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Unveiling the Potential of Non-Rhizobial Microorganisms to Improve Faba Bean Growth

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Abstract

The growing demand for sustainable agricultural practices has renewed interest in legumes such as faba bean (*Vicia faba* L.) due to their nutritional importance and capacity to enhance soil fertility through symbiotic nitrogen fixation. While nodulation is classically attributed to rhizobial genera, legume nodules are now recognized as complex microbial niches that also harbor non-rhizobial endophytes (NREs). Although NREs cannot induce nodulation independently, they can promote plant growth through nutrient acquisition, phytohormone production, and stress tolerance. In this study, bacterial communities associated with faba bean nodules collected from Aït Ourir were isolated and characterized, and their plant growth–promoting traits and co-inoculation effects were evaluated. A total of 80 isolates were obtained, comprising 9 rhizobia and 71 NREs. All rhizobial strains formed nodules, with strain F4.10 showing the highest symbiotic efficiency. Among the NREs, 65% exhibited nitrogen-fixation potential, while selected strains demonstrated phosphate solubilization (up to 17.76 mg/L) and indole-3-acetic acid production (up to 44.21 µg/mL). Co-inoculation of F4.10 with selected NREs significantly enhanced shoot length (29%), root length (47%), and root dry weight (152%) compared to single inoculation or non-inoculated controls. These results highlight the functional diversity of faba bean nodule microbiota and support the development of multifunctional biofertilizers for sustainable legume production.

1. Intorduction

The Green Revolution (GR) period (1960–2000) marked a transformative era in global agriculture, leading to substantial increases in food production and improved food security, particularly through the widespread cultivation of staple cereals such as wheat, rice, and maize [1, 2]. These productivity gains were largely driven by agricultural intensification, including expanded use of high-yielding crop varieties, mechanization, irrigation, and the extensive application of synthetic fertilizers and pesticides [3, 4]. Despite its success in enhancing food availability, the GR also generated considerable environmental impacts, including soil degradation, water pollution, greenhouse gas emissions, and biodiversity loss in many agrarian regions [5]. As global food demand continues to rise under increasing climate pressures, there is an urgent need to develop sustainable agricultural practices that maintain productivity while minimizing environmental impacts [6].

In this context, legumes represent a cornerstone of sustainable agriculture due to their capacity to enhance soil fertility, reduce dependence on synthetic nitrogen (N) fertilizers, and contribute to climate change mitigation [7]. Leguminous crops play a vital role in farming systems worldwide, serving as key sources of food, income, and livelihood for millions of smallholder farmers [8]. From a nutritional perspective, legumes are rich in proteins, essential amino acids, vitamins, and minerals, making them indispensable for human diets, particularly in low-income regions [9]. Among legumes, faba bean (*Vicia faba* L.) is of particular importance due to its high protein content, substantial nitrogen demand, adaptability to diverse agroecological conditions, and growing relevance in sustainable and low-input farming systems [10, 11].

A defining feature of legumes is their ability to establish a symbiotic association with nitrogen-fixing (N_2-fix) bacteria known as rhizobia, enabling symbitic nitrogen fixation (SNF) [12]. This symbiosis significantly reduces the need for synthetic nitrogen fertilizers by converting atmospheric nitrogen (N_2) into forms available for plant uptake [12]. Classical N_2-fix rhizobia include α-proteobacteria such as *Rhizobium* and *Bradyrhizobium*, as well as β-proteobacteria such as *Cupriavidus* and *Burkholderia*. These bacteria infect legume roots through root hairs and induce the formation of specialized organs called nodules, where SNF takes place [13, 14]. For decades, it was assumed that rhizobia were the exclusive microbial inhabitants of legume nodules.

However, recent advances in microbial ecology and high-throughput sequencing have revealed that legume nodules host a highly diverse community of non-rhizobial endophytes (NREs), collectively forming a complex nodule-associated phytomicrobiome [15–17]. Although NREs do not induce nodulation or fix N_2 independently, growing evidence suggests that they may contribute to plant growth promotion through mechanisms such as phytohormone production, nutrient solubilization, stress tolerance enhancement, and pathogen suppression [18]. Moreover, NREs may interact synergistically with rhizobia, potentially improving nodule functionality and overall plant performance.

Despite increasing recognition of their presence, the ecological role and functional significance of NREs within legume nodules remain poorly understood [18]. Only a limited number of studies have investigated their diversity and plant growth-promoting (PGP) potential, and their contribution to legume productivity and symbiotic efficiency is still largely unexplored [19, 20]. This knowledge gap limits the development of next-generation biofertilizers, as current formulations primarily rely on rhizobial inoculants and often show inconsistent performance under field conditions due to environmental stresses and microbial competition.

Addressing this gap is essential for advancing sustainable agriculture and improving biofertilizer efficiency. We hypothesize that NREs associated with faba bean nodules interact synergistically with rhizobia to enhance plant growth and yield. Therefore, this study aims to develop a biofertilizer based on both rhizobia and nodule-associated NREs to improve faba bean performance under controlled conditions. Specifically, the objectives of this study are to:

1. Isolate and characterize NREs from faba bean root nodules and assess their biodiversity;
2. Evaluate the PGP traits of the isolated NRE strains;
3. Assess the combined effects of rhizobia and NREs on faba bean growth and development under controlled experimental conditions.

2. Material and methods

2.1. Study site, Plant Selection, and Nodule Gathering

The faba bean root nodules (RNs) used in this study were collected from the Aït Ourir region (31° 33' 59.99" N; -7° 39' 59.99" W), located in the Al Haouz Province, Marrakech, Morocco. Five healthy plants were randomly selected from five distinct fields, all showing no visible disease symptoms (Fig. 1). The selection criterion focused on the presence of active RNs, identified by their characteristic pink coloration, indicating potential nitrogen fixation activity. After carefully removing the soil around the roots to avoid damage, the roots were pulled out. Subsequently, roots were transported in sterilme plastic bags to the laboratory and kept at 4°C. Once in the lab, the roots were carefully rinsed with tap water and sterile distilled water (SDW) to remove remaining adherent soil. Healthy nodules from each root were pulled out and were stored directly in 4°C until bacterial isolation.

2.2. Isolation of rhizobia and non-rhizobia endophytes

Healthy, non-ruptured RNs were desinfected by immersion in 70% ethanol for 30 s to remove any microbial epiphytes and washed, five times, using SDW to remove any residula disifectant

The desinfected nodules were aseptically macerated in 3 mL of sterile physiological water (0.9% NaCl) in a small sterile glass tube [21]. A 100 µL of the nodule suspension was further diluted in 3 mL of sterile physiological water to reduce microbial load and ensure isolation of individual colonies. Nodule suspensions were plated on five different media (ISP2 Medium, ISP3 Medium, Ganze No. 1 Medium, Yeast Mannitol Agar, YMA + Congo Red), to maximize the recovery of diverse bacterial taxa. The inoculated plates were incubated at 28–30°C and examined after four days for the emergence of endophytic bacteria. To confirm the efficiency of surface sterilization and to ensure that the isolated strains were truly endophytic rather than epiphytic, the final wash water was also streaked on the same media.

