofertilizers as complementary inputs within integrated fertilization strategies, aimed at enhancing nutrient efficiency and sustaining crop productivity rather than fully replacing conventional fertilization practices.

In this context, the present study aims to evaluate the effect of biofertilizers on wheat (*Triticum aestivum* L.), variety INIA 405, under the agroecological conditions of Cajamarca, Peru, with the objective of optimizing yield and promoting sustainable agricultural practices that mitigate the negative impact of chemical fertilizers on soil health and highland ecosystems.

2. Materials and Methods

2.1. Study Site

The study was conducted during the 2023–2024 growing season at the Sulluscocha Experimental Annex of the Baños del Inca Agricultural Experimental Station (INIA), located in the district of Llacanora, Cajamarca Province, Peru (7°12′8.06″ S, 78°22′18.10″ W; 2984 m a.s.l.) (Figure 1).

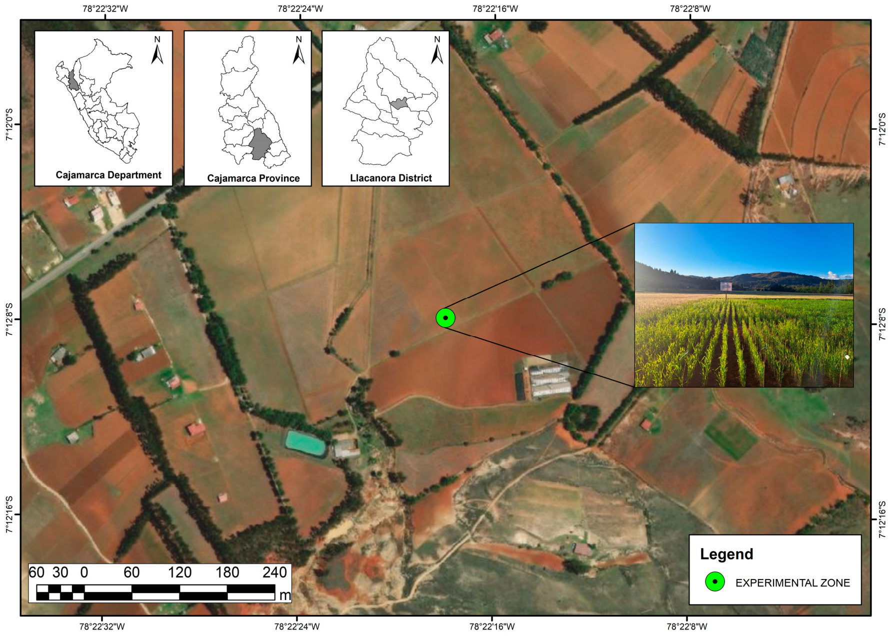


Figure 1. Geographic location of the experimental site. Field trials were conducted at the Sulluscocha Experimental Annex of the Baños del Inca Agricultural Experimental Station, National Institute for Agricultural Innovation (INIA), located in the Llacanora District, Cajamarca Province, Cajamarca-Peru. The grey-shaded areas in the inset maps indicate the geographic boundaries of the Cajamarca Department, the Cajamarca Province, and the Llacanora District, respectively.

Meteorological variables were obtained from SENAMHI. During the experimental period, recorded temperatures ranged from 7.9 °C to 22.5 °C, with mean daily values between 14.3 °C and 18.6 °C. The highest monthly precipitation was observed in February, reaching 120.3 mm. During the 2023–2024 agricultural campaign, rainfall in the experimental area remained within the typical range for the region, with no extreme events affecting crop development. Relative humidity was higher in the rainy months, reaching a maximum of 82% in January, and progressively decreasing to a minimum of 66% in August and September. The relative humidity exhibited higher values during the rainy season, with a maximum of 82% observed in January, and subsequently declined to a minimum of 66%

during August and September. On average, the relative humidity throughout the growing season was approximately 74.3% (Figure 2).

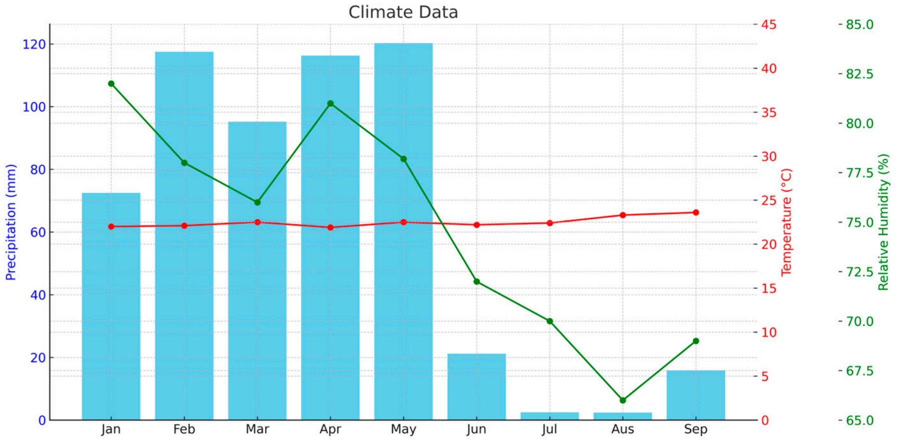


Figure 2. Monthly precipitation, maximum and minimum temperatures, and relative humidity during the 2023–2024 growing season at the Sulluscocha experimental annex, Baños del Inca Agricultural Experimental Station (INIA), Cajamarca, Peru.

2.2. Soil Physicochemical Properties

Before establishing the experiment, composite soil samples were collected from a depth of 0–40 cm and analyzed at the Soil Laboratory of the Baños del Inca Agricultural Experimental Station (INIA). The evaluated soil parameters included soil texture, pH, electrical conductivity, organic matter content, total nitrogen (N), available phosphorus (P), available potassium (K) and the concentration of exchangeable cations (Table 1).

Table 1. Soil physicochemical characteristics before planting the wheat.

Parameter	Result
pH	5.3
Electrical conductivity (S m^{-1})	11.5
Organic matter (%)	3.8
Total nitrogen (%)	0.19
Available phosphorus (mg kg^{-1})	15.2
Available potassium (mg kg^{-1})	233.6
Texture	Clay loam

2.3. Plant Material

The plant material was certified seed of wheat (*Triticum aestivum* L.) cv. INIA 405 ‘San Isidro’, supplied by the Instituto Nacional de Innovación Agraria (INIA, Peru). This cultivar exhibits a high average yield potential of approximately 5.2 t ha^{-1}, exceeding several traditional varieties, and shows good tolerance to yellow rust (*Puccinia striiformis*) and other foliar diseases prevalent in high-Andean environments. INIA 405 is adapted to 2500–3500 m a.s.l., particularly under the agroecological conditions of the central Peruvian highlands, and has an intermediate growth cycle of 130–140 days [55].

