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ARTICLE

Rhizobium Strain S2_8_1 Promotes Ryegrass Regrowth under Soil and Hydroponic Conditions

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ABSTRACT: (1) Purpose: Sustainable forage production requires strategies that accelerate plant regrowth while reducing reliance on synthetic nitrogen fertilizers. This study aimed to evaluate the potential multifunctionality of a plant growth-promoting microorganism, strain S2_8_1, hypothesized to enhance ryegrass regrowth in association with increased rhizosphere nitrification and cytokinin-related plant responses. (2) Methods: Comparative experiments were conducted using S2_8_1 and a cytokinin producing *Streptomyces* strain (Shan2) under both soil and hydroponic conditions. Treatments were evaluated for biomass production, soil NO_3^--N content, nitrification rate, and leaf zeatin riboside (ZR) levels. Multiple linear regression was applied to quantify the relative contributions of nitrification and cytokinin signaling to regrowth. (3) Results: S2_8_1 was associated with increased soil nitrification and leaf ZR accumulation, leading to a 52% improvement in total biomass after 14 days of regrowth. Regression analysis showed that nitrification rate and ZR content jointly explained over 90% of biomass variation (R^2 = 0.91–0.92), indicating a strong joint statistical association. Under hydroponic conditions, S2_8_1 retained functional activity, increasing ZR by 47% and biomass by 41% within 10 days. By contrast, Shan2 promoted regrowth primarily through cytokinin production, with limited impact on soil nitrogen dynamics. (4) Conclusions: S2_8_1 is associated with coordinated nitrification- and cytokinin-related responses contributing to ryegrass regrowth. Its plant growth-promoting effects suggest its potential as a multifunctional biofertilizer for reducing fertilizer dependence and supporting sustainable forage production.

KEYWORDS: Cytokinin; nitrification; biofertilizer; regrowth; plant microbe interaction

1 Introduction

Feeding a growing global population requires transformative strategies to reconcile agricultural productivity with environmental sustainability [1–3]. While chemical fertilizers have historically boosted crop yields, their low nutrient use efficiency has led to groundwater contamination, soil degradation, and greenhouse gas emissions, which issues that jeopardize the long-term viability of agroecosystems [4,5]. These concerns have intensified the search for microbial solutions that can enhance crop productivity while reducing environmental impact [6]. In this context, plant growth-promoting micro¥organisms (PGPM) that improve both nutrient cycling and phytohormone regulation are gaining attention as sustainable alternatives [7,8].

PGPMs are increasingly recognized for their role in supporting food production and plant-based industries [9,10]. However, most research has focused on either nutrient cycling or on modulating plant

hormone pathways, rather than both. Species from the genera *Bacillus* and *Pseudomonas* have been shown to improve soil nitrogen status, thereby enhancing nutrient uptake and promoting plant growth [8,11]. In contrast, many strains, particularly from *Streptomyces*, produce phytohormones such as cytokinins that stimulate regrowth and aid plant recovery following damage [12,13]. Despite these advances, the system-level mechanisms by which dual-functional rhizosphere microbes simultaneously influence nutrient cycling and hormone signaling remain largely unexplored.

Nitrogen is essential for all living organisms, yet its bioavailable forms are limited in many ecosystems. Regrowth following defoliation is a critical determinant of productivity in forage systems, particularly for species such as ryegrass that are subjected to frequent cutting or grazing. This process involves rapid re-establishment of photosynthetic capacity and biomass accumulation, which are tightly regulated by both nutrient availability and hormonal signaling. Although atmospheric dinitrogen is the largest nitrogen reservoir, it is biologically accessible only to a select group of nitrogen-fixing bacteria and archaea [14]. As a result, nitrogen availability in terrestrial ecosystems is primarily regulated by microbial transformations that modify its oxidation state. Among these, ammonia-oxidizing bacteria (AOB), such as Nitrosomonas and *Nitrosospira* species, catalyze the first step of nitrification by converting ammonia (NH_3) to nitrite (NO_2^-) under aerobic conditions [15,16]. Nitrite is subsequently oxidized to nitrate (NO_3^-), a plant-available form of nitrogen, by nitrite-oxidizing bacteria (NOB) with the nitrite oxidoreductase enzyme complex [17,18]. Field studies have confirmed the pivotal role of AOB in various ecosystems. For example, Di et al. (2009) [19] identified AOB as the main driver of soil nitrification in New Zealand grasslands, while Wang et al. (2024) [20] demonstrated their contribution to improved maize productivity. These findings highlight the importance of ammonia oxidizers in regulating nitrogen availability and linking microbial activity to plant growth. In parallel, hormonal signaling also plays a critical role in promoting regrowth.

Cytokinin biosynthesis plays a central role in plant–microbe interactions, acting as a key regulatory node in hormone signaling. As essential phytohormones, cytokinins govern cell division and differentiation, thereby shaping plant development and stress responses [21]. Their synthesis is typically catalyzed by isopentenyl transferase (IPT), a rate-limiting enzyme present in both plants and various microorganisms, including phytopathogens and PGPMs [22,23]. Recent studies have highlighted the role of microbial cytokinins in improving plant resilience under abiotic stress. For example, a *Bacillus subtilis* strain increased shoot cytokinin levels by 30% under drought conditions, enhancing leaf health in *Platycladus orientalis* [24]. Similarly, cytokinin production by *Pseudomonas fluorescens* improved drought tolerance in tomato plants [25]. Despite these findings, relatively few PGPMs or soil bacteria have been confirmed to synthesize cytokinins, making this a promising but underexplored area for agricultural applications.

