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Effect of L-Tryptophan and *Rhizobium* on Growth, Physiology, and Auxin Production of Maize (Zea mays L.)

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Abstract

This heavy reliance on chemical fertilizers has led to environmental problems and has prompted the adoption of sustainable practices to enhance crop production. The effect of L-tryptophan (L-TRP) and Rhizobium inoculants on the growth, physiological characteristics, indole-3-acetic acid (IAA) production and yield of maize (Zea mays L.) under controlled pot culture conditions was evaluated in this study. The experiment was conducted in a completely randomized design with six treatments and three replications, including an untreated control, Rhizobium alone, and two levels of L-TRP applied alone and with Rhizobium. Optimum concentration of L-TRP was found to be specific to each response, as at physiological maturity, the lower concentration of L-TRP in combination with Rhizobium resulted in the best shoot and root length, fresh weight of shoots and roots, and grain yield, while the higher concentration of L-TRP showed the best response for chlorophyll. IAA production was also greatest when the low concentration combined treatment was used, and combined treatments generally produced more IAA than either amendment alone, suggesting a synergistic effect between bacterial inoculation and precursor availability rather than an additive effect. There was no measurable difference in harvest index among treatments, with increased biomass accumulation being the primary driver of yield gains. These results demonstrate that the use of Rhizobium, along with an optimized concentration of L-tryptophan, can enhance the growth and yield of maize under controlled conditions, and that the effects are trait-specific, indicating the need for field testing, molecular identification of the strain, and replication in future studies.

Keywords: Plant growth-promoting rhizobacteria; Biofertilizer; Indole-3-acetic acid; Plant bio-stimulants; Sustainable nutrient management; Grain yield

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1. Introduction

Global food demand is constantly increasing due to population growth, urbanization, and changing dietary trends, putting pressure on agricultural systems in major maize-producing countries like Pakistan, where nitrogen management is a major constraint on productivity (Gul et al., 2021). Excessive fertilizer application over the decades has resulted in soil degradation, nutrient imbalances, groundwater contamination, greenhouse gas emissions and loss of beneficial soil microbes (Kayesh et al., 2023; Huang et al., 2024). These issues have driven an increased focus on sustainable nutrient management practices that optimize productivity while protecting soil and environmental quality, and beneficial soil microorganisms and natural plant bio-stimulants are becoming increasingly important supplements to chemical fertilizers (Baloch, 2025; Singh et al., 2025).

Plant growth-promoting rhizobacteria (PGPR) are capable of stimulating plant growth via direct or indirect pathways such as biological nitrogen fixation, phosphate solubilization, synthesis of the siderophore and the production of the phytohormone (Frankenberger & Arshad, 1995), ACC deaminase activity and induction of stress tolerance, which collectively improve nutrient acquisition, root elaboration, and photosynthetic efficiency in various environmental conditions (Aioub et al., 2022; Baloch, 2025). L-tryptophan (L-TRP), the main biochemical precursor of indole-3-acetic acid (IAA), the most abundant auxin involved in cell division, elongation, vascular differentiation, apical dominance, lateral root formation, and response to environmental stimuli, is the most important naturally occurring plant growth regulator (Vanneste & Friml, 2009). In maize and other crops, exogenous L-TRP has been found to stimulate endogenous auxin production, chlorophyll synthesis, nitrogen utilization, and biomass accumulation (Frankenberger & Brunner, 1983; Sarwar et al., 1992; Muneer et al., 2009).

While traditionally considered nitrogen-fixing symbionts of legumes, several *Rhizobium* strains can colonize non-leguminous crops, such as maize, and act as PGPR. In addition to biological nitrogen fixation, *Rhizobium* also promotes plant growth by producing phytohormones, mobilizing nutrients, and promoting root development (Mehboob et al., 2012; Khoso et al., 2025). The *Rhizobium* found in legume root nodules is able to produce IAA in the absence of the addition of a precursor, and the addition of L-TRP significantly boosts microbial IAA production (Datta & Basu, 2000; Sarwar et al., 1992), implying that the availability of the precursor directly regulates the microbial auxin synthesis. Similar precursor–microbe interactions have been reported in other auxin-producing soil bacteria like *Azospirillum* (Frankenberger et al., 1990) and *Azotobacter* (Zahir et al., 2000). Qureshi et al. (2013) observed that *Rhizobium* and L-tryptophan, when applied together, could produce synergistic effects, as maize growth and fodder yield were greater when both were applied than when applied individually.