2.4. Experimental Design and Agronomic Management

The field trial followed a 5 × 5 Latin square design, totaling 25 experimental units arranged in five rows and five columns to control orthogonal gradients of soil fertility and microclimate. This design was employed to minimize experimental error associated with field heterogeneity in two directions, ensuring that each treatment was evaluated once per row and once per column, thus statistically controlling for positional effects.

Treatments were randomly assigned within each row and column following a complete randomization scheme to avoid positional bias. Each experimental unit consisted of a 4.8 m^2 plot composed of four rows, each 4 m in length and spaced 0.30 m apart; plots were separated by 1 m alleys to facilitate access and management. The net experimental area was 120 m^2, and the gross area, including alleys, was 156 m^2. Sowing was conducted on 20 February 2024, within the recommended planting window for the site, under rainfed conditions. In each plot, four rows were established by continuous seeding along the furrows using 70 g of seed per plot.

Inoculation treatments were applied once at sowing, and each inoculant was handled independently to avoid cross-contamination. No mixed inoculations were performed; each treatment corresponded to a single inoculation applied separately at the time of seeding, using sterile equipment and separate containers for each microbial material to ensure experimental integrity.

Five treatments were evaluated. TR1 was an absolute control without mineral fertilization or bioinoculants. TR2 received 100% mineral fertilization according to soil-test recommendations; nitrogen was split-applied, with 50% at sowing and 50% at tillering. The total fertilizer inputs per plot were 210 g of diammonium phosphate (DAP), 120 g of potassium chloride (KCl), and 370 g of urea, with no inoculation applied. TR3–TR5 involved pre-sowing seed inoculation by direct impregnation with specific biofertilizers: Seeds in TR1 (absolute control) and TR2 (full mineral fertilization) did not receive any form of liquid treatment or simulated inoculation; they were sown completely dry.

TR3 used *A. brasilense*, TR4 used Trichomax (*T. viride*), and TR5 used an anchovy (*Engraulis ringens*)-based liquid biofertilizer produced via anaerobic biofermentation. After inoculation (TR3–TR5), seeds were shade-air-dried to ensure uniform coating and inoculant viability prior to sowing.

2.5. Inoculation

Seed inoculation procedures followed a standardized seed-coating protocol. The *A. brasilense* strain Az007 used in this study was supplied by the FOCAM project "Use of Microorganisms for the Sustainable Organic Production of Quinoa, Maize and Avocado in the Ayacucho Region" of the Universidad Nacional San Cristóbal de Huamanga (UNSCH) [56]. The inoculum was cultured in nitrogen-free (NFb) liquid medium at a bacterial concentration of 1×10^8 CFU g^{-1}, following the method described by Condori et al. [57].

For inoculation, 350 g of wheat seed were placed in a 4 L plastic container and mixed with 56 mL of the *A. brasilense* suspension. The suspension had a viable bacterial density of approximately 1.6×10^8 CFU mL^{-1}, resulting in an estimated 4.8×10^6 CFU per seed. The mixture was gently stirred until all seeds were uniformly coated, then air-dried in the shade to preserve inoculant viability before sowing.

The *T viride* inoculant used in TR4 was the commercial wettable powder (WP) formulation. For seed treatment, 1 L of water was added to a 4 L container, followed by 24 g of the *T. viride* formulation. Subsequently, 350 g of seed were submerged and stirred thoroughly to ensure uniform coating, then shade-dried. The product's declared potency was 1×10^{12} conidia kg^{-1}; the carrier was sterile corn, q.s. to 1 kg (C.S.P., 1 kg).

2.6. Preparation and Characterization of the Anchovy-Based Biofertilizer

The anchovy-based liquid biofertilizer used in treatment TR5 was obtained from a commercial anaerobic biofermentation process based on fresh residues of *Engraulis ringens*. According to the provider, the product is produced through a controlled anaerobic fermentation carried out in polyethylene containers for 45–60 days at ambient temperature (18–25 °C), with no aeration and without the addition of synthetic chemical stabilizers.

During fermentation, the pH gradually decreases to 4.5–5.2, inhibiting spoilage microorganisms and stabilizing the organic matrix. The final product consists of a liquid hydrolysate rich in soluble amino acids, peptides, micronutrients, and naturally occurring microbial metabolites. Each batch undergoes basic quality control, including measurement of pH, density, and absence of putrefactive odors, before packaging. For the anchovy-based biofertilizer treatment, 200 mL of an anaerobically biofermented anchovy (*Engraulis ringens*) liquid biofertilizer were diluted in 1 L of water within a 4 L container. Then, 350 g of seed were submerged, mixed to achieve complete coverage, and shade-dried.

The chemical composition of the applied product was determined from the manufacturer's certified analysis and is provided in Table S1. These values represent the nutrient concentrations per liter of the biofertilizer at the time of application.

2.7. Agronomic Parameters

Agronomic parameters were assessed at physiological maturity [58] (Zadoks growth scale, Z 9.5), 167 days after sowing. Sampling was conducted from the two central rows of each experimental unit to avoid border effects. For morphological and spike traits, plants or spikes were randomly collected within each plot; grain yield and test weight were measured on the harvested grain from the central rows of each plot.

Plant height (cm) was measured with a ruler from the stem base to the tip of the spike, excluding awns. Spike length (cm) was recorded from the base of the rachis to the spike apex using a graduated ruler. The number of grains per spike was determined by randomly selecting five spikes per plot, counting total grains per spike, and computing the arithmetic mean. Thousand-grain weight (g) was obtained by randomly selecting and weighing 1000 grains from the harvested seed of each plot using a high-precision balance (WLC 10/A2). Test weight (kg hL^{-1}) was measured with a grain moisture and test-weight analyzer (Ag–MAC Plus, Agra Tronix, Bradenton, FL, USA). Grain yield (t ha^{-1}) was calculated by weighing the grain harvested from the central rows of each plot, corresponding to a harvested area of 2.4 m^2, using a digital precision balance (WLC 10/A2) and scaling the plot weight (g) to a per-hectare basis. Root biomass (g) was quantified by carefully excavating twenty plants per plot to preserve root systems; roots were gently washed to remove adhering soil, oven-dried at 60 °C for 48 h (TIN TN-115) to constant mass, and weighed on an analytical balance. Root dry mass was subsequently used to compare belowground growth among treatments and to explore its association with agronomic and yield traits.