Colonies showing distinct morphological charactersitics, including pigementation, shape, and surface appearance, were slected and purified on on fresh ISP2 or YMA plates using the streak plate method. Repeated streaking was performed until pure cultures were obtained, confirmed by theuniformity of colony morphology.

2.3. Nodulation test in faba bean by rhizobia

Faba bean seeds (var. Aguadulce), kindly prvided by the National Istitute of Agricultural Research, were surface sterilized and placed on sterile Petri dishes lined with moistened Whatman filter paper. The dishes were incubated in the dark for 4 days at 22–28°C. Distilled water was added periodically to maintain adequate moisture.

After radicle emergence, germinated seeds were tranplanted in plastic cups filled with sterile, moistened perlite. Seeds were placed radicle-down and lightly covered with perlite.

Each seedling was then inoculated with one of the seven different rhizobial strains under investigation. The inoculation was performed by applying a standardized volume (3 mL) of rhizobial suspension (OD_{600} = 0.8), prepared in liquid YMA; directly onto the root zone to ensure effective colonization and nodulation

initiation. A non-inoculated control treatment was also included under the same conditions to check the capacity of the selected rhizobia strains to initiate and form symbiosis with faba bean. The cups were deposited in greenhouse and irrigated with SDW when required. Sloger's nutrient solution (without nitrogen) was supplied once or twice per week to support plant growth [22].

2.4. Plant growth promoting activities

The N_2-fix capacity of the isolated NREs was evaluated suing Jensen's modified method, following the medhology described by [23]. Breifly, the NREs strains were stricked onto the Jensen's nitrogen-free agar medium and incubated for 48h. NREs growth on this medium was taked and inicatin of postive results, and thus the presence of N_2-fix ability.

Phosphate (P)-solubilizing activity was evaluated using NBRIP liquid medium containin 5 g L^{-1} of $Ca_3(PO_4)_2$ as the only source of phophate [24]. To evalute the NRE capacity to solublize P, Erlenmeyer flasks containing 100 ml of NRIB borth were inoculated with 200 µL of the NRE suspension adjusted to an optical desnity of 0.1. Prior inoculation, the bacterial cells were wahsed three times to remove any residual pohosphate. Follwoing inoculation, the flask were incuabted for 96h at 28°C under agitation at 140 rpm. Solubilized phopshate was quantified following the method described by Raklami et al. [24].

IAA production was determined by growing bacterial strains in 100 mL of Luria–Bertani broth supplemented with 1.02 g L^{-1} L-tryptophan. Cultures were incubated at 30°C on a rotary shaker (140 rpm). After incubation, 1 mL of culture supernatant was mixed with 2 mL of Salkowski's reagent and two drops of phosphoric acid, then incubated in the dark for 30 min. Absorbance was measured at 530 nm to quantify IAA production [25].

2.5. Biological material, experimental design, and plant bioassay

The performance of the selected isolated strains, including both rhizobia and NREs, was evaluated in vivo using faba bean (*Vicia faba* L., cv. Aguadulce). The experiment was conducted using a randomized complete block design (RCBD) comprising three treatments, each with one plant per pot. The treatments were as follows: (i) uninoculated control (NI); (ii) plants inoculated solely with the rhizobial strain F410 (RI); and (iii) plants co-inoculated with F410 and a mixture of NRE strains (F33, F34, F510, and F222). Each treatment was replicated four times. The rhizobial strain was selected based on its nodulation efficiency during the nodulation test, whereas the NRE strains were selected according to their PGP performance.

Seeds of *V. faba* were surface-disinfected for five minutes using sodium hypochlorite (NaClO) diluted 1:5 (v/v) and then germinated as described above. After germination, seedlings were transplanted into 2-L pots previously filled with a sterile soil–peat mixture (70:30, w/w). The pots were maintained in a controlled greenhouse and irrigated twice weekly with 250 mL of distilled water (DW).

Bacterial inocula were prepared by growing the selected rhizobial strain in yeast extract mannitol (YEM) medium for three days and the NRE strains in tryptic soy agar (TSA) for 24 h at 28°C. Following incubation, bacterial cells were collected by centrifugation, rinsed once with sterile distilled water (SDW), and resuspended in DW to obtain a final optical density (OD_{600}) of 0.8. Each pot received 10 mL of the corresponding bacterial suspension, while control plants were supplied with DW only. For the co-inoculation treatment, the 10-mL inoculum was prepared by mixing equal volumes of each bacterial suspension.

At 20 days after sowing (DAS), faba bean plants were harvested, and the aerial parts were separated from the root systems. Roots were carefully washed with DW, and excess water was removed using paper towels. Growth parameters, including shoot and root length, as well as fresh and dry biomass, were measured using appropriate analytical methods.

2.6. Statitical analysis

The ability of the strains to produce IAA, solubilize P, data of nodulation test and influence of bacterioal treatment on plant growth parameters was analyzed using one-way analysis of variance (ANOVA) with COSTAT software. Mean comparisons were performed using the Least Significant Difference (LSD) test, and treatments sharing different letters were considered statistically significant at $p < 0.05$.

3. Results

3. 1. Isolation of rhizobia and non-rhizobia endophytes

Among the collection of bacterial isolates obtained from faba bean RNs, a total of 80 bacterial isolates were recovered. Based on their morphological characteristics on YEM agar, 71 isolates were classified as nonrhizobial endophytes (NREs). Specifically, 14 NRE strains were isolated from Field 1, 20 from Field 2, and 11 from Field 5 (Table 1).

Table 1
Faba bean nodule isolates from different fields in Ait-ourir region.

Sampling Site	Rhizobia strains	Count	NRE strains	Count	Total
Field 1	F1.04	1	F1.01, F1.02, F1.03, F1.05-F1.15	14	15
Field 2	F2.11, F2.12, F2.13	3	F2.01, F2.02- F2.10, F2.14, F2.15-F2.23	20	23
Field 3	F3.02	1	F3.01, F3.03, F3.04, F3.05-F3.14	13	14
Field 4	F4.10, F4.11, F4.12	3	F4.01, F4.02- F4.09, F4.13, F4.14, F4.15, F4.16, F4.17	14	17
Field 5	F5.03	1	F5.01, F5.02, F5.04, F5.05-F1.11	10	11
Total		9		71	80

In contrast, nine isolates were identified as rhizobia based on their typical morphology on YEM + CR agar, consistent with descriptions in the literature. These rhizobial colonies appeared smooth, translucent, glistening, round-shaped, and notably did not absorb Congo Red. Furthermore, these strains exhibited a slow growth pattern, with visible colonies forming after 3 to 5 days of incubation at 28°C.