However, there are still key knowledge gaps. Previous studies have assessed microbial inoculation or L-TRP application separately, and only a few have investigated their combined effects at different precursor concentrations throughout the maize crop cycle. The L-TRP concentration required to maximize microbial auxin production without disrupting hormonal balance remains unclear, and it is not known whether the early gains in IAA production are sustained through harvest and whether this will have a positive impact on yield. Hence, this study aimed to evaluate the influence of two levels of L-tryptophan (L-TRP) alone and in combination with *Rhizobium* inoculation on growth, physiological traits, chlorophyll accumulation, IAA production, and yield of maize under pot conditions. We postulated that the combined application would have a synergistic effect on maize growth by increasing the levels of microbial IAA; that the lower level of L-TRP will be a better source of auxin biosynthesis than the higher level; and that the effects observed in the seedling stage will carry over the entire growth of the crop to increased biomass accumulation and grain yield.

2. Materials and Methods

2.1 Experimental Site and Plant Material

The experiment was conducted under controlled greenhouse conditions at the Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad, Pakistan (31°26′N, 73°06′E). Maize (*Zea mays* L.) seeds of a locally adapted cultivar (FH-1041) were obtained from a certified seed source. Healthy, uniform seeds were selected, surface-sterilized with 70% ethanol for 2 minutes followed by 2% sodium hypochlorite for 5 minutes, and rinsed thoroughly with sterile distilled water before sowing. Pots were filled with 10 kg of air-dried, sieved soil collected from the experimental farm. The soil was characterized as sandy loam with pH 7.8, electrical conductivity 1.2 dS m^{-1}, organic matter 0.85%, total nitrogen 0.06%, available phosphorus 7.5 mg kg^{-1}, and exchangeable potassium 180 mg kg^{-1}. Visible plant residues and stones were removed, and soil was thoroughly mixed to ensure homogeneity. The study was arranged in a completely randomized design (CRD) with six treatments and three replications (R1, R2, and R3) (Figure 1). Pots were randomly positioned on greenhouse benches and repositioned weekly to minimize positional effects. Plants were irrigated with tap water as needed to maintain field capacity. Standard agronomic practices were uniformly applied to all pots.

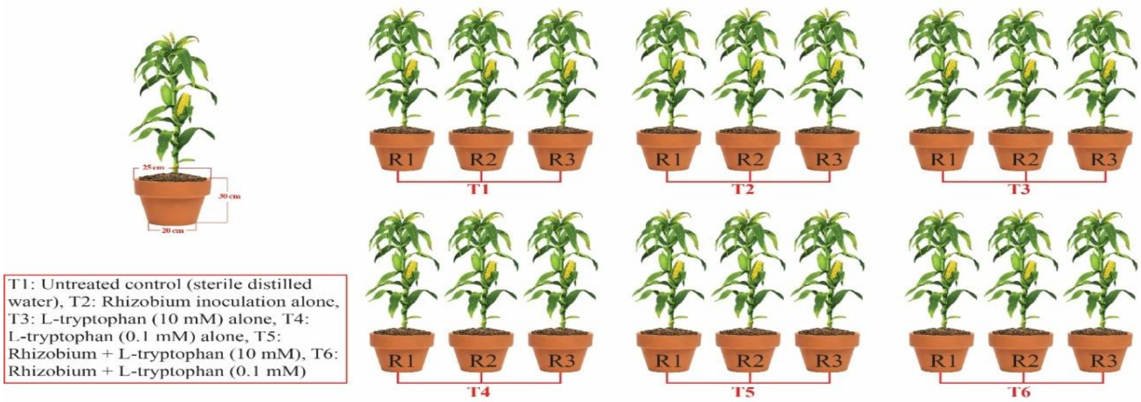

Figure 1. Experimental pot layout under greenhouse conditions.