2.8. Statistical Analysis

Data were analyzed according to a 5 × 5 Latin square design using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). The following additive linear model was fitted for each variable:

$$Y_{ijk} = \mu + F_i + C_j + T_k + \varepsilon_{ijk}$$

where Y_{ijk} is the observed response, μ is the overall mean, F_i is the effect of the i^{th} row, C_j is the effect of the j^{th} column, T_k is the fixed effect of the k^{th} treatment, and ε_{ijk} is the random experimental error.

Analyses of variance (ANOVA) were conducted using the PROC GLM procedure with type III sums of squares. Model assumptions of normality and homogeneity of variances were verified using the Shapiro–Wilk and Bartlett (or Levene) tests applied to residuals. When necessary, data were transformed to meet model assumptions. To validate model robustness, analyses were also confirmed using PROC MIXED, considering rows and columns as random effects.

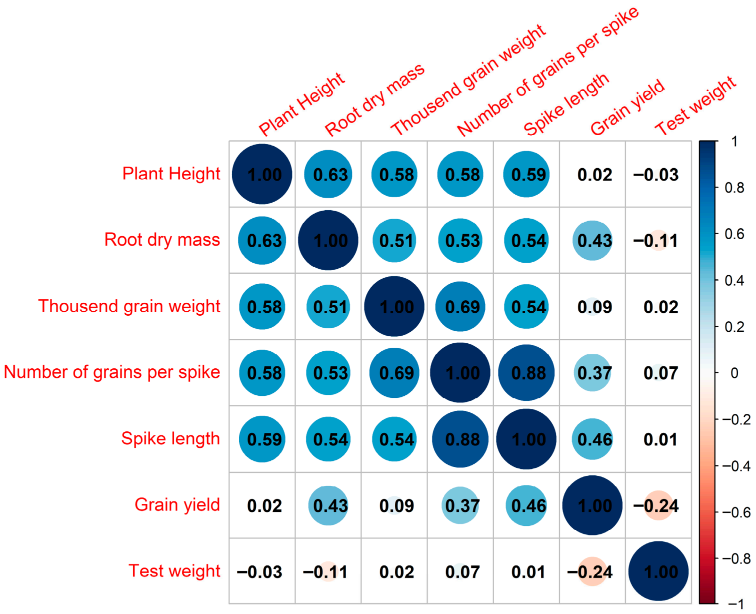
When the overall F-test was significant ($p \leq 0.05$), treatment means were compared using Tukey's Honest Significant Difference (HSD) test at $\alpha < 0.05$, applied to adjusted means (LSMeans).

Multivariate structure was examined by principal component analysis (PCA) using R software (Version 4.5),with FactoMineR (Version 2.12) and factoextra (Version 1.0.7) packages to summarize trait contributions and visualize treatment-related patterns. The quality of variable representation was evaluated with squared cosine ($\cos^2$) values, and variable contributions to each component were expressed as percentages.

3. Results

3.1. Correlation Analysis

The correlation matrix revealed coherent associations among agronomic and physiological traits (Figure 3). Plant height was positively correlated with root dry mass ($r = 0.63$), thousand-grain weight ($r = 0.58$), and spike length ($r = 0.59$), and root dry mass showed a positive association with grain yield ($r = 0.43$). Thousand-grain weight displayed moderate to strong correlations with grains per spike ($r = 0.69$) and spike length ($r = 0.54$), underscoring its relevance to yield potential. The strongest relationship was between grains per spike and spike length ($r = 0.88$), reinforcing the structural linkage between spike morphology and grain number. By contrast, grain yield exhibited generally weak correlations with most traits aside from root mass, and test weight showed a slight negative association with grain yield ($r = -0.24$).



	Plant Height	Root dry mass	Thousend grain weight	Number of grains per spike	Spike length	Grain yield	Test weight
Plant Height	1.00	0.63	0.58	0.58	0.59	0.02	−0.03
Root dry mass	0.63	1.00	0.51	0.53	0.54	0.43	−0.11
Thousend grain weight	0.58	0.51	1.00	0.69	0.54	0.09	0.02
Number of grains per spike	0.58	0.53	0.69	1.00	0.88	0.37	0.07
Spike length	0.59	0.54	0.54	0.88	1.00	0.46	0.01
Grain yield	0.02	0.43	0.09	0.37	0.46	1.00	−0.24
Test weight	−0.03	−0.11	0.02	0.07	0.01	−0.24	1.00

Figure 3. Correlation matrix among agronomic and physiological traits in wheat. Circle size and color intensity represent the strength of Pearson's correlation coefficients (r), with blue indicating positive and red negative associations.

3.2. Agronomic and Physiological Traits

3.2.1. Plant Height (cm)

Plant height was lowest in the unfertilized, uninoculated control (TR1: 53.05 ± 3.99 cm), while the highest means were observed in the anchovy-based biofertilizer (TR5: 57.77 ± 4.07 cm) and mineral fertilization (TR2: 57.06 ± 4.06 cm) treatments. Intermediate values occurred with seed inoculation by *A. brasilense* (TR3: 55.74 ± 4.04 cm) and *Trichoderma* (TR4: 54.27 ± 4.01 cm). Compared to the control, plant height increased by 7.6% under mineral fertilization, 8.9% with the anchovy-based biofertilizer, 5.1% with

A. brasilense, and 2.3% with *Trichoderma*. Collectively, these results indicate modest but consistent improvements in plant height with both chemical fertilization and biofertilizer application relative to the untreated control (Figure 4A, Table 2).