To confirm their identity, these putative rhizobial isolates were subjected to nodulation tests on faba bean under controlled conditions, aiming to verify their ability to induce nodule formation and thus validate their classification as true rhizobia.

3.2. Nodulation test in faba bean by rhizobia

Nodulation test results revealed that all nine strains successfully induced nodulation. Figure 2 highlights the performance of the seven most promising strains. Among them, strain F4.10 consistently outperformed the others across several parameters, including nodule number, shoot and root length, and total dry weight (Fig. 2). Specifically, F4.10 significantly increased the number of nodules (10 nodules per plant), enhanced shoot length (up to 66 cm), root length (up to 30 cm), and total dry biomass (≈ 1.026 g).

Strain F2.13 also demonstrated strong performance, producing a high number of nodules (≈ 8.33) and considerable dry weight, although it showed greater variability in shoot and root growth (Fig. 2). Strains F3.02 and F2.12 delivered moderate but consistent improvements across all measured parameters. In contrast, strains F4.11 and F4.12 exhibited weak nodulation capacity, and at least one replicate for each showed poor biomass accumulation, indicating limited overall effectiveness.

3.3. Plant growth promoting activities

The N_2-fixing potential of the NREs strains was evaluated using Jensen's nitrogen-free medium. Results revealed that 64.8% of the isolates (46 strains) were able to grow in the absence of any N source,

indicating their potential to fix N_2. However, growth intensity varied among the strains, suggesting differences in their nitrogen-fixing efficiency (Table 2). Among the 46 positive isolates, 11 strains exhibited notably strong growth, with five of these high-performing strains originating from field 2 (Table 2). The relatively high proportion of nitrogen-fixing NREs strains highlights their functional potential to contribute to plant nitrogen nutrition. Nevertheless, quantitative assessment of nitrogenase activity (such as through the acetylene reduction assay) is essential to confirm their nitrogen-fixing capabilities, differentiate among strains, and identify those with the highest nitrogen-fixation efficiency for potential application.

Table 2
Nitrogen Fixation Capacity of Purified Non-Rhizobial Endophytes Grouped by Field.

Field 1	N capacity	Field 2	N capacity	Field 3	N capacity	Field 4	N capacity	Field 5	N capacity
F1.01	++++	F2.01	+++	F3.01	++	F4.02	+	F5.01	+++
F1.02	++	F2.02	+++	F3.03	++++	F4.03	++	F5.05	+++
F1.03	++++	F2.03	+++	F3.04	++++	F4.04	+	F5.06	+++
F1.05	+++	F2.04	+	F3.05	++	F4.06	++	F5.08	++
F1.06	++++	F2.05	++	F3.06	+	F4.09	++	F5.09	+++
F1.07	+++	F2.07	++	F3.07	+++	F4.13	+	F5.10	++++
F1.08	+++	F2.08	+++	F3.09	+	F4.16	+++	F5.11	+++
		F2.10	+++	F3.10	++++				
		F2.14	+	F3.13	+				
		F2.16	++++						
		F2.17	++++						
		F2.20	++++						
		F2.21	++++						
		F2.22	++++						

+: Weak nitrogen fixation ++: Moderate nitrogen fixation +++: Strong nitrogen fixation ++++: Very strong nitrogen fixation.

Based on their nitrogen-fixing ability, 15 NREs strains were selected for further screening to assess their capacity to solubilize complex phosphate in the form of tri-calcium phosphate (Fig. 3). Phosphate solubilization activity varied widely among the isolates, ranging from 0.69 mg L^{-1} to 17.76 mg L^{-1}. The strains F3.10 (17.76 mg L^{-1}), F2.20 (14.59 mg L^{-1}), and F2.17 (14.38 mg L^{-1}) exhibited the highest solubilization potential, identifying them as strong candidates for biofertilizer applications, particularly in phosphorus deficient soils (Fig. 3). Other strains, including F3.04, F5.01, and F4.09, also showed

considerable P solubilizing ability. Moderate activity was observed in isolates such as F1.01, F2.03, F1.06, and F4.01 (10–12 mg L^{-1}), suggesting they may be beneficial when used in combination with more efficient strains (Fig. 3). In contrast, strains like F2.22, F5.10, F3.03, F1.03, and especially F4.02 (0.69 mg L^{-1}) displayed low solubilization capacity, indicating limited potential for independent use unless complemented by other PGP traits (Fig. 3).

All the 15 NREs strains showing higher N fixation ability were tested for IAA production. Results displayed that in addition to P solubilization, these strains were also able to produce IAA in presence of L-tryptophan. The relatively high IAA production was observed in isolate F3.04 (44.21 µg/ml), indicating a strong potential to enhance plant growth. Substantial levels were also recorded for F2.17 (23.87 µg/ml) and F3.03 (19.34 µg/ml), followed by F1.01 and F2.20 (> 15 µg/ml), supporting their relevance in plant growth promotion studies (Fig. 3). Moderate producers, including F2.03, F3.10, F4.09, and F5.01 (12–15 µg/ml), may contribute synergistically when used in consortia. In contrast, isolates such as F1.06, F2.22, F4.01, F4.02, and F5.10 exhibited low IAA levels (< 10 µg/ml), with F1.03 (3.06 ± 0.24 µg/ml) being the least active (Fig. 3).

3.4. The growth-promoting effect of NRE isolates on faba bean growth

Following the nodulation test, strain F4.10 was identified as the most effective rhizobial isolate, capable of forming functional nodules and significantly promoting faba bean growth. Based on their complementary PGP traits, four NREs strains (F3.03, F3.04, F5.10, and F2.22) were selected and tested in combination with F4.10 to assess the effects of co-inoculation on plant development (Fig. 4). As shown in Fig. 4, inoculation with rhizobia alone (F4.10) significantly enhanced root length compared to the control, although no notable improvement was observed in shoot length. In contrast, co-inoculation with rhizobia and the selected NREs strains led to a substantial increase in both shoot and root length, clearly outperforming all other treatments (Fig. 4). Specifically, co-inoculation resulted in a 29% increase in shoot length and a 47% increase in root length, demonstrating the synergistic effect of combining rhizobia with beneficial NREs strains on overall plant growth (Fig. 4).

In terms of biomass accumulation, no significant differences were observed in shoot or root fresh weight between the rhizobia-inoculated plants and those receiving co-inoculation with rhizobia and NRE strains (Fig. 5). However, a significant improvement was noted in dry weight following co-inoculation. Specifically, the shoot dry weight increased by 32% compared to the rhizobia-only treatment, while root dry weight showed a remarkable 152% increase compared to the non-inoculated control (NI) (Fig. 5). These findings highlight that co-inoculation enhances biomass allocation more effectively at the dry matter level, likely due to improved nutrient acquisition and metabolic efficiency driven by synergistic microbial interactions.