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2.2 *Preparation and Application of Rhizobium and L-Tryptophan*

A plant growth-promoting *Rhizobium* strain (earlier isolated and characterized for the production of IAA) was kept on yeast extract mannitol agar (YEMA) medium. Fresh bacterial cultures were prepared by inoculating 100 mL YEMA broth and incubating at 28 ± 2 °C, 120 rpm for 48 hr. The bacterial cells were centrifuged (5000 rpm for 10 minutes), washed twice with sterile phosphate-buffered saline (PBS) and resuspended in sterile distilled water to a final concentration of 10^8 colony-forming units (CFU) mL^{-1} (OD 600 = 0.5). Before sowing, the seeds were soaked in the bacterial inoculant for 2 hr. The analytical-grade L-tryptophan (Sigma-Aldrich, USA) was dissolved in sterile distilled water to prepare 10 mM and 0.1 mM stocks. Treatment combinations were applied as soil drenches at 0 and 15 days at a rate of 50 mL per pot.

2.3 *Growth Measurements*

Plant growth parameters were taken at the seedling stage (18 days after sowing) and at physiological maturity. Three plants per pot were carefully dug up, and the roots were washed with tap water. Shoot length, root length, shoot fresh weight and root fresh weight were taken.

2.4 *Chlorophyll Determination*

The seedling stage (18 days after sowing) leaf chlorophyll content was measured by the acetone extraction method (Lichtenthaler, 1987). A fresh leaf sample (0.5 g) was ground in 10 mL of 80% acetone, centrifuged (4000 rpm, 10 minutes), and the supernatant was spectrophotometrically measured (UV-Visible spectrometer, Shimadzu UV-1800, Japan) at 663, 645, and 470 nm.

2.5 *Indole-3-Acetic Acid (IAA) Determination*

IAA production by the *Rhizobium* strain was assessed in culture supernatant at the seedling stage (18 days after sowing), following the colorimetric Salkowski assay (Salkowski, 1885). Bacterial cultures (48 hr old) were centrifuged at 5000 rpm for 10 minutes, and 2 mL of cell-free supernatant was mixed with 4 mL of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$). After incubation in darkness for 30 minutes, absorbance was measured at 535 nm. IAA concentration was determined using a standard curve prepared with analytical grade IAA (0–100 µg mL^{-1}). This assay measures IAA in the bacterial culture supernatant rather than plant tissue IAA content; it reflects microbial auxin production potential rather than total plant auxin levels.

2.6 *Plant Yield Components*

At physiological maturity, ears were harvested manually, and grain yield was determined after threshing and drying (12% moisture content). Grain yield per plant was converted to kg ha^{-1} using a standard plant density of 75,000 plants ha^{-1}. Harvest index (%) was calculated as (grain yield / biological yield) × 100.

2.7 *Statistical Analysis*

Data were subjected to analysis of variance (ANOVA) using Statistix 8.1 software. Treatment means were compared using Fisher's Least Significant Difference (LSD) test at $P \leq 0.05$. Data are presented as mean ± standard error (SE) of three replications. Data were tested for normality using the Shapiro–Wilk test and homogeneity of variances using Levene's test. When assumptions were violated, the data were appropriately transformed before ANOVA.