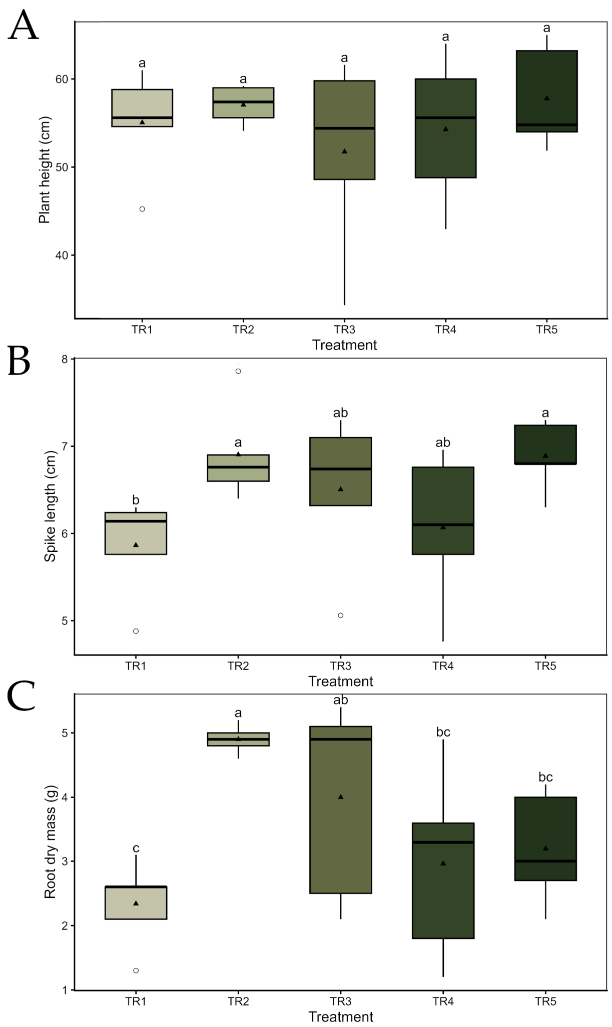


Figure 4. Effect of five treatments (TR1-TR5) on Wheat INIA 405 San Isidro: (**A**) Plant height (cm), (**B**) Spike length (cm) y (**C**) root dry mass (g). Box-and-whisker plots show the distribution of observations across the five treatments. Horizontal lines represent the medians, black triangles indicate the treatment means. Circles denote outlier values automatically identified by the boxplot algorithm (values beyond 1.5 × IQR). Different lowercase letters above the boxes indicate statistically significant differences according to Tukey's test ($p < 0.05$).

Table 2. Effect of five treatments (TR1–TR5) on agronomic traits of wheat (*Triticum aestivum* L.) INIA 405 San Isidro under the agroecological conditions of Cajamarca, Peru. Values are means ± standard error. Different lowercase letters within the same column indicate significant differences according to Tukey's test ($p < 0.05$).

Treatment	Spike Length (cm)	Number of Grains per Spike	Thousand Grain Weight (g)	Root Dry Mass (g)	Plant Height (cm)	Test Weight (kg hL^{-1})	Grain Yield (t ha^{-1})
	Mean ± E.E	Mean ± E.E	Mean ± E.E	Mean ± E.E	Mean ± E.E	Mean ± E.E	Mean ± E.E
TR1	5.9 ^{b} ± 0.58	24 ^{a} ± 3.22	42.24 ^{a} ± 1.83	2.34 ^{c} ± 1.21	53.05 ^{a} ± 3.99	75.28 ^{a} ± 4.33	0.6 ^{c} ± 0.47
TR2	6.9 ^{a} ± 0.56	30 ^{a} ± 3.43	42.66 ^{a} ± 2.30	4.90 ^{a} ± 1.77	57.06 ^{a} ± 4.06	68.10 ^{a} ± 4.24	1.2 ^{a} ± 0.79
TR3	6.5 ^{ab} ± 0.88	29 ^{a} ± 3.40	42.54 ^{a} ± 2.83	4.00 ^{ab} ± 1.61	55.74 ^{a} ± 4.04	70.58 ^{a} ± 4.27	0.9 ^{ab} ± 0.64
TR4	6.1 ^{ab} ± 0.87	23 ^{a} ± 3.18	41.90 ^{a} ± 2.52	2.96 ^{bc} ± 1.38	54.27 ^{a} ± 4.01	73.88 ^{a} ± 4.32	0.8 ^{bc} ± 0.59
TR5	6.9 ^{a} ± 0.40	28 ^{a} ± 3.37	42.00 ^{a} ± 3.25	3.20 ^{bc} ± 1.44	57.77 ^{a} ± 4.07	72.78 ^{a} ± 4.30	0.8 ^{bc} ± 0.59

E.E: Standard Error.

3.2.2. Spike Lenght (cm)

Spike length was greatest under mineral fertilization (TR2: 6.9 ± 0.56 cm) and the anchovy-based liquid biofertilizer (TR5: 6.9 ± 0.40 cm), followed by *A brasilense* (TR3: 6.5 ± 0.88 cm) and *Trichoderma* (TR4: 6.1 ± 0.87 cm), with the shortest spikes in the untreated control (TR1: 5.9 ± 0.58 cm). Relative to the control, spike length increased by 16.9% under mineral fertilization, 16.9% with the anchovy-based biofertilizer, 10.2% with *A. brasilense*, and 3.4% with *Trichoderma*. These patterns indicate that both chemical fertilization and the fish-derived biofertilizer were associated with longer spikes relative to the control. (Figure 4B, Table 2).

3.2.3. Root Dry Mass (g)

Root dry mass differed significantly among treatments ($p < 0.05$). Mineral fertilization achieved the highest mean (TR2: 4.90 ± 1.77 g), followed by seed inoculation with *A. brasilense* (TR3: 4.00 ± 1.61 g). Intermediate values were observed for *Trichoderma* (TR4: 2.96 ± 1.38 g) and the anchovy-based liquid biofertilizer (TR5: 3.20 ± 1.44 g), whereas the untreated control showed the lowest root mass (TR1: 2.34 ± 1.21 g). Relative to the control, root biomass increased by 109.4% under mineral fertilization, 70.9% with *A. brasilense*, 36.8% with the anchovy-based biofertilizer, and 26.5% with *Trichoderma*. Overall, both mineral fertilization and *A. brasilense* inoculation markedly enhanced belowground biomass compared to the untreated control (Figure 4C, Table 2).

3.2.4. Number of Grains per Spike

The highest number of grains per spike was observed under mineral fertilization (TR2: 30 ± 3.43) and *A brasilense* inoculation (TR3: 29 ± 3.40). Intermediate means occurred with the anchovy-based biofertilizer (TR5: 28 ± 3.37) and the untreated control (TR1: 24 ± 3.22), while the lowest value was recorded with *Trichoderma* (TR4: 23 ± 3.18). Relative to the control, the number of grains per spike increased by 25.0% under mineral fertilization, 20.8% with *A. brasilense*, and 16.7% with the anchovy-based biofertilizer, whereas *Trichoderma* showed a slight reduction of 4.2%. Although some treatments showed slight numerical increases in grain number per spike, these differences were not statistically significant (Figure 5A, Table 2).