This study provides an integrative, stage-specific, and cross-system evaluation of microbial functional effects, offering new insight into how multiple processes may operate concurrently during plant regrowth. To address this gap, we investigated two PGPMs: the ammonia-oxidizing strain S2_8_1 [26] and a cytokinin-producing *Streptomyces* strain (Shan2). By comparing S2_8_1 with Shan2, we aimed to clarify the respective roles of microbial nitrogen transformation and cytokinin signaling in promoting plant regrowth. In addition to soil-based experiments, we employed a hydroponic system to enable more precise measurement of microbial effects on cytokinin dynamics and plant responses [27,28]. We hypothesized that (1) S2_8_1 enhances ryegrass regrowth through the combined influence of nitrification-related processes and cytokinin-associated responses, and (2) it remains functionally active in soilless conditions. This dual-system approach provides a robust framework for evaluating PGPMs with multiple functional traits in the context of sustainable, low-input agriculture.

2 Materials and Methods

2.1 Experimental Design

Two plant growth-promoting microorganisms, S2_8_1 and Shan2, were used in this study. Both strains were isolated from the experimental site of Henan University of Science and Technology (Luoyang, China) using a selective medium for ammonia-oxidizing bacteria. S2_8_1 was selected based on its strong ammonia-oxidizing capacity in preliminary screening. Shan2, a *Streptomyces* strain with cytokinin-producing potential, was subsequently selected from the isolate collection based on its hormone-related activity. These two strains represent distinct functional traits associated with nitrogen transformation and plant growth regulation. Molecular identification revealed that S2_8_1 belongs to the genus Rhizobium (GenBank accession: ON667919.1; CCTCC NO: M2021374), and Shan2 to *Streptomyces* (GenBank accession: PQ605713; CCTCC NO: M20242569). Both are preserved at the China Center for Type Culture Collection (Wuhan University, China). Strains were cultured in enrichment medium containing 3.79 mM $(NH_4)_2SO_4$, 5.51 mM KH_2PO_4, 1.81 mM NaH_2PO_4, 0.059 mM $MnSO_4$ $4H_2O$, 0.12 mM $MgSO_4$ $7H_2O$, and 50.0 mM $CaCO_3$ (pH 7.2). LB medium (5 g yeast extract, 10 g NaCl, and 10 g tryptone per liter, pH 7.0) was used in control treatments. Before application, bacterial suspensions were centrifuged and resuspended to standardize inoculum concentration. The final bacterial suspension contained approximately 2.16×10^6 CFU mL^{-1}.

The experiment took place at the university experimental farm in Luoyang, which has a temperate continental monsoon climate, with average annual rainfall of 601 mm and a mean temperature of 14.2°C. The test plant was annual multiflora ryegrass (*Lolium multiflorum*) cultivar 'Tetragold', selected for its rapid growth, strong regenerative capacity, and high fresh biomass yield, traits ideal for forage production and root-soil interaction studies.

In September 2024, plastic pots (20 cm diameter × 25 cm height; 13.5 L) were filled with 5.80 kg of brown soil. Soil properties were: 23.8 g kg^{-1} organic carbon, 2.1 g kg^{-1} total nitrogen, 10.3 mg kg^{-1} phosphorus, 120.5 mg kg^{-1} potassium, 269.7 mg kg^{-1} magnesium, and pH 7.7. Pots were kept in a naturally lit, open-sided greenhouse (25–30°C), sheltered from rain, and watered every 2–3 days to ensure sufficient soil moisture. Ryegrass seeds were sown and grown for three weeks. Uniform, healthy seedlings were then transplanted into 100 pots. Two parallel experiments were conducted: Experiment 1 (Exp-1) used soil-based cultivation with PGPM and nitrate treatments (54 pots), while Experiment 2 (Exp-2) used a hydroponic system with S2_8_1 and nitrate applied directly to the roots (36 pots). To reduce variability, only uniform seedlings were selected. All plants received adequate water and light throughout the study.

2.2 Experiment-1 (Exp-1)

In Exp-1, 54 pots were randomly assigned to six treatment groups (n = 9 per group), each subdivided into three replicates (three pots per subgroup). Treatments included: (1) no addition (GW), (2) blank enriched medium (BT), (3) S2_8_1 bacterial suspension (JI), (4) LB medium (LB), (5) Shan2 bacterial suspension (JS), and (6) NO_3^-–N combined with Shan2 (NS). The primary objective of this study was to evaluate plant physiological responses to microbial inoculation. Based on previous studies conducted by our group [29] indicating that plant responses are mainly associated with microbial activity, the present experiment focused on the integrated effects of functional strains. Therefore, a treatment group with only nitrate addition was not set up in the experiment.

On day 0 of regrowth, 200 mL of the designated solution was applied to each pot: blank medium (BT), S2_8_1 suspension (JI), LB medium (LB), Shan2 suspension (JS), or 35 mmol L-1 NO_3^-–N (NS). The

GW group received no treatment. The NO_3^-–N concentration, previously shown to be optimal [29], was maintained throughout via replenishment. All pots received equal liquid volumes (200 mL), adjusted with deionized water. Soil moisture was maintained at 65–70% field capacity. After treatment, subgroup 1 was sampled for physiological and biochemical analyses. Subgroups 2 and 3 were clipped at a height of 5 cm, and biomass was dried for dry matter determination. On day 7, subgroup 2 was sampled; subgroup 3 was clipped again. Final measurements were taken from subgroup 3 on day 14.