3. Results

3.1 *Effect of Rhizobium and L-Tryptophan on Seedling Growth*

The shoot length, root length and root fresh weight did not significantly differ between the treatments at 18 days after sowing (Figure 2a, b, d). The only significant treatment effect was shoot fresh weight (Figure 2c). The shoot fresh weight was significantly lower in T5 (0.30 g) than in all other treatments (0.51–0.63 g), which were not significantly different from each other. These were T1 (0.53 g) and T6 (0.63 g), numerically but not statistically the highest. T4 and T2 showed the highest number of values for shoot length and root length, respectively, and T5 and T3 showed the lowest number of values for each of the two measurements, respectively, but none of those differences was significant. There was minimal difference in root fresh weight among treatments (0.26–0.35 g). In general, the effects of *Rhizobium* and L-TRP, applied alone or in combination, were minimal in the seedling stage, and the low-concentration combination (T6) did not yield any significant difference from the control treatment; however, the effect of combination application of the two treatments was significant at this stage in reducing shoot fresh weight under the high-concentration combination (T5).

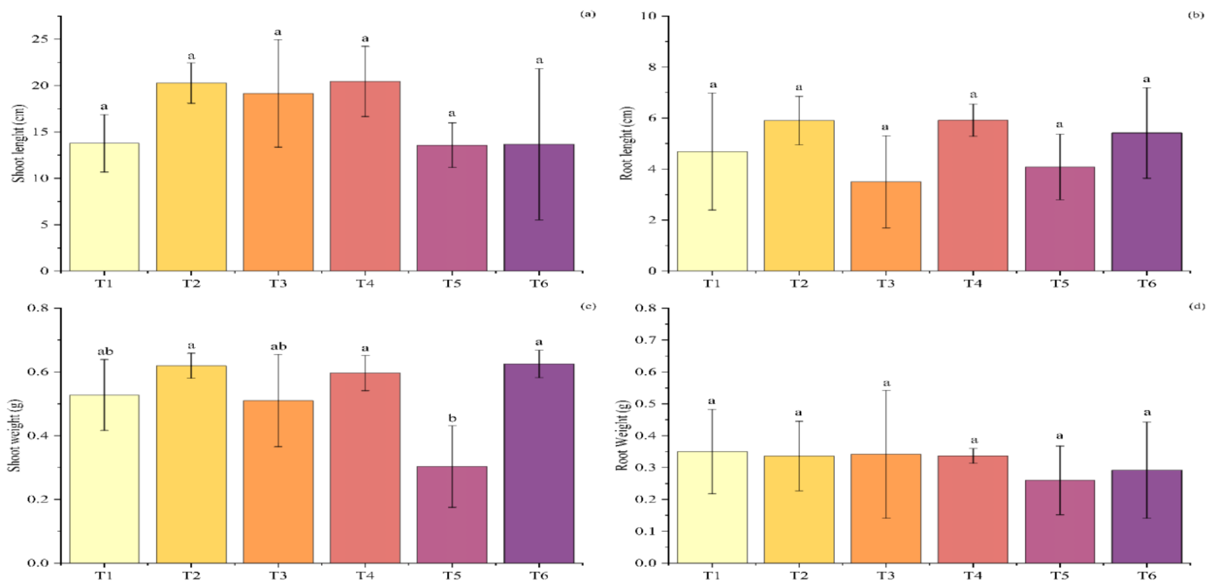

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Figure 2. Growth parameters (a) shoot length, (b) root length, (c) shoot fresh weight, and (d) root fresh weight of maize at the initial (seedling) stage. Bars represent means ± SE (n = 3); bars not sharing a common letter differ significantly (Fisher's LSD test, P ≤ 0.05); ns = not significant. Control = T1, Rhiz = T2 (*Rhizobium* alone), T10 = T3 (L-TRP 10 mM), T0.1 = T4 (L-TRP 0.1 mM), Rhiz+T10 = T5, Rhiz+T0.1 = T6.

3.2 Effect of Rhizobium and L-Tryptophan on Growth at Physiological Maturity

All growth parameters showed highly significant treatment effects at physiological maturity (Figure 3). T6 showed the highest shoot length (200.50 cm), root length (83.83 cm), shoot fresh weight (99.27 g), and root fresh weight (53.40 g). It was found that increases were 21.6% (shoot length), 50.3% (root length), 53.3% (shoot fresh weight), and 63.3% (root fresh weight) compared with the control. Also, T5 contributed substantially to growth, although its values were always lower than those of T6. *Rhizobium* or L-TRP applied singly showed significant improvement in growth compared to the control, but not as good as when applied together.