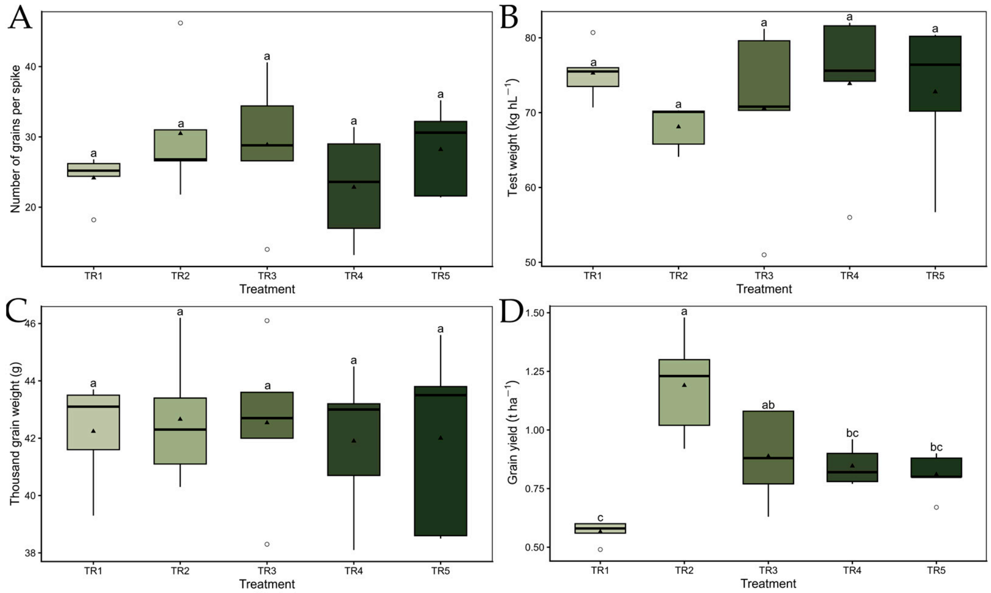


Figure 5. Effect of five treatments (TR1-TR5) on Wheat: (**A**) Number of grains per spike, (**B**) Test weight (cm), (**C**) Thousand grain weight (g) and (**D**) Grain yield (t ha^{-1}). Box-and-whisker plots show

the distribution of observations across the five treatments. Horizontal lines represent the medians, black triangles indicate the treatment means. Circles denote outlier values automatically identified by the boxplot algorithm (values beyond 1.5 × IQR). Different lowercase letters above the boxes indicate statistically significant differences according to Tukey's test ($p < 0.05$).

3.2.5. Test Weight (kg hL^{-1})

Test weight was highest in the untreated control (TR1: 75.28 ± 4.33 kg hL^{-1}), followed by *Trichoderma* (TR4: 73.88 ± 4.32 kg hL^{-1}) and the anchovy-based biofertilizer (TR5: 72.78 ± 4.30 kg hL^{-1}), with slightly lower means for *A. brasilense* (TR3: 70.58 ± 4.27 kg hL^{-1}) and mineral fertilization (TR2: 68.10 ± 4.24 kg hL^{-1}). Overall, grain physical quality appeared uniform across treatments, as all means fell within the same statistical group (Figure 5B, Table 2).

3.2.6. Thousand Grain Weight (g)

Thousand-grain weight showed minimal variation among treatments. The highest mean was recorded under mineral fertilization (TR2: 42.66 ± 2.30 g), closely followed by *A. brasilense* (TR3: 42.54 ± 2.83 g), the anchovy-based biofertilizer (TR5: 42.00 ± 3.25 g), and the untreated control (TR1: 42.24 ± 1.83 g), with the lowest value under *Trichoderma* (TR4: 41.90 ± 2.52 g). Overall, thousand-grain weight remained stable across treatments, indicating no appreciable effect of fertilization or inoculation on this grain physical attribute (Figure 5C, Table 2).

3.2.7. Grain Yield (t ha^{-1})

Grain yield was highest under mineral fertilization (TR2: 1.20 ± 0.79 t ha^{-1}) representing a 100% increase compared with the untreated control (TR1: 0.60 ± 0.47 t ha^{-1}), which had the lowest mean. Intermediate yields were observed with *A. brasilense* (TR3: 0.90 ± 0.64 t ha^{-1}), *Trichoderma* (TR4: 0.85 ± 0.59 t ha^{-1}), and the anchovy-based biofertilizer (TR5: 0.81 ± 0.59 t ha^{-1}), corresponding to yield improvements of 50.0%, 41.7%, and 35.0%, respectively, relative to the control (Figure 5D, Table 2).

3.3. PCA

Principal component analysis (PCA) clarified the structure of trait covariation and the major contributors to variability among treatments. The scree plot indicated that the first two components Dim-1 (PC1) and Dim-2 (PC2) captured the dominant share of variance (69.4%). PC1 was primarily associated with spike fertility and vegetative growth, with strong positive contributions from grains per spike, spike length, root dry mass, and plant height (Figure S1). In contrast, PC2 was defined mainly by yield-related attributes, particularly grain yield and test weight, which showed the highest loadings along this axis (Figure S2). Squared cosine ($\cos^2$) values confirmed that spike length and grains per spike were well represented on PC1, whereas grain yield and test weight dominated PC2 (Figures S3 and S4).

The PCA biplot made these relationships explicit: grains per spike and spike length clustered in the positive direction of PC1, reflecting their tight correlation and shared influence on spike productivity, while grain yield and test weight aligned with PC2, indicating their distinct contribution to the second axis. Thousand-grain weight and plant height occupied intermediate positions, with moderate contributions across both components. The strongest variable contributions were observed for grains per spike ($r^2 = 0.668$) and spike length ($r^2 = 0.610$) on PC1, and for grain yield ($r^2 = 0.535$) and root dry mass ($r^2 = 0.413$) on PC2 (Figure 6).

