agriculture

MDPI

Article

Competitiveness and Nitrogen Fixation Efficiency Analysis of *Rhizobium leguminosarum* Strains in Different Field Pea (*Pisum sativum* L.) Genotypes

Justina Kaziūnienė [1,*], Audrius Gegeckas [2], Laura Lapinskienė [3], Kristyna Razbadauskienė [1], Raimonda Mažylytė [2] and Skaidrė Supronienė [1]

[1] Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry, LT-58344 Akademija, Lithuania; skaidre.suproniene@lammc.lt (S.S.)
[2] Life Sciences Center, Institute of Biosciences, Vilnius University, LT-10257 Vilnius, Lithuania
[3] Department of Biology, Faculty of Natural Sciences, Vytautas Magnus University, LT-53361 Kaunas, Lithuania
* Correspondence: justina.kaziuniene@lammc.lt

Abstract

The uneven effectiveness of rhizobia inoculants has increased interest in developing specific inoculants for each genotype. This study investigated the biological nitrogen fixation efficiency and competition between different *Rhizobium leguminosarum* strains in different pea genotypes, namely, "Egle DS" and "Respect". The results showed that plant genotype was a significant factor determining competition and nitrogen fixation among *R. leguminosarum* strains. The most competitive *R. leguminosarum* LIN06 strain in the pea genotype "Egle DS" was characterized by a low nitrogen fixation efficiency, while the most competitive *R. leguminosarum* EGLE10 strain in the "Respect" genotype was characterized by a high biological nitrogen fixation efficiency. It was also found that the "Respect" genotype may prefer and form symbiotic relationships with more efficient nitrogen fixing strains, while the "Egle DS" genotype formed symbiotic relationships with less efficient strains. However, even less efficient strains had a significant positive effect on nitrogen accumulation in plants under natural conditions. Finally, our study showed that sophisticated tests and methods are not necessary to analyze the competitiveness of rhizobia; it is sufficient to analyze the effectiveness of bacterial strains on plants in unsterilized soil.

Keywords: *R. leguminosarum*; competition; biological nitrogen fixation; *Pisum sativum* L.

Academic Editors: Martin Weih and Tingting Chen

Received: 4 July 2025
Revised: 1 August 2025
Accepted: 16 August 2025
Published: 20 August 2025

Citation: Kaziūnienė, J.; Gegeckas, A.; Lapinskienė, L.; Razbadauskienė, K.; Mažylytė, R.; Supronienė, S. Competitiveness and Nitrogen Fixation Efficiency Analysis of *Rhizobium leguminosarum* Strains in Different Field Pea (*Pisum sativum* L.) Genotypes. *Agriculture* **2025**, *15*, 1784. https://doi.org/10.3390/agriculture15161784

1. Introduction

Pea (*P. sativum* L.) is a significant grain legume plant widely used in the human food and animal feed industry. It is the fourth most produced legume plant around the globe, behind beans, peanuts, and soybeans [1–3]. Pea grains are a valuable source of protein, carbs, fiber, amino acids, minerals, and vitamins [4,5]. Additionally, field pea is also useful as a rotation crop and cover plant because of its ability to increase soil quality through various mechanisms. Pea cultivation can improve soil structure by incorporating green biomass in the soil [6]. It can make weed control easier and break pest cycles [7–9], and it also can reduce soil pH and increase the availability of some mineral nutrients in the soil, such as Ca, Mg, K, and P [10]. Most importantly, pea plants can form a symbiotic relationship with nitrogen fixing bacteria called rhizobia [11,12].

Rhizobia are well known because of their ability to promote biological nitrogen fixation (BNF) and to enhance yield potentials in legumes [13–16]. Additionally, different

studies show that rhizobia inoculation can increase plant biomass formation and nitrogen accumulation in pea biomass [17–19]. Pea biomass incorporation increases organic carbon and nitrogen content in the soil, and rhizobia inoculant application also has an indirect positive influence on soil fertility and structure improvements [20–25]. Some research shows that rhizobia inoculation can also significantly increase available phosphorus and potassium content in the soil given rhizobia's ability to solubilize phosphate and to promote organic matter decomposition by enhancing soil organic matter and nitrogen content. Soil organic matter serves as the primary energy source for soil microorganisms [26,27]. Thus, rhizobia inoculation positively affects legume plants, soil, and even soil microorganisms.