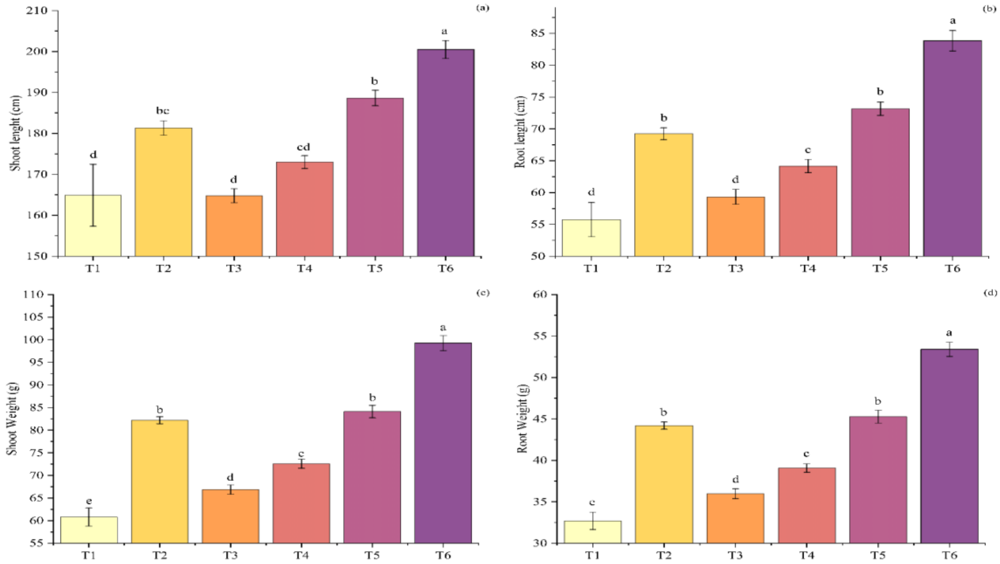

Figure 3. Growth parameters (a) shoot length, (b) root length, (c) shoot fresh weight, and (d) root fresh weight of maize at the harvest stage. Bars represent means ± SE (n = 3); bars not sharing a common letter differ significantly (Fisher's LSD test, P ≤ 0.05); ns = not significant. Control = T1, Rhiz = T2 (*Rhizobium* alone), T10 = T3 (L-TRP 10 mM), T0.1 = T4 (L-TRP 0.1 mM), Rhiz+T10 = T5, Rhiz+T0.1 = T6.

3.3 Chlorophyll Pigments

Chlorophyll a, b and total chlorophyll content showed significant treatment effects at the seedling stage (Figure 4). Total chlorophyll was highest under T5 (0.174 mg g^{-1} FW), followed by T4 (0.164 mg g^{-1}), with increases of 28.9% and 21.5% over T1 (0.135 mg g^{-1}), respectively. T6 had a slight increase in total chlorophyll (0.143 mg g^{-1} or 5.9% more than control) while the lowest total chlorophyll was found in T3 (0.119 mg g^{-1} or 11.9% less than control). Chlorophyll a increased the highest under treatment T1 (0.103 mg g^{-1}) and decreased with all treatments except T6 (0.098 mg g^{-1}), while the other fraction, chlorophyll b, showed a reverse trend, increasing most under T5 (0.110 mg g^{-1}) and T4 (0.102 mg g^{-1}) compared to the control (0.032 mg g^{-1}). These results suggest that L-TRP and *Rhizobium* treatments did not always result in greater chlorophyll in the plants; in this case, the highest L-TRP concentration with *Rhizobium* (T5) treatment showed the greatest pigment response at this stage.