2.3 Experiment-2 (Exp-2)

In Exp-2, 36 pots with ryegrass seedlings were transferred to a hydroponic system maintained at 25°C under greenhouse conditions. Pots were randomly assigned to four treatment groups (n = 9 each), subdivided into three replicates (three pots per subgroup, n = 3 independent pots per treatment at each sampling time point). Treatments included: (1) distilled water (GW), (2) 100 mL of 35 mmol L-1 NO_3^-–N solution (NT), (3) 100 mL of blank enriched medium (BT), and (4) 100 mL of S2_8_1 bacterial suspension (JI).

Due to the slower regrowth rate of hydroponically grown ryegrass, a 10-day regrowth period was used. On day 0, subgroup 1 was sampled for physiological and biochemical measurements, while subgroups 2 and 3 were clipped at a height of 5 cm. Clipped biomass was weighed and dried to determine dry matter content. A 5-day regrowth cycle was adopted to detect early growth differences. On day 5, subgroup 2 was sampled; subgroup 3 was clipped again under the same conditions. Final sampling of subgroup 3 was conducted on day 10.

2.4 Biomass and Transpiration Rate

Roots were gently washed to remove adhering soil, and surface moisture was blotted before recording fresh weight. Roots, stems, and leaves were then oven-dried at 65°C for 72 h to determine dry biomass. Regrowth biomass was defined as the dry weight of newly grown leaves after clipping, and total biomass was calculated as the sum of root, stem, and leaf dry weights.

Transpiration rate (Tr) was measured between 11:00 and 12:00 using a LI-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA) under controlled conditions: 1000 μmol m^{-2} s^{-1} light intensity, 28°C temperature, and 400 ppm CO_2. Due to the narrow leaf blades of ryegrass, two to three leaves were overlapped to fully cover the chamber area and ensure accurate measurements.

2.5 Soil NO_3^-–N Content and Soil Nitrification Rate

Ryegrass plants were gently removed from pots, and rhizosphere soil was collected by brushing soil particles from roots 5–10 times using a clean brush. Fine soil was retained, labeled, and stored in separate bags to avoid cross-contamination. Soil NO_3^-–N concentration and net nitrification rate were determined using rhizosphere soil samples. Nitrate content was measured by the phenol disulfonic acid colorimetric method [30], using phenol disulfonic acid reagent, calcium sulfate, distilled water, a spectrophotometer, a constant-temperature water bath, and porcelain evaporating dishes. To assess net nitrification, soil samples were incubated in the dark at 25°C for 4 days. NO_3^-–N was measured before and after incubation. The daily nitrification rate was calculated as:

$$\text{Nitrification rate} = \frac{NO_3^-\text{-}N_{post} - NO_3^-\text{-}N_{pre}}{\text{Day}} \quad (1)$$

$$NO_3^-\text{-}N = \frac{(C \times V \times V_2)}{V_1 \times m} \quad (2)$$

where C is the NO_3^-–N content in the test solution (mg mL^{-1}), determined using a standard calibration curve; V is the total volume of the sample solution (mL); V_1 is the volume of the filtrate used for color development (mL); V_2 is the volume of the color developed solution (mL); and m is the mass of the air-dried soil sample (g).

2.6 Zeatin Riboside

Zeatin riboside (ZR), a stable derivative of zeatin, serves as a key indicator of cytokinin signaling. For hormone extraction, 0.50 g of fresh leaf tissue was cut into small pieces and ground with quartz sand in a mortar precooled to −40°C using liquid nitrogen. During grinding, 5 mL of 80% methanol (with 1 mmol L^{-1} butylated hydroxytoluene, BHT) was added three times. The homogenate was transferred to 10 mL centrifuge tubes and extracted at 4°C for 12 h. After centrifugation (10 min), the supernatant was filtered through a C18 column. A secondary extraction was performed with 1 mL of the same solvent, incubated for 1 h, centrifuged, and filtered again. Combined filtrates were vacuum-dried and stored at −80°C.

Xylem sap was collected by wrapping 0.200 g of absorbent cotton around the clipped stem, secured with a zip-lock bag and rubber band to prevent evaporation. After 12 h, sap volume was estimated by weight gain. Cotton was extracted twice with 1 mL of 80% methanol (1 mmol L^{-1} BHT), first for 12 h at 4°C and again for 1 h. Extracts were combined, dried under vacuum, and stored at −80°C.

ZR content in leaves and xylem sap extracts were quantified using ELISA according to the manufacturer protocol (Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China). Absorbance was measured at 490 nm ZR levels in leaves were expressed per gram of fresh weight, while xylem ZR (C_{ZR}) was expressed per milliliter of sap. Cytokinin transport rates were estimated under dark (B_{ZR}) and light (L_{ZR}) conditions using:

$$B_{ZR} = C_{ZR} \times X_r \tag{3}$$

$$L_{ZR} = C_{ZR} \times T_r \times M \times SLA \tag{4}$$

where C_{ZR} is the ZR concentration in xylem sap (ng mL^{-1}), X_r denotes the rate of xylem sap transport in darkness (mL h^{-1}), T_r represents the transpiration rate (mL m^{-2} s^{-1}), M is the biomass of regrown leaves (g), and specific leaf area (SLA, cm^2 g^{-1}) is the ratio of leaf surface area to leaf dry weight, which is determined beforehand.