According to Regulation (EU) 2019/1009 of the European Parliament and of the Council, *Rhizobium* spp. is one of four microorganism genera that can be registered and CE-marked as microbial plant biostimulants. As a result, biostimulants containing rhizobia are common in the EU market [28]. The main challenges facing biostimulant manufacturers and growers are the short shelf-life and uneven efficiency of rhizobia products. Rhizobium cells do not form endospores, making it difficult to prevent product expiration. The shelf-life of rhizobia biostimulants is usually 3–12 months and depends on the product formulation, the product's physical state, and the use of preservatives. Rhizobia biostimulants can be liquid formulations (with and without additives), solid carrier-based formulations (organic and inorganic), metabolite-based formulations, or synthetic polymer-based formulations, but there still is a great room for improvement [29,30].

Uneven rhizobia biostimulant efficiency depends on symbiosis efficiency between rhizobia and plant. Symbiosis efficiency varies and depends on many factors, such as rhizobia nitrogen fixation efficiency and competitiveness, host genotype, soil structure, pH and composition, meteorological conditions, and indigenous rhizobia diversity in the soil [31–34]. Soil structure and composition, meteorological conditions, and local rhizobia diversity cannot be controlled by humans, but a rhizobia strain can be chosen as the inoculum. It is not sufficient to have an intensive nitrogen-fixing rhizobia strain. Additional research needs to be performed to analyze the strain's genetic and physical properties, adaptation, compatibility with plant genotypes, and competitiveness against indigenous strains to develop a high-efficiency inoculum. Thus, although rhizobia inoculants have been used for more than a century, research to optimize their efficiency is still ongoing [35].

R. leguminosarum strain competitiveness with different pea genotypes is less studied, and additional investigations are necessary to understand the interaction between rhizobia strains and diverse pea genotypes to create high-efficiency rhizobia inoculants. Different marker-based methods, such as antibiotic resistance markers (rifampicin, streptomycin), enzyme reporters (*gusA*, *celB*, *lacZ)*, fluorescent proteins (GFP, RFP), or PCR based on *NodC* or other housekeeping genes, can be applied for rhizobia competition analyses [36–39]. Research objectives, conditions, available equipment, and resources are important elements to be assessed before selecting a method for rhizobia competition analysis. The *GusA* reporter gene system is easily applicable to large-scale experimental studies. The nodule-staining protocol is simple, and the method is considered safe for humans and the environment and highly reliable. Pea root nodules infected with tagged rhizobia strains can be visually observed based on color changes upon contact with chromogenic substrate, without the need for additional equipment to monitor changes [40–42]. *GusA* reporter gene expression is quite stable over time and under variable conditions, including different temperatures and pH values. Wildtype *GusA* lose expression at temperatures above 50 °C and some thermostable GUS mutants do not lose activity at 70 °C or higher [41,43]. Optimal pH diapason for *GusA* reporter gene expression is around pH 5.5–7.0, but some modified versions of *GusA* showed activity in more drastic pH ranges, such as 4.0–8.0 [39,43–45]. *GusA* markers, like other methods, have some drawbacks; the use of different nitrogen

forms for fertilization, nutrient deficiency or excess, phytohormones, and metals and heavy metals can promote or inhibit *GusA* expression [46–49]. The large-scale nature of this study, the strongly controlled investigation conditions, and the *GusA* application options led to the selection of this marker for rhizobia competition analysis.