Similar to shoot and root fresh weight, leaf area was not significantly affected by any of the applied treatments. This lack of improvement may be attributed to the short duration of the experiment, which lasted only 20 days. It is well known that the establishment of effective symbiosis between rhizobia and

leguminous plants requires time, particularly for nodule formation and functional nitrogen fixation. Moreover, the entry and activity of NREs within nodules or root tissues may also be delayed, further limiting the extent of their interaction with the plant during this short period (Fig. 6).

Therefore, longer-duration experiments are needed to fully capture the dynamics and potential benefits of co-inoculation with rhizobia and NREs. Such studies would allow for a more comprehensive evaluation of their effects not only on vegetative growth but also on faba bean yield and reproductive traits, which are essential for practical agricultural applications.

4. Discussion

Root nodules represent a specific ecological environements within the root systems of leguminous plants, including faba bean, because of the highly specific symbiotic relation between rhizobia and leguminous plant's systems. In this context, this study aims to develop a biofertilizer based on rhizobia and NREs associated with faba bean (*Vicia faba* L.) to improve plant growth.

This study confirms and demonstrates that RNs of faba bean harbor not only typical rhizobia but also a diverse range of NREs that do not belong to the α-proteobacteria group. A total of nine rhizobial strains and 71 NREs were recovered from the nodules of faba bean plants collected in the Aït Ourir region. In harmony with these results, Mowafy et al., [26] reported the isolation of four NRE strains from faba bean nodules, nbamely *Bacillus* sp. MAP3, *Brevibacillus* sp. MAP4, and *Pseudomonas* sp. MAP5 and MAP8. Simalalry, NREs have been recovered from the nodules of other leguminous plants, including cowpea (*Vigna unguiculata* L.), mung bean (*Vigna radiata* L.), lentil (*Lens culinaris* L.), chickpea (*Cicer aeietinum* L.) and faba bean [16, 17, 27]. These findings confirm the growing body of evidence that legume nodules can host a diverse microbial community and highlight the essential roles of NREs in enhancing plant growth and stress resilience through mechanisms that may complement rhizobial symbiosis.

Nodulation serves as a critical indicator of plant growth promotion and authenticates rhizobial strain effectiveness. Our nodulation assays revealed significant differences among rhizobia strains in faba bean. Strain F4.10 demonstrated superior performance, producing 10.00 ± 5.51 nodules per plant, significantly outperforming F2.13 (8.00 ± 7.94) and F2.12 (6.00 ± 3.05). In contrast, strains F4.11 showed minimal nodulation capability (2.00 ± 2,08).

Plant growth parameters strongly correlated with nodulation efficiency: F4.10-inoculated plants achieved maximal shoot length (60.33 ± 5.51 cm) and dry weight (1.03 ± 0.10 g), while poorly nodulating strain F4.11 resulted in stunted growth (27.33 ± 21.12 cm shoot length, ≤ 0.50 = g biomass). Our results are in harmony with those of Batra et al., (2020), who reported that out of 24 mesorhizobial isolates tested, 21 (87.5%) rhizobial strains showed positive nodulation in sterilized cups.

Plant growth-promoting traits are considered necessary traits to screen and select the best performing strains during the process of producing a biofertilizer. In this context, we have evaluated the efficiency of our NREs to fix nitrogen, solubilize phosphorus, and produce IAA. Among the 71 NREs tested,

approximately 65% demonstrated the ability to fix N_2, with positive strains distributed across all sampled. This widespread presence underscores the significant functional potential of these endophytes in supporting plant nitrogen nutrition. In a recent study, [28], dmonstrated that N_2-fix species belonging to *Bacillus* were isolated from the nodules of common pea (*Pisum sativum* L.). Those strains were able to actively grow on N free mediuù and also display various PGP traits. Simlalary, N_2-fix were isolated from the nodules of other legumes species such soybean (*Glycine max* L.); groundnut (*Arachis hypogea L.);* and mung bean *(Vigna radiata*) [29–31].

Phosphate solubilization and nitrogen fixation are equally important PGP mechanisms exhibited by the studied isolates. The P solubilization capacity of our NREs strains varied among the 15 tested isolates, ranging from 0.69 to 17.76 mg/L. Notably, isolates F3.10, F2.20, and F2.17 exhibited the highest solubilization activity, highlighting their promise as biofertilizers in phosphorus-deficient soils, while others showed moderate to low activity, suggesting their use may be more effective in consortia with stronger strains. Compared to the findings of Batra et al., (2019), who rdemonstrated that only 12% of chickpea endophytic isolates could solubilize more than 40 µg/mL of $Ca_3(PO_4)_2$, 6% showed moderate P solubilization (20–40 µg/mL), and 34% exhibited low P solubilization (4.0–25 µg/mL), our isolates generally displayed lower solubilization levels, none exceeding 17.76 mg/L. This suggests that while a few of our strains possess promising P-solubilizing abilities, they fall within the lower range of efficiency compared to those previously reported. Similarly, Kumar et al., [32] observed that 56% of NREs from legume nodules were capable of P solubilization, thereby contributing to improved plant growth.

Based on the categorization of IAA production levels among the tested isolates, 33% were classified as high IAA producers (> 15 µg/ml), including F3.04, F2.17, F3.03, F1.01, and F2.20. Approximately 27% fell into the moderate category (12–15 µg/ml), comprising isolates such as F2.03, F3.10, F4.09, and F5.01. The remaining 40% of isolates exhibited low IAA production (< 10 µg/ml), with F1.03 showing the lowest level (3.06 ± 0.24 µg/ml). Alinging with these results, [33] showed all tested NREs produced IAA, with concentrations reaching as high as 38 µg/ml and 71 µg/ml, indicating strong IAA producing potential. While in-vitro assays highlight the functional potential of these endophytes, their true effectiveness as bioinoculants ultimately depends on their interaction with host plants. In the present research, co-inoculation of the most promising PGPR isolates (F3.03, F3.04, F5.10, and F2.22) with the highly effective rhizobial strain F4.10 resulted in improved plant performance compared to inoculation with F4.10 alone. Significant increases were observed in root and shoot length. These findings further support the potential of tailored co-inoculation strategies to enhance legume growth through synergistic microbial interactions. For exemple, Bahroun et al. [34] proved that fababean inoculation with *Rahnella aquatilis* increased shoot/root length and weight. In another experiment, Fox et al., [35] reported the significant improvement of the growth of *Medicago truncatula* through the co-inoculation with *Ensifer* (*Sinorhizobium*) *medicae* and *Pseudomonas fluorescens*. This could be explained by the synergetic cooperation between typical symbiotic rhizobia and NREs. This research demonstrates that NREs are efficient plant growth promoters, particularly when interacting synergistically with symbiotic root-nodule bacteria such as rhizobia. In this context, co-inoculation strategies involving NREs and rhizobia may yield superior results compared to single-strain applications. For instance, certain NREs can not only fix

atmospheric nitrogen independently or synergistically but may also enhance rhizobial colonization, increase nodule number and size, and improve nitrogen assimilation efficiency. However, inoculation with NREs is not always postive, since negative effect was observed by the ionoculation with *Agrobacteriu*m strains that reduce the nodulation in common bean by *Rhizobium gallicum* [36].