2.7 Statistical Analysis

All data are presented as means ± standard deviation (SD). One-way ANOVA was performed using SPSS (version 16.0; SPSS Inc., Chicago, IL, USA), followed by Duncan's multiple range test for post hoc comparisons. Differences were considered significant at $p < 0.05$. To evaluate the combined effects of soil nitrification and leaf ZR content on biomass, multiple linear regression models were constructed using all-subsets regression. Both additive (nitrification and ZR as independent predictors) and interaction models (including a nitrification × ZR term) were tested. Model selection was based on the Akaike Information Criterion (AIC), with lower AIC indicating a better fit [31,32]. Model performance was assessed using the coefficient of determination (R^2). Regression and visualization were conducted in R [33] (version 4.4.2), using the 'lavaan' package for regression, and 'ggplot2', 'dplyr', 'tidyr', and 'plotly' for data processing and plotting.

3 Results

3.1 Exp-1

As shown in Fig. 1, the GW, BT, and LB treatments showed no significant differences in clipping or cumulative biomass during regrowth. In contrast, both JI and NS significantly enhanced ryegrass biomass compared to GW. Notably, the biomass increases in the JI treatment were comparable to those in the NS treatment, with JI achieving a 3.6-fold increase in clipping biomass and a 52% improvement in total biomass by day 14, indicating a strong growth-promoting effect. NS outperformed JS, suggesting that Shan2 impact is amplified when combined with nitrate. JS still led to moderate biomass gains, highlighting the role of cytokinin production in regrowth.

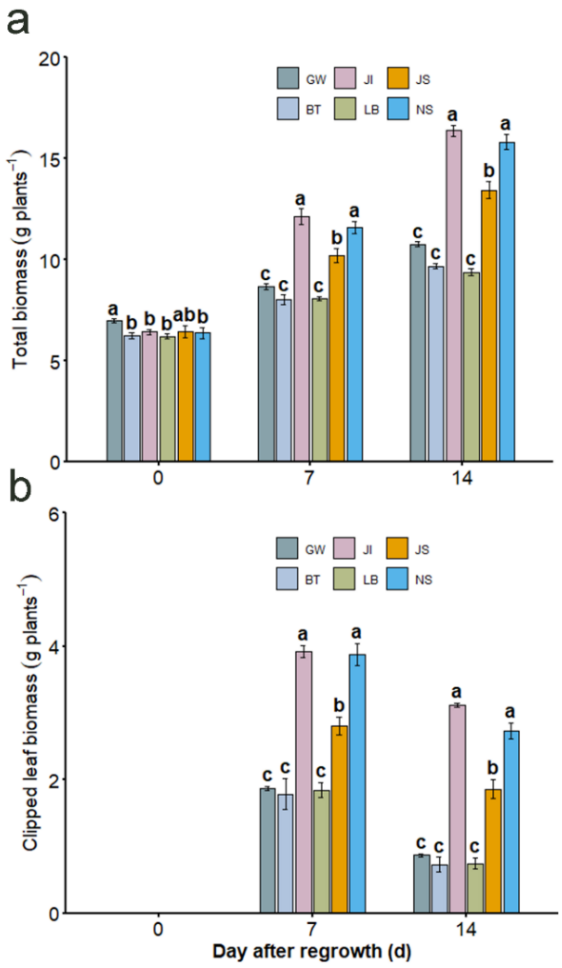

Figure 1: Total biomass (**a**) and clipped leaf biomass (**b**) of *Lolium multiflorum* under different treatments. Time points 0, 7, and 14 represent the first day of the experiment and the 7th and 14th days of regrowth, respectively. GW, BT, JI, LB, JS, and NS represent the following treatments: no addition (GW), blank medium (BT), inoculation with S2_8_1 strain (JI), LB medium (LB), inoculation with Shan2 strain (JS), and combined application of NO_3^-–N and Shan2 strain (NS). Values are presented as mean ± standard deviation (n = 3). Different lowercase letters indicate significant differences among treatments at the same time point ($p < 0.05$).

S2_8_1 also significantly increased soil NO_3^-–N content and nitrification rate after 7 days (Fig. 2; $p < 0.05$), with values similar to those in NS by day 14. This indicates that S2_8_1 treatment was associated

with higher net nitrification under the present experimental conditions. The JS treatment showed a smaller but significant increase in NO_3^-–N ($p < 0.05$), potentially through indirect microbial interactions with nitrogen cycling.