The aim of this research was to select the most competitive and efficient *R. leguminosarum* strains for the biostimulation of two different pea genotypes: "Respect", which is of medium–early maturity and highly resistant to lodging and seed shedding from pods, a productive and popular genotype in Central and Northern European countries, including Lithuania; and the new genotype "Egle DS", developed by Lithuania scientists, which is of late maturity, resistant to lodging and to seeds falling out of pods, and also demonstrates high productivity, and high protein and essential amino acid contents [50]. Nineteen *R. leguminosarum* strains, genetically characterized in a previous study, were selected for this investigation because of their ability to significantly increase biomass accumulation in pea plants [24]. Some *R. leguminosarum* strains analyzed in this work were isolated from the "Respect" and "Egle DS" pea genotypes. In this research, all 19 *R. leguminosarum* strains' influence on total nitrogen accumulation in pea biomass was tested in a sterile vermiculite–sand mixture and non-sterile soil. Finally, five *R. leguminosarum* strains with the highest influence on total nitrogen accumulation in pea biomass were selected for competition analysis in experiments with "Respect" and "Egle DS" genotypes.

2. Materials and Methods

2.1. R. leguminosarum Strains Biological Nitrogen Fixation Efficiency Evaluation

In 2022, fifty rhizobia isolates were isolated from five different pea genotypes, which were grown in the Kedainiai district, Dotnuva, Lithuania [55.396450, 23.866748]. All five pea genotypes—"Bagoo", "Respect, "Astronaute", "Lina DS" and "Egle DS"—used for rhizobia isolation were grown in the same field, in which commercial rhizobia inoculants had never been used before. All fifty rhizobia isolates were genetically characterized by 16S rRNA, *recA*, *atpD*, and *nodC* genes and investigated with pea plants into 2022–2023.

Nineteen isolates that showed the highest efficiency on pea biomass accumulation in a previous study were selected for biological nitrogen fixation efficiency analysis on field pea genotype "Egle DS". Based on housekeeping *recA* and *atpD* genes' analysis results, all 19 isolates were identified as *R. leguminosarum*. Eleven of the nineteen *R. leguminosarum* strains were genetically similar based on the genes investigated; the remaining eight strains were genetically different (Table 1). Primary experiments were performed in 2024 and carried out in sterile vermiculite–sand mixture and in unsterilized soil to select the five most efficient biological nitrogen fixing *R. leguminosarum* strains for competition analysis.

2.1.1. *R. leguminosarum* Strains' Biological Nitrogen Fixation Efficiency Evaluation in Sterile Vermiculite–Sand Mixture

Pea seeds were sterilized in a 70% ethyl alcohol solution, washed with sterile deionized water (SDW), and additionally disinfected with sodium hypochlorite solution containing 5% active chlorine for 5 min. Finally, sterile seeds were rinsed five times with SDW. Sterile seeds were germinated in Petri plates on sterile filter paper Whatman 1 (Cytiva, Marlborough, MA, USA) at room temperature, in the dark, for 4 days. One pea seedling was planted in a 500 mL vegetative pot which was filled with a sterile mixture of vermiculite and sand in a ratio of 3:1 and supplied with 100 mL of a nitrogen-free nutrient medium [1 mM $CaCl_2 \times 2H_2O$, 0.1 mM KCl, 0.8 mM $MgSO_4 \times 7H_2O$, 10 µM Fe EDTA, 35 µM H_3BO_3, 9 µM $MnCl_2 \times 4H_2O$, 0.8 µM $ZnCl_2$, 0.5 µM $Na_2MoO_4 \times 2H_2O$, 0.3 µM $CuSO_4 \times 5H_2O$, 368 µM KH_2PO_4 and 400 µM Na_2HPO_4, pH = 6.5] (Sigma-Aldrich, St. Louis, MO, USA) [39,51].

Table 1. Characterization of *R. leguminosarum* strains used in this study. Type strains are indicated with a superscript T.