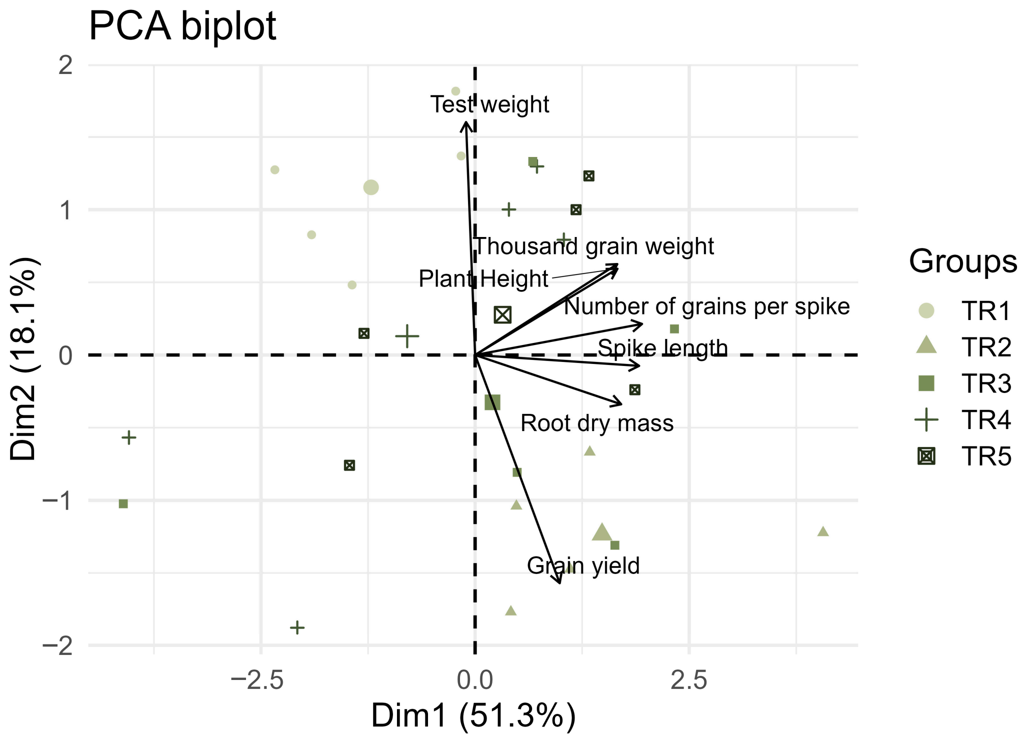


Figure 6. Principal component analysis (PCA) biplot showing the distribution of treatments and trait vectors (Black arrows) for grain yield, test weight, thousand grain weight, plant height, root dry mass, spike length, and number of grains per spike. PC1 and PC2 together explained more than 70% of the total variance.

4. Discussions

Our field results demonstrate that biofertilizer inoculation can significantly enhance wheat performance in high-altitude rainfed systems. The application of microbial and organic biofertilizers—*A brasilense*, *T viride*, and an anchovy-based liquid fertilizer—improved both physiological traits and yield outcomes of wheat (cv. INIA 405) under realistic farming conditions. In particular, *A. brasilense* seed inoculation stimulated vigorous belowground growth, evidenced by a marked increase in root biomass (relative to the control). This is consistent with the well-documented root-promoting activity of *Azospirillum*, which produces phytohormones (e.g., auxins, gibberellins) that spur root development and thereby expand the plant's capacity for water and nutrient uptake [59,60]. A more developed root system in the inoculated plants likely underpinned their improved shoot growth and yield [61,62]. Indeed, *A. brasilense* treatment in our trial raised grain yield to ~0.90 t ha^{-1}, about a 50% increase over the unfertilized control (0.60 t ha^{-1}) and approaching the yield obtained with full NPK fertilization (~1.2 t ha^{-1}). Because inoculated treatments received no mineral fertilizers yet achieved ~70–75% of the yield of NPK fertilization, our results support biofertilizers as complementary inputs that can reduce mineral fertilizer requirements under low-input conditions, not as full substitutes for mineral nutrient supply.

The anchovy-derived biofertilizer also showed notable aboveground benefits: it significantly increased spike length and grain number per spike compared to the control, indicating an improvement in spike architecture [63,64]. This finding aligns with reports that fish protein hydrolysate biostimulants can promote the development of larger wheat ears with more grains [65]. *T. viride* inoculation yielded a more moderate improvement in grain yield and did not markedly change root or spike metrics in our study; however, the positive yield response suggests that *Trichoderma*'s benefits may manifest through subtler mechanisms such as enhanced nutrient mobilization or stress alleviation (even without dramatic changes in morphology) [66–69]. In sum, all three biofertilizers boosted wheat growth and productivity relative to unfertilized plants, with *A. brasilense* showing the strongest effect on root biomass and the fish-based fertilizer most enhancing spike traits [70–72].

Our findings are broadly in agreement with recent studies on biofertilizer impacts in cereals. Numerous field trials have documented that inoculation with plant growth-promoting rhizobacteria can increase grain yields, though the magnitude varies with environmental context [40,73–75]. Under favorable fertility conditions, yield gains tend to be modest—for example, *A. brasilense* inoculation in well-fertilized wheat plots still led to about a 10% yield uplift in a Brazilian study [60]. By contrast, under nutrient-limited or stress conditions, biofertilizers can elicit larger relative increases. We note that reports of increased kernel weight with microbial inoculants are context-dependent and not universal; for example, Raza et al. [76] reported that co-inoculating wheat with *Azospirillum* and *Agrobacterium* raised grain yield by 36% and also improved the thousand-kernel weight by 17%. In our trial, although microbial persistence was not assessed, the yield advantages observed with *Azospirillum* and *Trichoderma* relative to the control align with the positive effects reported in the literature. The yield benefits of *Trichoderma* inoculants observed in our study (~40–45% over the control) are in line with those reported in other wheat-growing regions [77–80]. For instance, *T. harzianum* strains tested in India boosted wheat yields by ~29–36% across semi-arid and humid locations [81], and *T. viride* biofertilizer increased grain yield by 36.5% in field trials in Nepal [82]. Similar efficacy has been noted in other crops as well; e.g., cucumber and cabbage yields were improved by approximately 25–35% with *Trichoderma*-based treatments [83,84]. These literature comparisons reinforce that the yield gains we observed in the Peruvian Andes (on the order of tens of percent) are credible and within the range reported for biofertilizer-induced increases in cereal production worldwide.