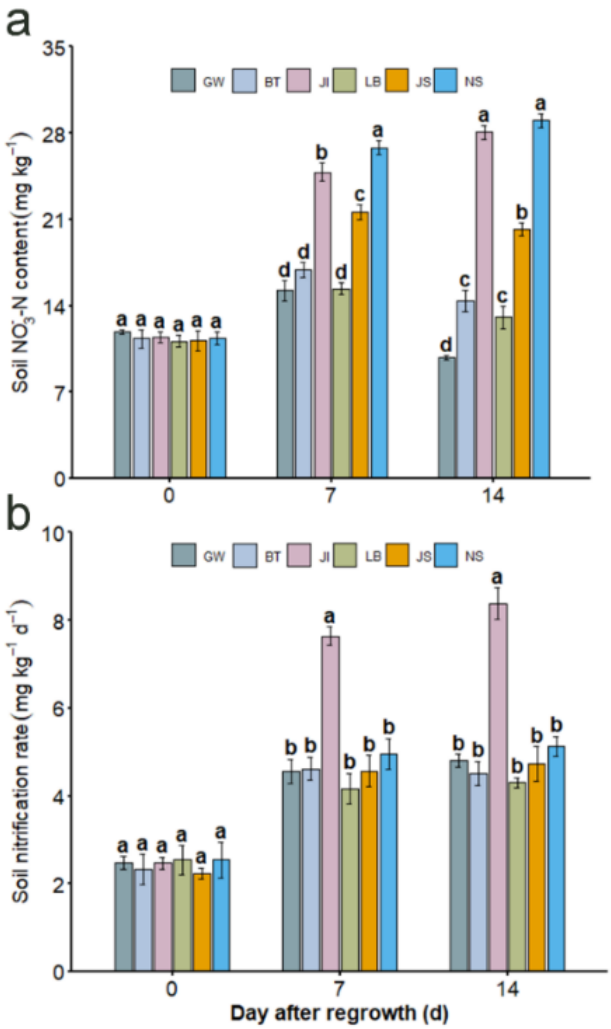

Figure 2: Soil NO_3^-–N content (**a**) and soil nitrification rate (**b**) under different treatments. Time points 0, 7, and 14 represent the first day of the experiment and the 7th and 14th days, respectively. GW, BT, JI, LB, JS, and NS refer to the following treatments: no addition (GW), blank medium (BT), inoculation with S2_8_1 strain (JI), LB medium (LB), inoculation with Shan2 strain (JS), and combined application of NO_3^-–N and Shan2 strain (NS). Values are expressed as mean ± standard deviation (n = 3). Different lowercase letters indicate significant differences among treatments at the same time point ($p < 0.05$).

As shown in Table 1, no treatment differences in leaf ZR, B_{ZR}, or L_{ZR} were detected at day 0. However, during regrowth, ZR transport from roots to shoots increased significantly in JI, JS, and NS treatments under both light and dark conditions ($p < 0.05$). By days 7 and 14, NS showed the highest ZR content, followed by JI. These results indicate that S2_8_1 promotes both nitrification and cytokinin translocation, supporting its dual functionality and potential as a microbial strategy for enhancing plant regrowth.

Table 1: The ZR, B_{ZR} and L_{ZR} of different treatments.

Items	Days after Regrowth (d)	Treatments					
		GW	BT	JI	LB	JS	NS
ZR content in leaves (ng g^{-1})	0	61.43 ± 3.42^{a}	59.58 ± 3.07^{a}	61.24 ± 2.94^{a}	59.51 ± 2.22^{a}	61.13 ± 2.07^{a}	61.05 ± 1.46^{a}
	7	71.40 ± 3.81^{c}	69.44 ± 3.09^{c}	84.96 ± 3.74^{b}	70.77 ± 1.62^{c}	88.70 ± 2.62^{b}	97.39 ± 3.52^{a}
	14	72.71 ± 2.96^{c}	71.93 ± 2.87^{c}	90.03 ± 5.35^{b}	68.24 ± 3.43^{c}	92.69 ± 3.91^{b}	100.91 ± 1.56^{a}
B_{ZR} (ng $plant^{-1}$ d^{-1})	0	7.13 ± 0.37^{a}	6.93 ± 0.12^{a}	6.98 ± 0.11^{a}	7.00 ± 0.36^{a}	7.04 ± 0.15^{a}	6.98 ± 0.16^{a}
	7	6.85 ± 0.27^{c}	6.85 ± 0.25^{c}	10.23 ± 0.52^{b}	6.81 ± 0.31^{c}	10.79 ± 0.35^{b}	13.12 ± 0.37^{a}
	14	7.05 ± 0.26^{c}	7.12 ± 0.36^{c}	12.36 ± 0.42^{b}	7.10 ± 0.31^{c}	12.84 ± 0.39^{b}	14.68 ± 0.34^{a}
L_{ZR} (ng $plant^{-1}$ d^{-1})	0	21.47 ± 0.65^{a}	21.16 ± 0.41^{a}	21.29 ± 0.53^{a}	21.10 ± 0.45^{a}	21.24 ± 0.53^{a}	21.30 ± 0.19^{a}
	7	20.52 ± 0.60^{c}	21.03 ± 0.10^{c}	38.26 ± 0.78^{b}	20.78 ± 0.73^{c}	39.39 ± 0.22^{b}	44.83 ± 0.56^{a}
	14	20.81 ± 0.70^{c}	21.33 ± 0.67^{c}	34.82 ± 0.61^{b}	21.12 ± 0.39^{c}	35.82 ± 0.30^{b}	42.24 ± 0.73^{a}

Note: Values are presented as mean ± standard deviation (n = 3). Different lowercase letters within each row indicate significant differences among treatments ($p < 0.05$). GW, BT, JI, LB, JS, and NS represent the following treatments: no addition (GW), blank medium (BT), inoculation with S2_8_1 strain (JI), LB medium (LB), inoculation with Shan2 strain (JS), and combined application of NO_3^-–N and Shan2 strain (NS). ZR refers to zeatin riboside content in leaf tissue; B_{ZR} and L_{ZR} denote the transport rate of ZR from root to leaf under dark and light conditions, respectively.