R. leguminosarum Strain	Host Plant	Host Plant Genotype	Symbiovar	Genospecies Based on Concatenated Alignment of *recA* and *atpD* Gene Sequences	Genetica Relation Based on *nodC* Gene Phylogenetic Analysis	Reference
ASTR03	*Pisum sativum* L.	"Astronaute"	*R. leguminosarum* sv. *viciae*	*R. leguminosarum* bv. *viciae* 3841 (B)	*R. ruizaguesonis* UPM1133	[24]
ASTR08		"Astronaute"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1164	
BAGOO07		"Bagoo"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1164	
EGLE03		"Egle DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1164	
EGLE04		"Egle DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1164	
EGLE05		"Egle DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1164	
EGLE06		"Egle DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* li29	
EGLE07		"Egle DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1164	
EGLE09		"Egle DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* vd14	
EGLE10		"Egle DS"		*R. leguminosarum* USDA 2370^{T} (E)	*R.* KNa13	
LIN03		"Lina DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1164	
LIN04		"Lina DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1164	
LIN06		"Lina DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv *viciae* 3841	
LIN07		"Lina DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1192	
LIN08		"Lina DS"		*R. leguminosarum* USDA 2370^{T} (E)	*R. leguminosarum* bv. *viciae* BIHB 1164	
LIN09		"Lina DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* 3841	
LIN10		"Lina DS"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1164	
RSP05		"Respect"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1164	
RSP08		"Respect"		*R. leguminosarum* bv. *viciae* 3841 (B)	*R. leguminosarum* bv. *viciae* BIHB 1164	

R. leguminosarum isolates were grown in TY broth [5 g tryptone (Organotechnie S.A.S., La Courneuve, France), 3 g yeast extract (Organotechnie S.A.S., La Courneuve, France) and 1.3 g $CaCl_2 \times 6H_2O$ (Sigma-Aldrich, St. Louis, MO, USA)] [52] for 24 h until optical density (OD600) reached 0.6–0.8. Then, isolate suspensions OD600 were adjusted to =0.05 using sterile 0.8% NaCl solution. The number of cells in 1 mL of prepared rhizobia isolate suspension was obtained according to the serial dilution method, plating suspensions on a solid YEMA medium [53]. For each replicate, a fresh rhizobia suspension was prepared and used to inoculate individual plants. Each variant was grown in four replicates; in total, four independent inoculations were carried out per treatment. Pea seedlings were inoculated at the leaf development stage (BBCH 07) with 1 mL of fresh rhizobia isolate suspension and grown in the plant growth chamber GC40 (Canden Products LLC, USA) for 1 month (BBCH 37) using a 16/8 h day/night photoperiod and 20/16 °C day/night temperature modes [54]. Uninoculated plants were used as controls. A total nitrogen analysis was performed of the dry plant biomass after harvest.

2.1.2. *R. leguminosarum* Strains Biological Nitrogen Fixation Efficiency Evaluation in Non–Sterilized Soil

Pea seeds sterilization, germination, *R. leguminosarum* strain cultivation, and plant growing experiments were performed according to the same procedures as in Section 2.1.1. Unsterilized soil was used for the investigation (Table 2). Nitrogen-free nutrient medium was not supplied for plant feeding. Plants were watered with SDW according to the

demand. Each variant was grown in four replicates. The total nitrogen analysis was performed in dry plant biomass after harvest.

Table 2. Soil properties.

Properties						
Sample	**Granulometric Composition**	**Humus, %**	**pH**	**Nmin, mg kg^{-1}**	**Mobile Phosphorus P_2O_5, mg kg^{-1}**	**Mobile Potassium K_2O, mg kg^{-1}**
SOIL15, 0–30 cm	Heavy loam	2.80	6.7	10.42	230	220

2.2. Rhizobium leguminosarum Strains Competition Analysis

Five of the most efficient biological nitrogen fixing *R. leguminosarum* strains were selected for competition analysis. Competition between *R. leguminosarum* strains was performed with two field pea genotypes, "Respect" and "Egle DS", in sterile vermiculite–sand mixture. All five *R. leguminosarum* strains were marked with *gusA* reporter gene by bi-parental mating and investigated in pea experiments in the same year, 2024.

2.2.1. *R. leguminosarum* Strains Marking with *gusA* Reporter Gene

The *R. leguminosarum* strains were marked with *gusA* reporter gene. Plasmid carrying the *gusA* reporter gene (Plasmid #133228 (pOPS0263)) was taken from Addgene collection "https://www.addgene.org (accessed on 6 April 2024)". The antibiotic sensitivity of *R. leguminosarum* strains and *Escherichia coli* carrying the *gusA* reporter gene was determined and a transconjugants screening was performed according to the methodology of Marcela Mendoza-Suárez and colleagues, with the only exception being that X–GlcA (5–Bromo–4-chloro–3-indolyl β–D–glucuronide sodium salt) (Sigma-Aldrich, St. Louis, MO, USA) solution 200 μg/mL was used as a substrate [55].