It is noteworthy that the biofertilizers in our study achieved a substantial proportion of the chemically fertilized yield under very low-input conditions. The wider spread observed for inoculation-only treatments (TR3–TR5) reflects the intrinsic biological variability of microbial inoculants under rainfed highland field conditions: as living organisms, their establishment, rhizosphere colonization, and metabolic activity vary across microenvironments (pH, moisture, root exudates, competition with native microbiota), yielding more heterogeneous plot-level effects than typically seen with mineral fertilization. While the full NPK treatment unsurprisingly produced the highest grain yield, the *Azospirillum*, *Trichoderma* and fish fertilizer treatments each produced ~70–75% of that maximal yield despite receiving no mineral nutrients. This outcome underscores the potential of biofertilizers to partially substitute for synthetic fertilizers in resource-limited settings. A key mode of action is through improved nutrient use efficiency [59,60]. In our trial, inoculated plants showed greater nitrogen uptake and utilization efficiency than uninoculated plants (e.g., Azospirillum-treated wheat had higher nitrogen recovery efficiency than the control), which agrees with other studies reporting enhanced agronomic N use efficiency and uptake with *A. brasilense* inoculation [60]. *Trichoderma* can also facilitate plant nutrition—for example, *T. harzianum* has been shown to increase crop N acquisition by stimulating root growth and nutrient solubilization [85]. By boosting the availability and uptake of nutrients naturally, biofertilizers allow plants to attain higher yields per unit of soil fertility. In practical terms, this means they can reduce but not eliminate the need for synthetic inputs, helping optimize fertilizer efficiency rather than replace it outright. Galindo et al. [60] demonstrated this principle: inoculating wheat with *A. brasilense* enabled a 50% reduction in N fertilizer (from 100 kg ha^{-1} to 50 kg ha^{-1}) with no yield penalty, even resulting in a slight profit increase. Our results support this idea—the biofertilized wheat plots, with zero synthetic NPK added, still far outperformed the unfertilized control and yielded robustly, highlighting that a considerable fraction of the crop's nutrient demand can be met through biological inputs and native soil fertility processes.

Although the present experiment focused primarily on plant responses, several well-documented soil and rhizosphere mechanisms help explain the observed yield and biomass improvements under *Azospirillum*, *Trichoderma*, and anchovy-based biofertilizer treatments. For *Trichoderma*, multiple studies demonstrate that this fungus enhances root development, nutrient mobilization, and physiological resilience through mechanisms such as improved rhizospheric enzyme activities, enhanced nutrient solubilization, and modulation of plant stress responses. For example, *T. viride* has been shown to significantly increase plant height, root length, and spike length in wheat, especially when combined with organic substrates such as humic acids [82,86]. Other studies report increased yield components (up to +36.5% grain yield) associated with enhanced nutrient acquisition and improved root physiology [82]. In addition, *T. viride* can activate host antioxidant enzymes such as catalase, peroxidases, and superoxide dismutase, thereby reducing oxidative stress and improving plant health under biotic challenges [87]. These rhizosphere and physiological effects are consistent with the moderate but positive yield responses we observed for *T. viride* inoculation.

The comparatively weaker performance of *T. viride* in our field trial may reflect the interaction between its physiological mechanisms and the edaphic constraints of high-altitude soils. Although *Trichoderma* is well known to promote plant growth through auxin and gibberellin production, enhancement of nutrient utilization efficiency, and stimulation of root-associated enzymatic activities, the expression of these functions is strongly conditioned by environmental factors. According to recent comprehensive syntheses, *Trichoderma* relies on multiple modes of action such as competition, antibiosis, mycoparasitism, secretion of cell-wall-degrading enzymes, volatile secondary metabolites, and induction of systemic resistance to support plant performance [88–90]. However, the activity of these mechanisms decreases under suboptimal pH, low temperatures, or nutrient-limited soils, which can reduce conidial germination, metabolic activity, and the production of key hydrolytic enzymes [91]. Such environmental sensitivity may have constrained the ability of *T. viride* to colonize roots effectively or to express its full biostimulatory potential under our high-Andean conditions, thereby explaining its more moderate yield response compared with *A. brasilense* and the anchovy-based biofertilizer.

A. brasilense also exerts well-characterized mechanistic effects on wheat that align with our observations. Experimental evidence shows that *A. brasilense* enhances nitrogen uptake and accumulation, increases grain protein concentration, and stimulates root proliferation through hormonal pathways such as auxin production [92]. Field studies further demonstrate that inoculation improves nutrient uptake efficiency across macro- and micronutrients and can increase wheat yields by 13–31% depending on the strain and environmental conditions [93]. More recent trials confirm that *A. brasilense* enhances agronomic N-use efficiency, increases N recovery, and raises yield by >10% even under varying N fertilization regimes [60].

Beyond nutrient uptake, the hormonal activity of *A brasilense* provides an additional mechanistic explanation for the strong root proliferation observed in our trial. A hallmark trait of *Azospirillum* is its ability to synthesize indole-3-acetic acid (IAA), the primary auxin regulating cell division, elongation, and root system architecture. Recent evidence demonstrates that IAA biosynthesis in *A. brasilense* is highly plastic and responsive to environmental cues: daylight, osmotic stress (PEG), abscisic acid, salicylic acid, chitosan, and fungal elicitors (e.g., *Fusarium oxysporum*) all significantly increase IAA production and *ipdC* gene expression [94]. This suggests that under stressful highland rainfed conditions, *A. brasilense* would likely enhance its auxin output, thereby intensifying its plant-growth-promoting effects. The centrality of IAA to wheat growth promotion is further confirmed by experiments using *A. brasilense* mutants impaired in *ipdC* expression, which produce only ~10% of normal auxin levels; these mutants show dramatically reduced ability to stimulate root and shoot growth compared with the wild-type strain [95]. Conversely, strains engineered to overexpress *ipdC* induce even greater improvements in plant biomass. These mechanisms provide a strong physiological basis for the substantial root biomass and yield improvements detected in our *A. brasilense* treatment.

The anchovy-based biofertilizer used in our study likely acted through mechanisms related to organic matter enrichment, amino acid supply, and stimulation of root system development. Recent analyses of anchovy-derived fertilizers reveal that these products are exceptionally rich in essential and non-essential amino acids such as leucine, glycine, alanine, and glutamic acid, which are known to enhance microbial metabolism, nutrient mineralization, and rhizosphere activity [96]. Beyond their amino acid profile, anchovy residues processed through circular bioeconomy pathways (e.g., "AnchoisFert") contain substantial amounts of organic carbon, proteins, flavonoids, magnesium, potassium, phosphate, and sulfate, making them powerful slow-release organic fertilizers capable of outperforming conventional mineral and organic fertilizers in crop systems [64,97]. These biochemical attributes are consistent with enhanced nutrient cycling and increased metabolic activity in the rhizosphere, which can result in improved shoot growth and reproductive traits

Together, the mechanistic evidence from the literature supports the interpretation that the yield gains observed in our study arise from improved nutrient acquisition, enhanced rhizosphere activity, and strengthened physiological resilience—despite the fact that post-harvest soil indicators such as SOC, aggregation, or enzymatic activity were not measured.