3.2 Exp-2

At day 0 of regrowth, no significant differences in total biomass were observed among treatments. However, by days 5 and 10, ryegrass treated with S2_8_1 (JI) showed significantly higher biomass than both the GW and NT controls (Fig. 3a), confirming the growth-promoting effect of S2_8_1 under hydroponic conditions. To explore the underlying mechanism, ZR content in leaves was assessed during regrowth. As shown in Fig. 3b, ZR levels did not differ among treatments at day 0 but increased significantly in the JI group by day 5, with further enhancement by day 10. By the end of the experiment, leaf ZR content in JI exceeded that of GW by 47%, indicating enhanced ZR accumulation and transport in treated plants. These results demonstrate that S2_8_1 remains metabolically active in a soilless environment, enhancing both cytokinin accumulation and its movement from roots to shoots. This dual function supports robust regrowth of hydroponically cultivated ryegrass and highlights the strain's functional versatility beyond soil-based systems. The hydroponic system, by minimizing soil-mediated nitrification, provides a simplified framework to assess plant–microbe interactions independent of nitrogen transformation processes.

3.3 Synergistic Effects of Nitrification and Cytokinin on Biomass

For S2_8_1, models that included both soil nitrification rate and leaf ZR content explained ryegrass biomass more effectively than single-factor models (Table 2). The interaction model yielded the best fit, with R^2 values of 0.91 for total biomass and 0.92 for aboveground biomass. Among individual predictors, nitrification rate showed a lower AIC than leaf ZR, indicating a stronger association with biomass variation. Importantly, the improved performance of the interaction model suggests a joint association and a positive statistical interaction between nitrification rate and cytokinin status in relation to biomass accumulation, rather than independent additive effects. In contrast, for Shan2, leaf ZR content alone best predicted total biomass (AIC = 34.26). For aboveground biomass, all models except the ZR-only model showed similar explanatory power (R^2 = 0.87). As illustrated in Fig. 4, total biomass under S2_8_1 treatment was positively correlated with both enhanced nitrification and elevated cytokinin levels (Fig. 4a). By comparison, in the Shan2 treatment, biomass was more strongly associated with ZR content than with nitrification (Fig. 4b). Regression analyses indicated that nitrification rate and leaf ZR jointly explained biomass variation under S2_8_1 treatment. This statistical interaction is consistent with, but does not by itself prove, a physiological synergy between nitrogen transformation and cytokinin-related responses.

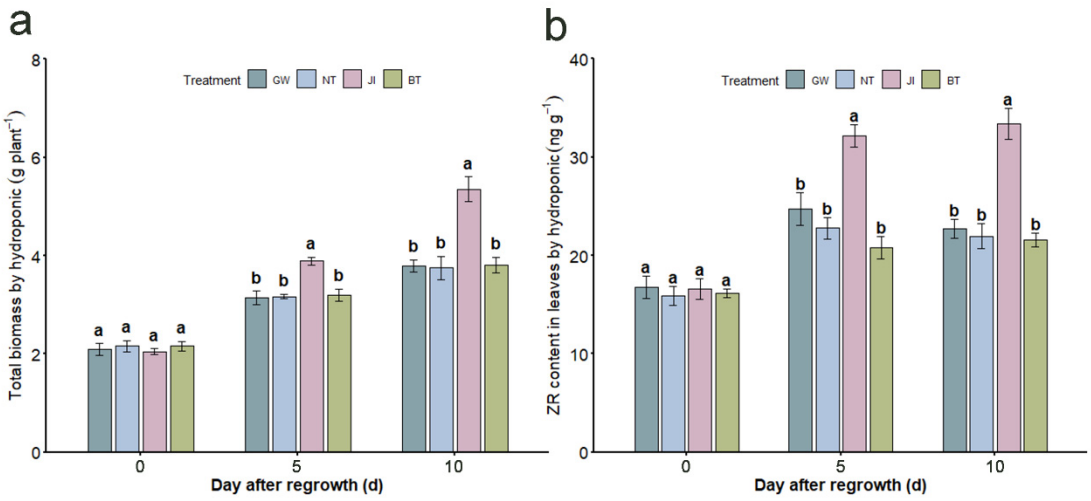

Figure 3: Total biomass (**a**) and ZR content in leaves (**b**) of ryegrass under hydroponic conditions across different treatments. Time points 0, 5, and 10 correspond to the first, fifth, and tenth days of *Lolium multiflorum* regrowth, respectively. GW, NT, JI, and BT represent the following treatments: growth in distilled water (GW), growth in 35 mmol L^{-1} NO_3^-–N solution (NT), growth in S2_8_1 bacterial solution (JI), and growth in blank culture medium (BT). Values are expressed as mean ± standard deviation (n = 3). Different lowercase letters indicate statistically significant differences among treatments ($p < 0.05$).

Table 2: Multiple linear regression analysis of the effects of soil nitrification rate and ZR content of leaves on ryegrass biomass under treatments with strains S2_8_1 (JI) and Shan2 (JS).

Treatments	Response Trait	Explanatory Traits of the Model	*p*	R^2	AIC
JI	Total biomass	×	0.005	0.91	39.57
		soil nitrification rate	<0.001	0.88	37.51
		ZR content of leaves	<0.001	0.86	39.36
		+	<0.001	0.89	39.22
JI	Above ground biomass	×	0.004	0.92	29.19
		soil nitrification rate	<0.001	0.89	28.32
		ZR	<0.001	0.84	31.22
		+	0.001	0.89	30.28
JS	Total biomass	×	0.009	0.88	35.71
		soil nitrification rate	0.001	0.79	36.80
		ZR	<0.001	0.84	34.26
		+	0.003	0.84	36.15
JS	Above ground biomass	×	0.012	0.87	32.93
		soil nitrification rate	<0.001	0.87	29.19
		ZR	0.002	0.77	33.95
		+	0.002	0.87	31.12

Note: The interaction model (×) includes both soil nitrification rate and ZR content with an interaction term, while the additive model (+) includes both variables without interaction. *p* values indicate statistical significance, R^2 represents the model explanatory power, and AIC reflects model quality and complexity.