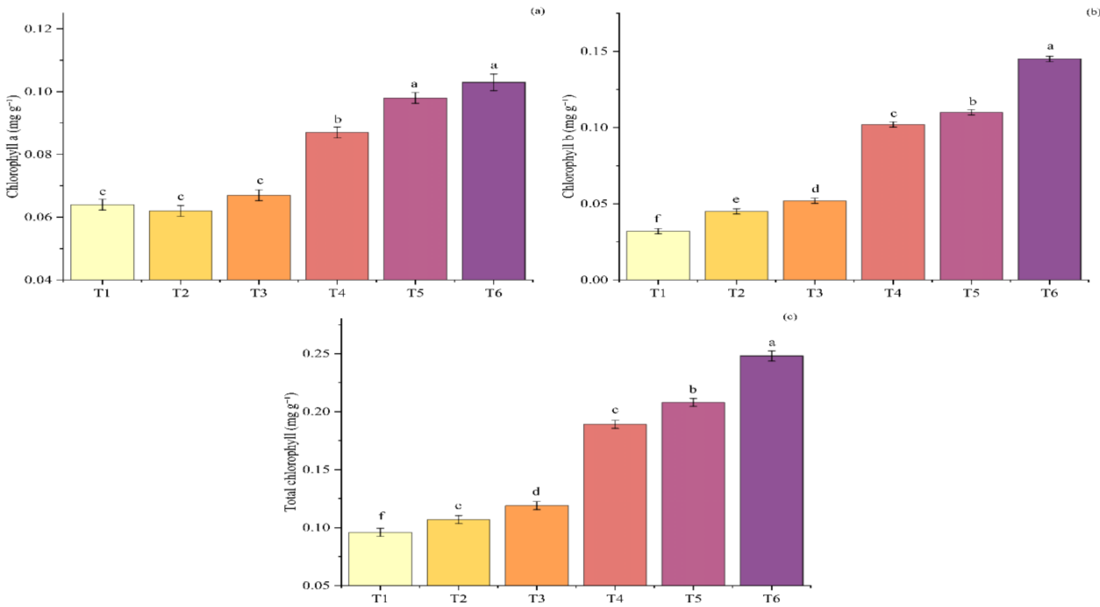

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Figure 4. (a) Chlorophyll a, (b) chlorophyll b, and (c) total chlorophyll content (mg g^{-1} FW) of maize at the seedling stage (18 days after sowing). Bars represent means ± SE (n = 3); for each pigment, bars not sharing a common letter differ significantly (LSD, $P \leq 0.05$).

3.4 Indole-3-Acetic Acid (IAA) Production

At the seedling stage, IAA production was greatly affected by treatments (Figure 5). The highest concentration of IAA (7.50 μg mL^{-1}) was observed in T6, which was 400% more than the control (1.50 μg mL^{-1}). The concentration of T5 was the second highest (6.20 μg mL^{-1}), followed by *Rhizobium* alone (T2, 5.80 μg mL^{-1}). Increased IAA production was observed in individual applications of *Rhizobium* or L-TRP compared to the control, and the combined application of *Rhizobium* and L-TRP showed a synergistic effect in all cases, indicating that at this concentration, the availability of L-TRP and *Rhizobium* inoculation enhanced IAA production. This early auxin stimulus was not re-evaluated at physiological maturity, so it is not possible to confirm that this early auxin stimulus continues throughout the growth cycle; the continued superiority of T6 in growth and yield at harvest (Sections 3.2, 3.5) is consistent with the early auxin stimulus continuing through the growth cycle, but it is not confirmed.