Additionally, their ability to solubilize phosphates, produce phytohormones, or suppress pathogens complements the symbiotic activity of rhizobia, resulting in improved nutrient uptake and overall plant vigor. Therefore, the observed benefits of co-inoculation are likely due to these cooperative interactions, where each microbial partner contributes distinct but complementary functions that enhance plant performance under field conditions [37]. Establishing a functional symbiosis between rhizobia and legumes is a timedependent process, especially when it comes to nodule formation and effective nitrogen fixation. Similarly, the colonization and physiological activity of NREs within root tissues or nodules may occur gradually, potentially limiting their influence during short-term experiments.

As a result, longer experimental durations are necessary to better understand the dynamics and full benefits of co-inoculation strategies. Extended trials would enable a more thorough assessment of their impact, not only on early vegetative growth but also on yield and reproductive parameters of faba bean key indicators for real-world agricultural applications.

5. Conclusion

This study demonstrates that faba bean root nodules (RNs) harbor diverse community of both rhizobial and non-rhizobial endophytic bacteria (NREs) with significant plant growth–promoting potential. The isolation of nine rhizobial strains and 71 non-rhizobial endophytes displayed the diversity and the complexity of the fababean nodule microbiome and its functional relevance to plant performance. Several isolates exhibited key plant growth promoting traits, including nitrogen fixation, phosphate solubilization, and indole-3-acetic acid production. Among rhizobial isolates, strain F4.10 showed superior nodulation efficiency and consistently enhanced plant growth. In addition, selected NREs demonstrated strong plant growth–promoting capacities and significantly improved faba bean growth when co-inoculated with the rhizobial strain. These synergistic interactions resulted in higher shoot and root growth and increased biomass compared with single inoculations, emphasizing the advantage of using microbial consortia rather than individual strains.

Overall, the findings provide compelling evidence that combining rhizobia with functionally complementary non-rhizobial endophytes can enhance faba bean growth and productivity.

Declarations

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Conflict of interests

There is no conflict of interest between the authors.

Data availaibility

The authors declare that the results of this study are included in the manuscript and are available upon request from the corresponding author.

Consent to Publish declaration

Not applicable

Ethics and Consent to Participate

The study was conducted following the national guidelines.

Permissions to collect the plants/plant parts

Not applicable

Author Contributions

Conceptualization, A.R.; supervision, A.N, L.E., and A.R.; validation, A.R. and L.E.; investigation, S.N, E. O., and A.A.; methodology, S.N, E. O., and A.A..; data curation, S.N and A.R.; formal analysis, A.R.; software, A.R.; writing—original draft preparation, S.N; writing—review and editing, E.O., A.N., L.E., and A.R.; project administration, A.R.; funding acquisition, A.R. All authors have read and agreed to the published version of the manuscript.