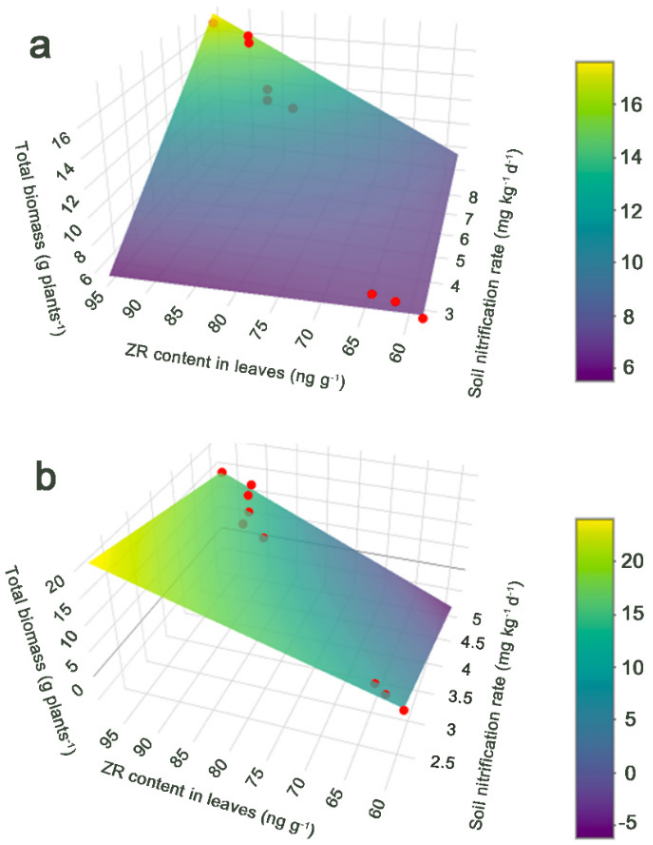

Figure 4: Three dimensional plots illustrating the relationships among total biomass, soil nitrification rate, and ZR content in leaves under the JI (S2_8_1) treatment (**a**) and the JS (Shan2) treatment (**b**), based on the interaction models (×) presented in Table 2. The color gradient of the surface plot indicates total biomass levels, with corresponding values represented by the color scale bar.

4 Discussion

4.1 Dual Functional Mechanisms of S2_8_1 in Promoting Ryegrass Regrowth

This study shows that strain S2_8_1 promotes ryegrass regrowth in association with coordinated changes in rhizosphere nitrification and cytokinin-related plant responses: enhanced nitrogen cycling and cytokinin signaling. Compared with most PGPMs that influence either nutrient availability or hormone production, S2_8_1 boosts both soil nitrification and zeatin riboside accumulation and translocation. This dual effect highlights its potential as a promising candidate for next-generation PGPMs aimed at improving forage regrowth.

The concurrent increases in soil NO_3^- availability and cytokinin activity were consistently associated with improvements across multiple growth traits. Multiple linear regression revealed that the interaction between nitrification rate and leaf ZR content explained over 90% of biomass variation (Table 2; R^2 = 0.91–0.92). This result indicates a positive statistical interaction and joint association between the two variables in relation to plant growth, rather than independent additive effects. While previous studies have reported PGPMs enhancing either nitrogen cycling [19] or cytokinin production [24], S2_8_1 uniquely combines both. This trait is particularly valuable in intensively managed forage systems, where frequent cutting demands rapid nutrient uptake and shoot recovery [34,35]. By enhancing both rhizosphere nitrogen

availability and cytokinin transport, S2_8_1 promotes faster regrowth and reduces reliance on synthetic nitrogen, aligning with sustainable agriculture goals [8,36].

Mechanistically, this synergy may involve co-expression of genes related to ammonia oxidation (amoA) and cytokinin biosynthesis (IPT like sequences) [37,38]. S2_8_1 may also activate root signaling in response to localized nitrate, triggering feedback that enhances both nutrient uptake and shoot development [29]. However, these interpretations remain hypothetical, and further genomic and transcriptomic analyses will be required to establish causal relationships and clarify the underlying regulatory mechanisms.

4.2 Comparative Efficacy of S2_8_1 and Shan2 Strains

Although both S2_8_1 and Shan2 enhanced ryegrass regrowth, their mechanisms and overall efficacy differed markedly. Shan2, a *Streptomyces* strain, primarily promoted regrowth through cytokinin production, as evidenced by elevated ZR levels in treated plants. This aligns with previous reports highlighting the strong cytokinin-producing capacity of *Streptomyces* spp. [12,39]. However, Shan2 had smaller influence on soil nitrification and contributed less to biomass accumulation compared to S2_8_1. In contrast, S2_8_1 displayed broader functionality, enhancing both nitrogen cycling and cytokinin signaling. By day 7, S2_8_1 inoculated plants exhibited a 63.2% increase in soil NO_3^-–N, a 19% increase in leaf ZR, and an 86% rise in L_{ZR} relative to controls (Table 1; Fig. 2). These results align with findings that nitrate availability can stimulate cytokinin biosynthesis and translocation [40,41], reinforcing the dual role consistent with physiological synergy of S2_8_1.