2.2.2. Coinoculation with Two *R. leguminosarum* Strains

The coinoculation investigation with two field pie genotypes was performed in a sterile vermiculite–sand mixture according to the procedures and experiment conditions described in Section 2.1.1.

R. leguminosarum transconjugants were cultivated in TY broth supplemented with nitrofurantoin 10 μgmL^{-1} (Sigma-Aldrich, St. Louis, MO, USA) and kanamycin 50 μgmL^{-1} (Sigma-Aldrich, St. Louis, MO, USA) for 3 days. *R. leguminosarum* transconjugant suspensions were centrifuged and washed with fresh TY broth three times to remove antibiotics. The final suspensions' optical density (OD600) was adjusted with TY broth till 0.6–0.8. The number of cells in 1 mL of prepared rhizobia isolate suspension was obtained according to the serial dilution method, plating suspensions on a solid YEMA medium. Pea seedlings at the leaf development stage (BBCH 07) were inoculated with 1 mL unmarked rhizobia isolate suspension and with 1 mL *gusA* marked rhizobia strain (Table 3). Uninoculated plants and plants inoculated with only one strain *R. leguminosarum* (1 mL per seedling) were used as controls. Control variants with one strain inoculation were used to evaluate the efficiency difference between unmarked and genetically modified *R. leguminosarum* strains in preliminary plant experiments. It was observed that the *gusA* marker had no effect on the symbiotic performance of *R. leguminosarum* strains. Each variant was grown in four replicates, and for each replicate a fresh rhizobia suspension was prepared and used to inoculate individual plants. Plants were grown in the plant growth chamber GC40 (Canden Products LLC, USA) for 1 month (BBCH 37) using a 16/8 h day/night photoperiod and 20/16 °C day/night temperature modes [54]. A Competitiveness Index evaluation and total nitrogen analysis of dry plant biomass were performed after harvest, and nodules were

counted. The nodule-staining with X–GlcA (5–Bromo–4-chloro–3-indolyl β–D–glucuronide sodium salt) (Sigma-Aldrich, St. Louis, MO, USA) solution 200 µg/mL as substrate was performed to detect β–Glucuronidase activity; roots with nodules were incubated overnight at 28 °C [55]. The Competitiveness Index was calculated using the following formula: CI of Y = (nodulesY/nodulesX)/(cfuY/cfuX). cfuY and cfuX are the number of colony-forming units of co-inoculated strains Y and X per ml of inoculant suspension applied to plants, and nodulesY and nodulesX are the number of nodules occupied by the respective strains [56]. The total nitrogen in plant biomass was analyzed using the Kjeldal method [57].

Table 3. Scheme of *R. leguminosarum* strains' competition investigation. "x" indicates inoculation with the respective strain.

			Field Pea				
			Strains marked with *gusA*				
		K	EGLE07	BAGOO07	ASTR08	EGLE10	LIN06
	K	Not inoculated	x	x	x	x	x
Not marked strains	LIN06	x	x	x	x	x	
	EGLE07	x		x	x	x	
	BAGOO07	x			x	x	
	ASTR08	x				x	
	EGLE10	x					

2.3. Statistical Analysis

The statistical analyses were performed using the statistical software SAS 9.4 (SAS Institute, Cary, NC, USA) [58]. Correlation and one-way and two-way analyses of variance (ANOVA) statistical tests were used for data analysis. Tukey's HSD test was applied to analyze mean comparisons between different variants. The smallest significant difference was calculated using a probability level of $p \geq 0.05$. The values marked with the same letter showed no significant difference at $p \geq 0.05$.