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Figures

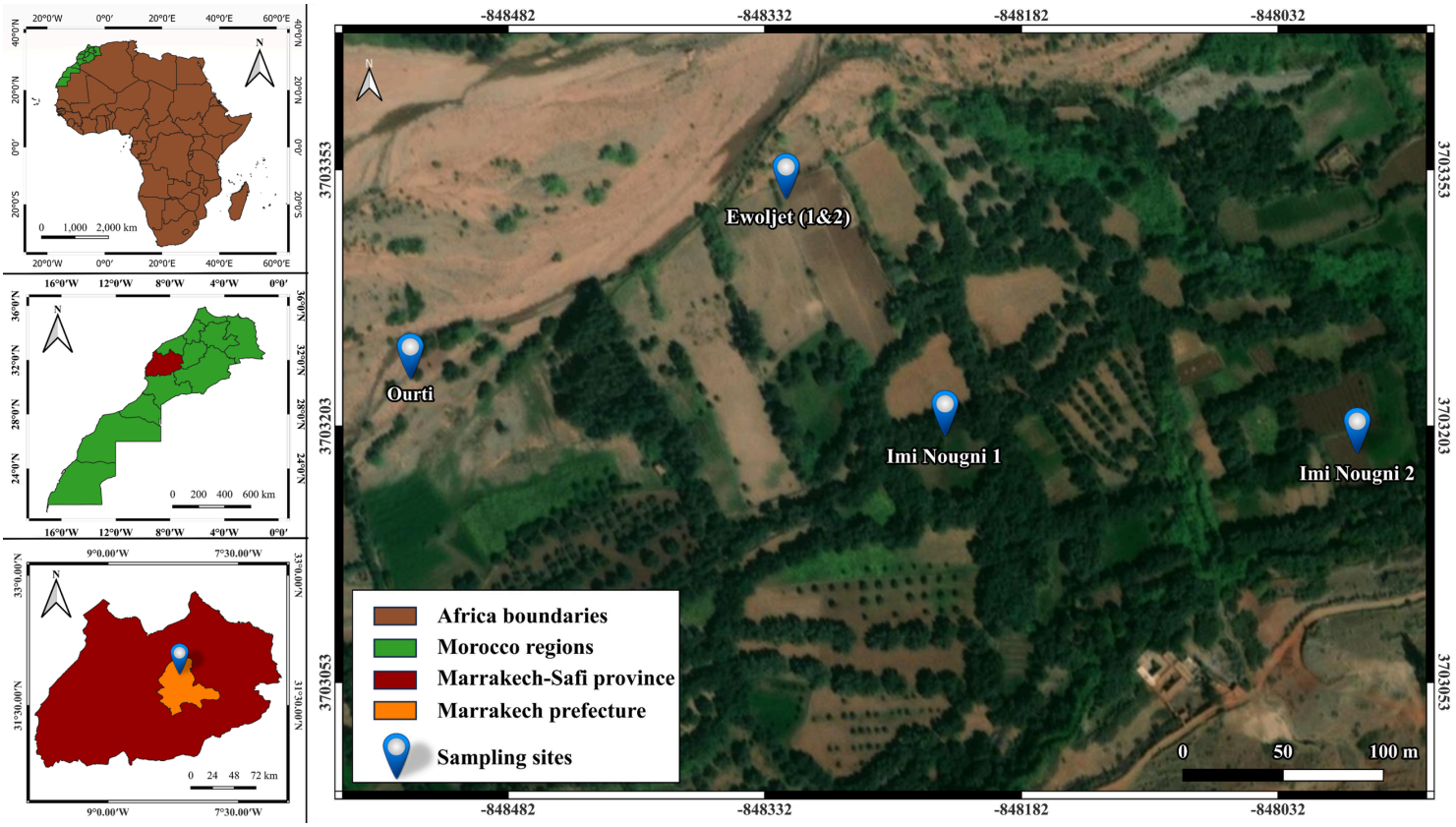


Figure 1

Geographical Distribution of Sampling Points

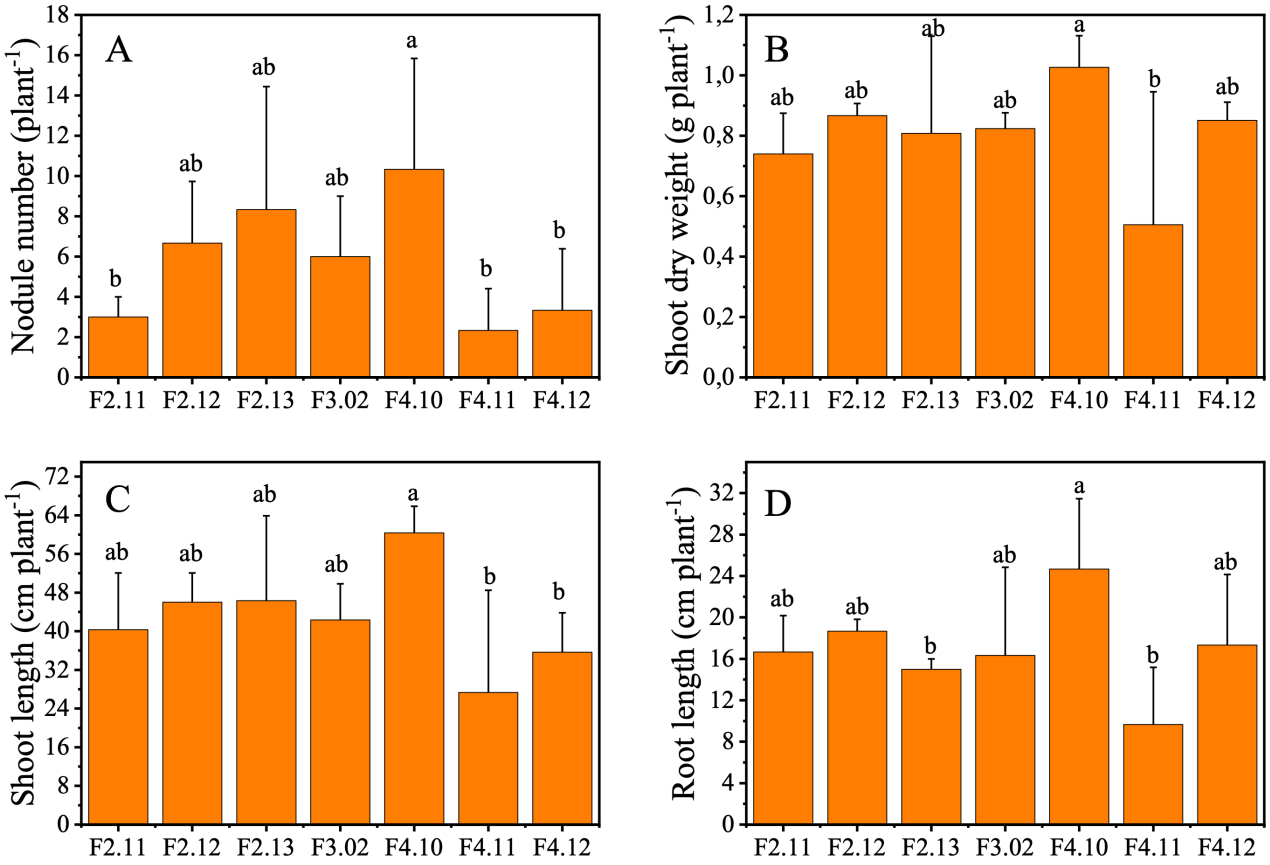


Figure 2

Effect of different rhizobia treatments on (A) nodule number, (B) shoot dry weight, (C) shoot length, and (D) root length of *Vicia faba* plants