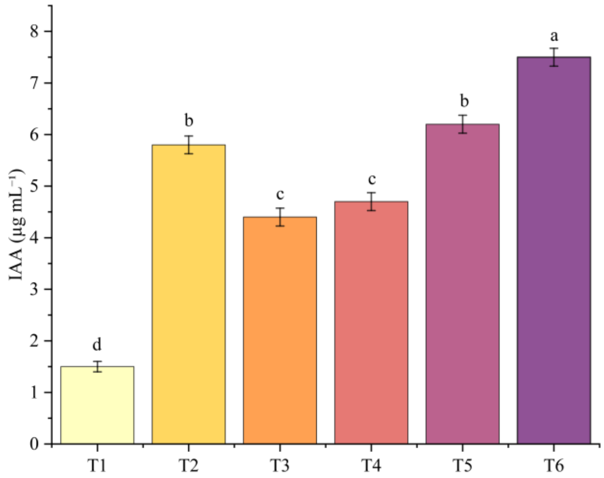

Figure 5. Indole-3-acetic acid (IAA) content of maize at the seedling stage (18 days after sowing). Bars represent means ± SE (n = 3); bars not sharing a common letter differ significantly (LSD, $P \leq 0.05$).

3.5 Grain Yield and Harvest Index

The treatments resulted in significantly different grain yield, but not harvest index (Table 1). The highest grain yield was achieved in the treatment T6, which was 62.5% higher than the control treatment. Individual treatments were followed by T5. Harvest index showed small differences among individual replicates but no significant differences, indicating that higher yields were due to increased biomass production rather than changes in biomass distribution.

Table 1. Effect of treatments on yield and yield-related traits of maize. Control = T1, Rhiz = T2 (*Rhizobium* alone), T10 = T3 (L-TRP 10 mM), T0.1 = T4 (L-TRP 0.1 mM), Rhiz+T10 = T5, Rhiz+T0.1 = T6. Values are mean ± SE (n = 3); within a column, means not sharing a common letter differ significantly (Fisher's LSD test, $P \leq 0.05$).

Treatment	Cob Weight	Grain Weight per Cob	1000-Grain Weight	Grain Yield per Plant	Harvest Index	Grain Yield
	(g)	(g)	(g)	(g)	(%)	(kg ha^{-1})
T1	45.0 ± 1.73 d	32.0 ± 1.00 d	220.0 ± 4.36 d	32.0 ± 1.00 d	34.2 ± 0.62 a	2400 ± 75 d
T2	58.5 ± 1.32 b	43.5 ± 1.32 b	252.0 ± 3.61 b	43.5 ± 1.32 b	34.4 ± 0.72 a	3263 ± 99 b
T3	49.5 ± 1.32 c	36.0 ± 0.87 c	230.0 ± 4.36 d	36.0 ± 0.87 c	35.0 ± 0.30 a	2700 ± 65 c
T4	53.2 ± 1.04 c	39.0 ± 0.87 c	238.0 ± 2.65 c	39.0 ± 0.87 c	34.9 ± 0.40 a	2925 ± 65 c
T5	60.0 ± 1.73 b	45.0 ± 0.87 b	258.0 ± 3.46 b	45.0 ± 0.87 b	34.8 ± 0.21 a	3375 ± 65 b
T6	68.0 ± 1.73 a	52.0 ± 1.80 a	278.0 ± 5.29 a	52.0 ± 1.80 a	34.0 ± 0.64 a	3900 ± 135 a
ANOVA (P-value)	<0.001	<0.001	<0.001	<0.001	0.210	<0.001

4. Discussion

The synergistic effect of *Rhizobium* and L-tryptophan on the growth of maize, its chlorophyll content, IAA production, and grain yield under both conditions, compared to either treatment alone, supported the hypothesis that precursor-mediated stimulation of microbial phytohormone biosynthesis may improve plant performance. That is, the treatment that led to the highest vegetative growth, L-TRP production, and yield was not the same as the one that resulted in the greatest chlorophyll accumulation. This suggests that the optimal concentration of L-TRP might differ across physiological traits and depend on the process being optimized.

The clear positive effect of combined treatments over individual amendments suggests a synergistic effect between both in which the availability of the precursors can stimulate the production of IAA by the microbes, and the bacteria can improve the plant's uptake of nutrients and root architecture on their own (Spaepen & Vanderleyden, 2011; Vanneste & Friml, 2009). This trend is in line with previous findings regarding PGPR use in maize cultivation (Zahir et al., 2005; Mehboob et al., 2012) and extends this to show that the benefit is concentration-dependent, but caution must be

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exercised when making comparisons with field-based studies, as there are differences in scale and environmental variability.