Regression analysis further highlighted this distinction (Table 2; Fig. 4). In S2_8_1-treated plants, biomass positively correlated with both nitrification rate and ZR content, supporting a synergistic effect. In contrast, under Shan2 treatment, soil nitrification explained only 79% of biomass variation, lower than the 91% in S2_8_1, and ZR content alone was a weak predictor. This likely reflects the limited role of cytokinins in root development, which depends more on nutrient availability and auxin signaling [42–44].

These contrasting mechanisms underscore the importance of selecting PGPM based on functional complementarity. While cytokinin-producing strains like Shan2 can support shoot regrowth, their limited contribution to nutrient cycling may reduce their effectiveness in nutrient-deficient systems. In contrast, multifunctional strains like S2_8_1 offer coordinated promotion of both shoot and root growth, outperforming single-function PGPMs [45,46]. Similar synergy has been observed in *Pseudomonas fluorescens*, where quorum sensing regulates both nitrogen and hormone pathways [5], suggesting a comparable regulatory mechanism may operate in S2_8_1.

4.3 Hydroponic Relevance and Functional of S2_8_1

Many soil derived microbes fail to function effectively in hydroponic systems due to the absence of soil structure and microbial networks [47,48]. In contrast, S2_8_1 retained its growth-promoting activity under hydroponic conditions, significantly increasing both total biomass and leaf ZR content. By day 10, ZR levels in S2_8_1 treated plants were 47% higher than in water controls, alongside substantial biomass gains (Fig. 3), demonstrating that the strain remains metabolically active in soilless environments. However, because nitrification-related variables were not quantified in the hydroponic system, these results do not directly support a nitrification-mediated mechanism under hydroponics. Instead, they demonstrate that the growth-promoting effect of S2_8_1 extends beyond soil-grown systems. S2_8_1 consistent performance across soil and hydroponic systems suggests adaptive traits such as efficient root colonization, secretion of bioactive compounds, and osmotic stress tolerance [16]. However, these

interpretations remain speculative and require further investigation to elucidate the physiological and molecular basis of its broad-spectrum functionality.

This functional robustness is particularly relevant to emerging soilless agriculture systems, including vertical farming and greenhouse-based forage production, where biologically compatible inputs are increasingly sought [49,50]. While hydroponics offers advantages like high nutrient availability, reduced water use, and faster growth [27,28], the success of PGPMs in these systems depends on their ability to function independently of soil-based interactions. The ability of S2_8_1 to do so highlights its potential as a reliable bioinoculant for controlled-environment agriculture.

4.4 Ecological and Agronomic Implications

The dual functionality of S2_8_1 positions it as a promising biofertilizer that supports both productivity and ecological sustainability. By enhancing ryegrass regrowth in both soil and hydroponic systems, S2_8_1 improves nitrate availability, reduces reliance on synthetic nitrogen, and accelerates biomass recovery, benefits especially relevant to intensively managed grasslands where nutrient depletion and yield instability are persistent challenges [51,52].

Despite these promising outcomes, several limitations warrant consideration. This study was conducted under controlled conditions, which, while valuable for mechanistic insights, may not fully represent field complexity. Field-scale trials are essential to evaluate the consistency and robustness of S2_8_1 under variable environmental and edaphic conditions. Additionally, the genetic and molecular basis of its dual function remains to be elucidated. Genomic studies could reveal key genes involved in nitrification enhancement and cytokinin biosynthesis. The proposed dual-function mechanism was inferred from physiological measurements and statistical associations rather than direct molecular evidence. We did not quantify amoA, IPT-like genes, nitrifier abundance, gene expression, or enzymatic activity. Therefore, our results should be interpreted as evidence that S2_8_1 treatment was associated with increased net nitrification and elevated ZR-related responses, rather than direct proof of the underlying genetic mechanism. Future work should combine genome annotation, targeted qPCR, transcriptomics, enzyme assays, and direct metabolite profiling to verify the molecular basis of these effects. As interest in microbial consortia grows, S2_8_1 may serve as a foundational strain in engineered inoculants that combine nutrient cycling and hormone-mediated growth regulation [46,53]. Together, these findings contribute to the broader understanding of multifunctional PGPMs and highlight the potential of S2_8_1 for future exploration as a bioformulation component targeting improved crop performance and sustainability.

5 Conclusions

This study demonstrates that the multifunctional PGPM strain S2_8_1 significantly enhances ryegrass regrowth, in association with simultaneous increases in soil nitrification and cytokinin-related processes. Compared to a cytokinin-producing *Streptomyces* strain, S2_8_1 showed greater effectiveness in biomass accumulation, consistent with its ability to influence both nutrient cycling and hormone-related responses. Notably, S2_8_1 maintained its growth-promoting effects under both soil and hydroponic conditions, underscoring its functional robustness and adaptability across cultivation systems. These findings highlight the novelty and versatility of S2_8_1 as a next generation microbial biofertilizer with potential for future application pending field validation. Its ability to enhance regrowth, reduce dependence on synthetic nitrogen inputs, and function consistently across environments highlights its relevance for ecological intensification of forage production and other cropping systems. The coordinated variation observed between nitrification and cytokinin-related responses suggests a potential functional coupling at the system

level, which may contribute to improved plant recovery and resilience. However, this relationship should be interpreted with caution, as the underlying causal mechanisms remain to be established. Future research should focus on field-scale validation, molecular characterization, and integration with complementary microbial consortia to fully realize its application in sustainable crop management.

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Availability of Data and Materials: The data generated and/or analyzed during this study are available from the corresponding author on reasonable request. The S2_8_1 and Shan2 genome sequence has been deposited into NCBI with accession number ON667919.1 and PQ605713.

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