Crucially, the yield improvements from biofertilization did not come at the cost of grain quality in our experiment. Grain physical parameters, including thousand-grain weight and test weight, remained statistically similar across all treatments. The 1000-grain weight hovered around ~42 g in both biofertilized and control plots, and test weight was around 68.10–75.28 kg hL^{-1} for all, indicating that grains from biofertilizer-treated plants were as well-filled and dense as those from untreated or fully fertilized plants. This stability in grain size and density suggests that the additional grains produced under biofertilizer treatments were not smaller or shriveled—an encouraging outcome implying no trade-off between yield quantity and quality. In some cases, biofertilizers may even enhance certain quality aspects: for instance, the fish protein hydrolysate used by Mironenko et al. [65] led to slight increases in wheat grain protein (about +2% protein content and +5% gluten content) alongside a ~5% yield boost. We did not measure protein content in our study, but maintaining high test weight and kernel weight is itself a positive sign of grain quality. Overall, our findings concur with the notion that biofertilizers primarily increase yield by increasing the number of fertile spikes or grains (via improved growth and nutrient uptake), rather than by markedly altering individual grain mass [98–100]. Even where modest increases in grain weight have been observed with PGPR or fungal inoculants, the dominant effect remains an increase in grain number per area [76]. The use of these biofertilizers improved yield components (spike size, grain number) without detrimental effects on grain filling.

From an agronomic and ecological perspective, these results are highly promising. Biofertilizers are not a complete replacement for mineral fertilizers, but our study illustrates that they can reduce the requirement for synthetic inputs while improving crop performance—a win-win for sustainability [101,102]. Farmers in highland areas could utilize products like *Azospirillum* and *Trichoderma* inoculants or fish-based fertilizers to boost their wheat yields and stability, cutting down on costly chemical fertilizer usage. This not only has economic benefits (lower input costs, potentially higher net profits) but also environmental ones. Reduced application of chemical N/P fertilizers means lower risk of nitrogen leaching and runoff, and a smaller carbon footprint associated with fertilizer production and use [103,104]. By leveraging naturally occurring soil microbes and organic nutrient sources, this approach aligns with the principles of agroecological intensification—enhancing soil health and fertility through biological means rather than solely through industrial inputs [105,106]. Notably, *A. brasilense* and *T. viride* are both benign organisms that pose minimal risk to the environment, making them safe and sustainable tools for crop management [60]. Their use can also contribute to long-term soil quality (e.g., through better root growth and organic matter inputs from increased residue), thereby fostering a more resilient agroecosystem in the face of climatic stresses [40].

Finally, our study provides original field evidence of biofertilizer efficacy in the Andean highlands, under realistic rainfed farming conditions. To date, most research on biofertilizers in wheat has been conducted in temperate or subtropical regions, often under controlled or irrigated conditions. In contrast, the high Andes present a challenging environment with marginal soils, drought-prone climate, and limited access to inputs—a context where sustainable yield improvements are critically needed. Potential legacy fertility from historical management is a known feature of on-farm soils; randomization within a Latin square and the control mitigate this, yet cannot eliminate all residual heterogeneity. Demonstrating that inoculants like *Azospirillum* and *Trichoderma* can significantly improve wheat root growth, spike productivity, and grain yield in this setting is therefore a noteworthy contribution. It confirms that the benefits of biofertilization are not confined to lowland intensive systems but are also attainable in subsistence-oriented, rainfed mountain agriculture. This is particularly pertinent for Peru and other Andean countries, where enhancing the productivity of staple crops must be balanced with preserving fragile ecosystems and reducing farmers' dependence on imported agrochemicals. Our results highlight a viable strategy: integrating biofertilizers with conventional practices to achieve more with less. In practical terms, a farmer in the high Andes could inoculate wheat seeds with *A. brasilense* or *T. viride*, or apply an organic fish-based amendment, and expect a considerable yield increase along with improved root vigor and no loss in grain quality—all while using lower amounts of synthetic fertilizer. Such an approach improves the sustainability of wheat production by contributing to nutrient cycling, reducing external inputs, and enhancing agroecosystem health. In conclusion, this study provides strong evidence that biofertilizers can augment wheat productivity in high-altitude rainfed systems, and it underscores their role as complementary inputs that can partially replace chemical fertilizers to foster more sustainable and agroecologically sound cereal production in the Andes. The positive outcomes observed serve as an encouraging example of how harnessing beneficial microbes and organic nutrients can drive agricultural innovation in traditionally under-served farming regions.

5. Conclusions

This study provides the first field-based evidence from the Peruvian Andes that biofertilizers can substantially enhance wheat performance under rainfed, high-altitude conditions. Unlike most previous trials conducted in irrigated or temperate regions, these results were obtained under the challenging agroecological context of the Andes, where soils are marginal and synthetic inputs are limited. The demonstrated improvements in root biomass, spike architecture, and yield highlight the potential of *A. brasilense*, *T. viride*, and fish-based organic fertilizers to strengthen crop productivity within these resource-constrained environments. Importantly, these biofertilizers should be viewed as complementary components of integrated nutrient management rather than complete substitutes for mineral fertilizers. Their application can improve nutrient use efficiency and soil biological activity, allowing partial reductions in synthetic fertilizer use without compromising grain quality. This integrative approach offers a realistic pathway toward more sustainable, low-input wheat production systems across the Andean highlands, combining traditional practices with modern microbial and organic technologies to enhance both yield and environmental resilience.

To support broader adoption of biofertilizers in Andean agriculture, targeted promotion strategies are essential. Demonstration plots and participatory field schools are essential for building farmer confidence, as visual evidence of improved crop vigor and reduced fertilizer requirements strongly influences adoption in smallholder systems. To ensure consistent field performance, we recommend that extension programs provide clear protocols for seed inoculation (e.g., dosage, dilution, drying time, and storage conditions) and promote the co-application of biofertilizers with minimal starter mineral fertilization under highly depleted soils. Strengthening local production of microbial inoculants, particularly *Azospirillum*, *Trichoderma* and fish-based organic fertilizers through community-level biofactories can reduce costs and improve access in remote areas

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol6010013/s1, Table S1. Chemical composition of the biofermented anchovy-based fertilizer (*Engraulis ringens*); Figure S1. Variable contributions to PC1; Figure S2. Variable contributions to PC2; Figure S3. Variable cos2 on PC1; Figure S4. Variable cos2 on PC2.

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Data Availability Statement: The data are available by contacting corresponding authors for collaboration.

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Conflicts of Interest: The authors declare no conflicts of interest.

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