The better growth and yield results in lower L-TRP concentrations imply that microbial auxin biosynthesis that supports these growth parameters is highly controlled; that is, surplus L-TRP may result in saturation of the microbial metabolic pathway, or in the production of auxin levels that are inhibitory instead of stimulatory (Spaepen & Vanderleyden, 2011; Chen & Khoso, 2025). This dose sensitivity was not seen in the case of chlorophyll accumulation, with the higher dose (especially in combination with *Rhizobium*) showing the highest chlorophyll response, suggesting that there is no single "optimal" dose for all measured traits, and that this will need to be optimized on a trait-by-trait basis for practical application. Concentration-dependent responses have been reported for other auxin-producing rhizobacteria (Zahir et al., 2000; Khalid et al., 2001).

The relationship between chlorophyll response and auxin availability does not seem to be the only factor involved because there was no statistically significant association between the levels of IAA and total chlorophyll (across treatment means) and differences in chlorophyll content existed even at the same IAA concentration during treatment, suggesting that other physiological factors such as nitrogen availability and utilization (Arshad & Frankenberger, 1998) might have also played a role in inducing the differences in pigment. Most of the treatments also resulted in a decrease in chlorophyll a level and an increase in chlorophyll b, suggesting that L-TRP and *Rhizobium* changed the partitioning of the pigments, which could be related to the regulation of the chlorophyll cycle (Tanaka & Tanaka, 2011; Khoso et al., 2024), but not uniformly stimulating total pigment biosynthesis, as the type of regulation was not investigated here.

The modest treatment difference observed at the seedling stage, compared with the marked differences at physiological maturity, suggests that the benefits of *Rhizobium* and L-TRP are cumulative, and that the early sampling time may have been before the treatment differences became apparent. The pattern highlights the need to evaluate the effect of PGPR/Bio-stimulants across the entire crop stage, not just at the seedling stage, as results at the seedling stage would have greatly underestimated the final measured effect at harvest.

The results highlight the potential of using PGPR and natural bio-stimulants together in sustainable maize production systems to reduce synthetic fertilizer use and confirm that the benefits depend on the concentration of the precursor used and the desired outcome. The practical consequence is that combined inoculation strategies seem to work mainly by increasing the total pool of resources available to the plant, rather than by changing how the plant allocates its resources to grain yield, as yield improvement was not due to a change in partitioning.

These findings are subject to several caveats. The *Rhizobium* strain was not identified to the species level and was not deposited in a public culture collection, reducing the ability to reproduce and make cross-study comparisons. The Salkowski assay, which quantified IAA, was performed on the microbial culture supernatant rather than on plant tissue and therefore does not directly reflect the plant's IAA level. The available statistical power to detect smaller effects, especially at the seedling stage, was limited (only three replications per treatment); the controlled pot environment also does not reflect the variability in the environment and microorganisms that affect inoculant performance in the field. These limitations could be addressed through molecularly characterized strains in field trials and by replicating the trials, which appears to be a logical next step towards the practical translation of the results into biofertilizer recommendations.

5. Conclusion

The present study showed that *Rhizobium* sp. inoculation, combined with L-tryptophan, enhanced the growth, physiological performance, IAA production, and grain yield of maize under greenhouse conditions. *Rhizobium* with 0.1 mM L-tryptophan had the highest vegetative growth and grain yield; *Rhizobium* with 10 mM L-tryptophan had the highest response in chlorophyll, indicating that the response was dependent on concentration and trait. The results indicate that the use of PGPR and natural bio-stimulants could complement synthetic fertilizers.

The generalizability of these results, however, is limited by the identified bacterial strain, limited replication (n = 3), and controlled pot conditions. The results should be confirmed under field conditions, molecular identification of the *Rhizobium* strain should be done, and the mechanisms of concentration-dependent and trait-specific responses should be explored in future studies. These studies will help develop biofertilizer strategies for sustainable maize production that can be implemented in the field.

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