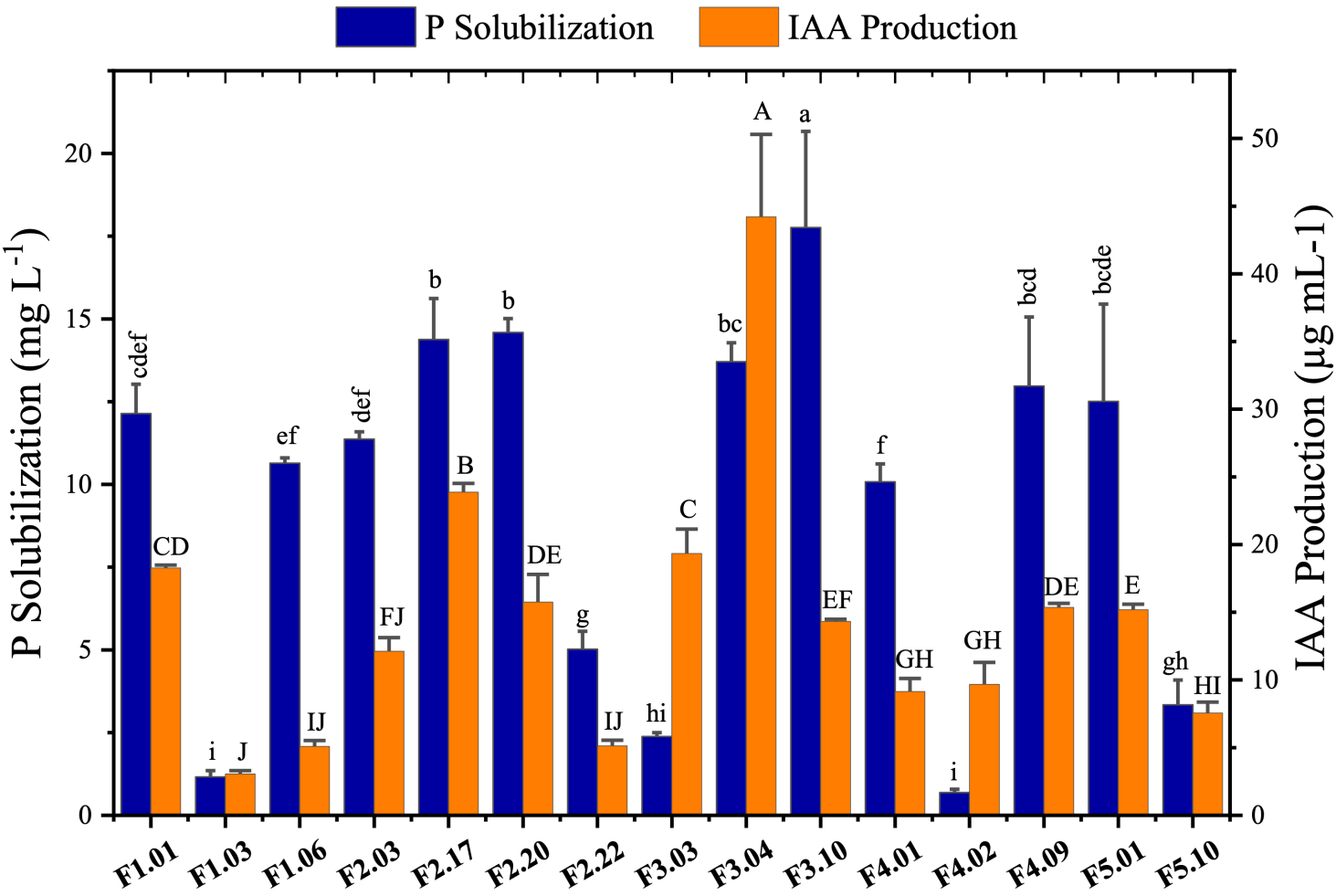


Figure 3

Phosphate Solubilization and IAA Production Profiles of non-rhizobial endophytes isolated from the nodules of *Vicia faba*

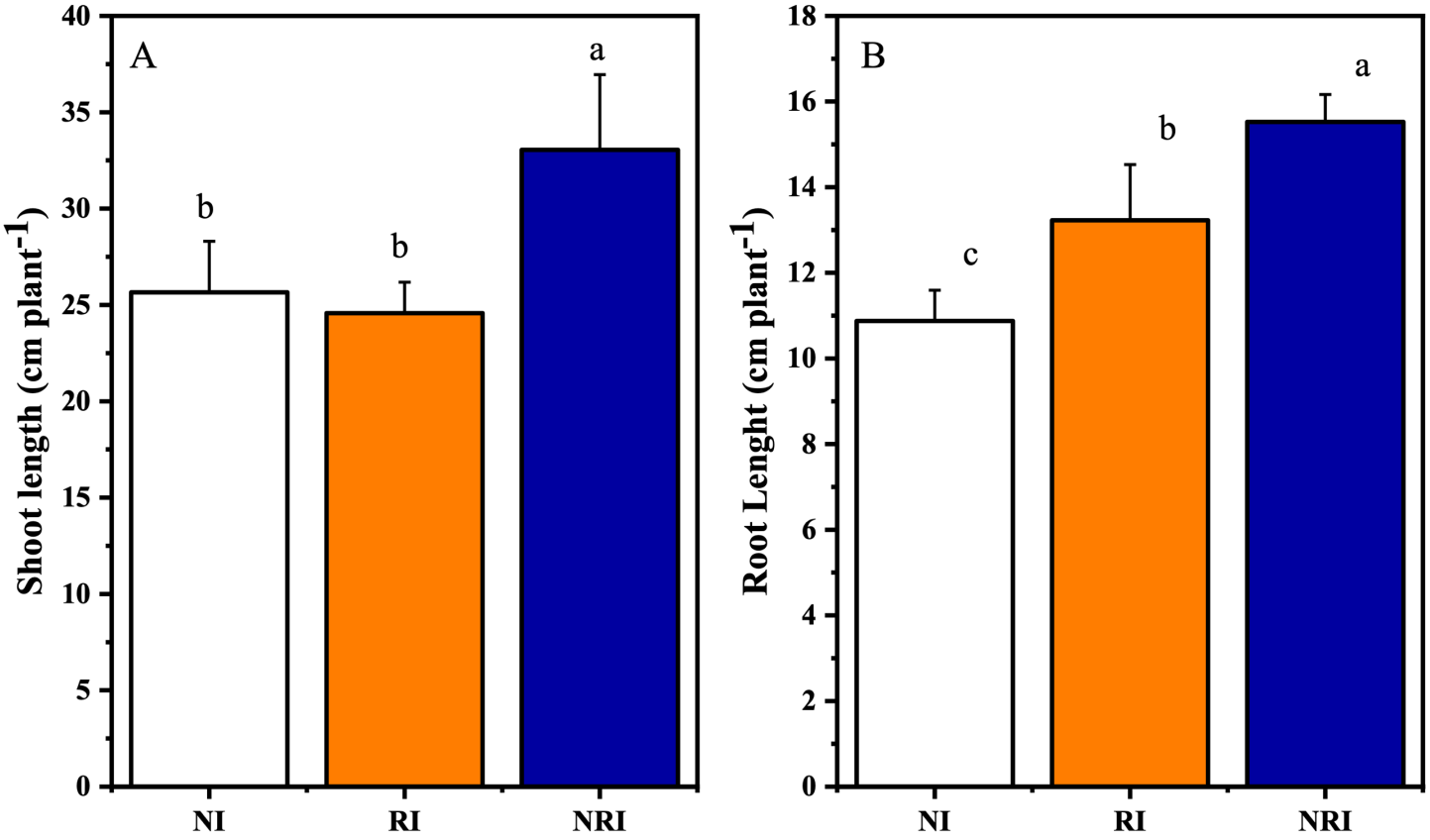


Figure 4

Effect of Rhizobia and Co-inoculation treatments on shoot and root length of *Vicia faba*Plants. NI = Non-inoculated, RI = Rhizobia-inoculated, NRI = Co-inoculated with Rhizobia and Non-Rhizobial Endophytes (NREs)

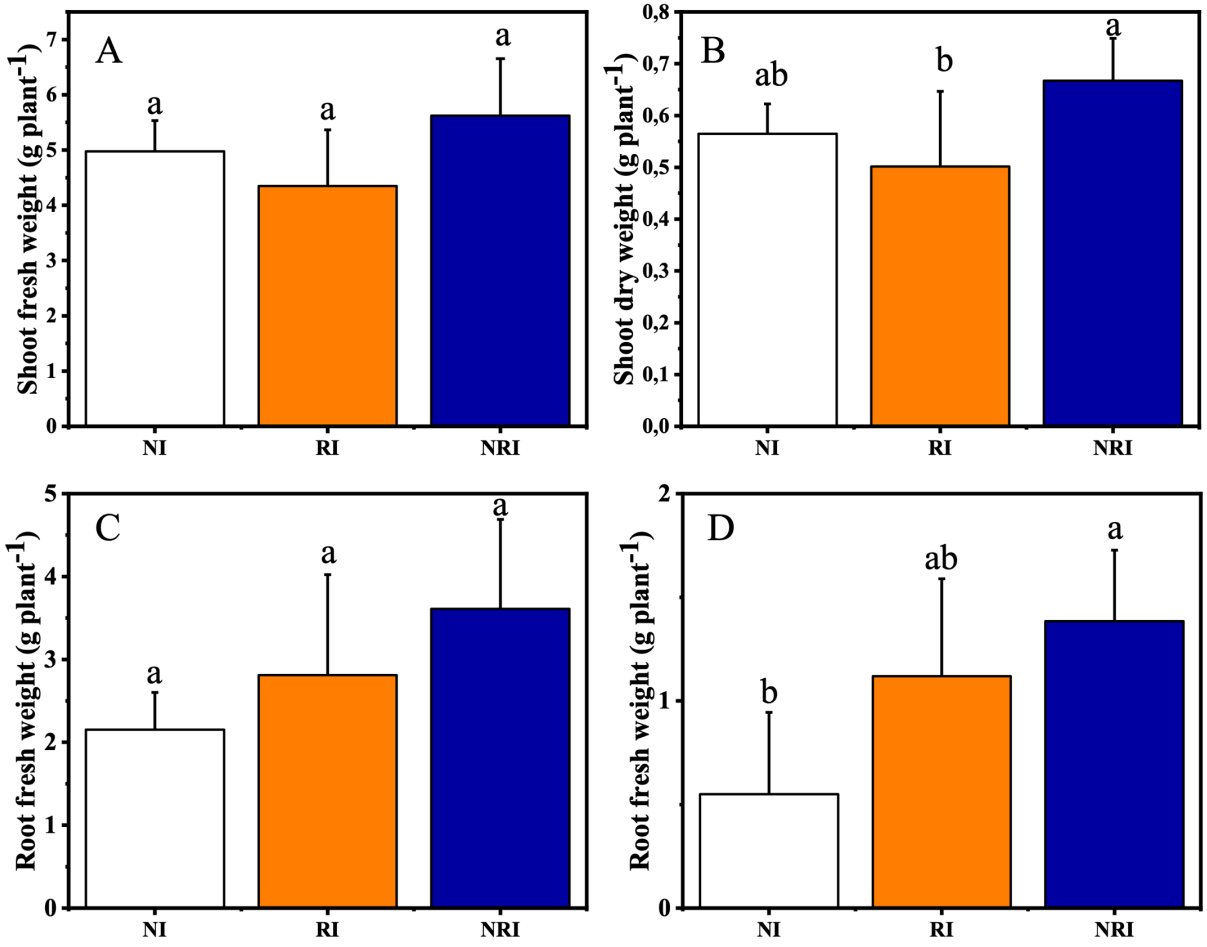


Figure 5

Effect of Rhizobia and Co-inoculation treatments plant on growth performance of

*Vicia faba*Plants. (A) shoot fresh weight, (B) shoot dry weight, (C) root fresh weight, and (D) root dry weight. NI = Non-inoculated, RI = Rhizobia-inoculated, NRI = Co-inoculated with Rhizobia and Non-Rhizobial Endophytes (NREs)

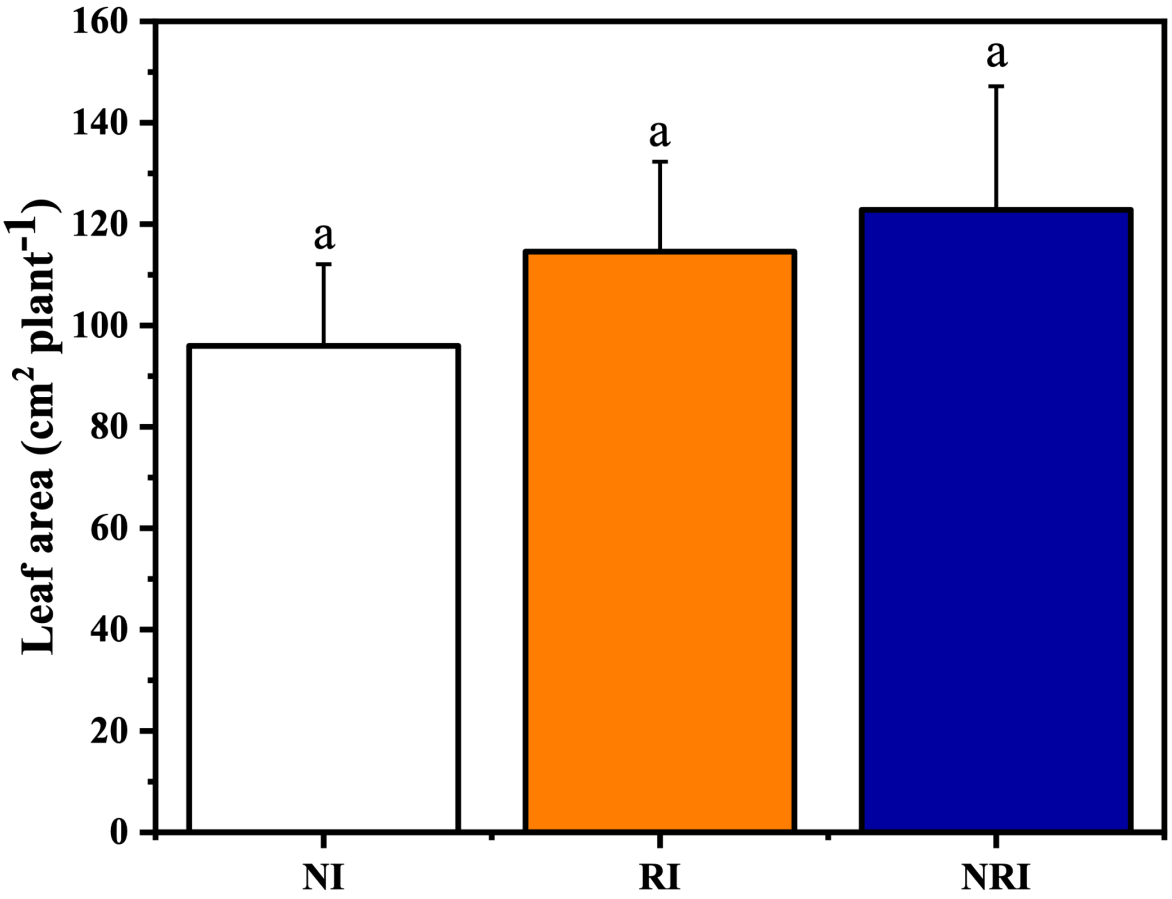


Figure 6

Effect of Rhizobia and Co-inoculation treatments plant on leaf area of *Vicia faba*Plants. (A) shoot fresh weight, (B) shoot dry weight, (C) root fresh weight, and (D) root dry weight. NI = Non-inoculated, RI = Rhizobia-inoculated, NRI = Co-inoculated with Rhizobia and Non-Rhizobial Endophytes (NREs)