3. Results

3.1. R. leguminosarum Strains' Biological Nitrogen Fixation Efficiency Evaluation in Different Substrates

Plant experiments with pea genotype "Egle DS" and 19 *R. leguminosarum* strains showed that all strains examined had different influences on total nitrogen accumulation in the plants. It was also found that the same *R. leguminosarum* strains' influence on plant total nitrogen accumulation was different in the vermiculite–sand mixture (sterilized) and soil (not sterilized) substrates (Figure 1).

The highest total nitrogen in the pea plants was observed accumulated in the vermiculite–sand mixture. Total nitrogen varied from 2.9% to 7.6% in variants where *R. leguminosarum* strains were applied. The results showed that all pea plants where *R. leguminosarum* strains were applied formed nodules on pea roots and accumulated a statistically significant higher amount of total nitrogen compared to the control without bacterial application. No nodules were found on control plant roots. The highest total nitrogen accumulated in pea plants where *R. leguminosarum* EGLE07, *R. leguminosarum* BAGOO07, *R. leguminosarum* EGLE05, and *R. leguminosarum* ASTR08 strains were applied. Differences in the amount of nitrogen accumulated in the plant biomass were not statistically significant. Total nitrogen accumulated in these plants was higher than 7% and was more than 4.8-fold higher compared to the control.

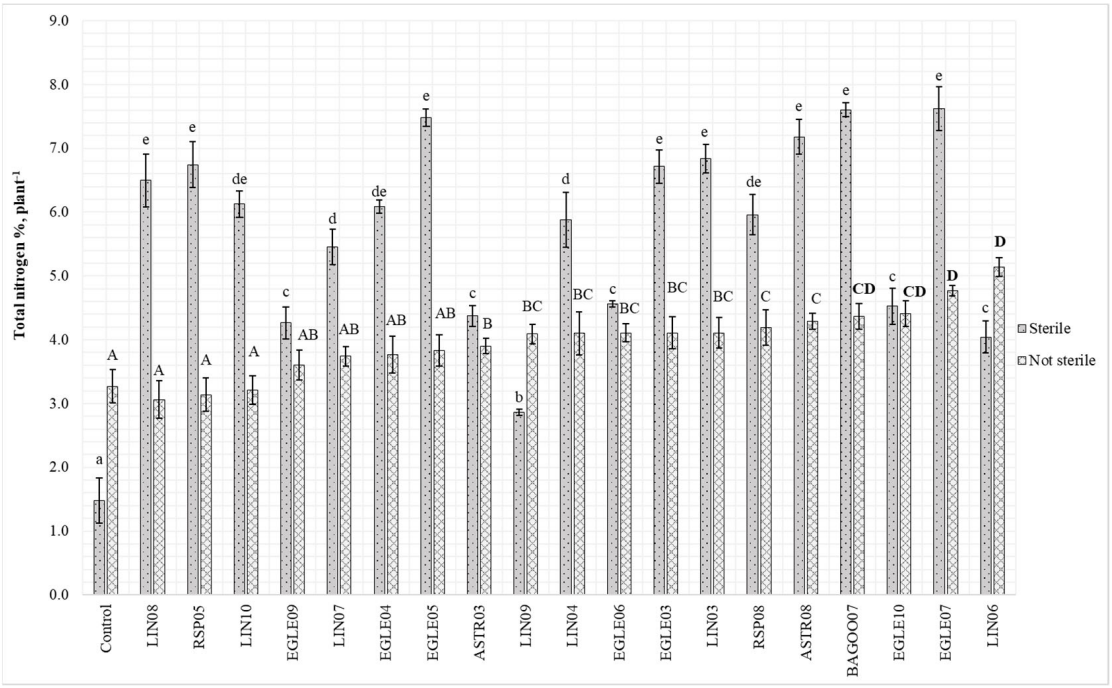


Figure 1. Total nitrogen accumulated in "Egle DS" genotype pea biomass after the application of different *R. leguminosarum* strains. Plants were grown in sterilized vermiculite–sand mixture (sterile) and unsterilized soil (not sterile). Error bars indicate the standard deviations within four biological replications at each treatment. Lowercase letters indicate statistical differences in vermiculite–sand mixture (sterile) and uppercase letters represent statistical differences in unsterilized soil (not sterile). Values marked with the same letter are not significantly different at $p \geq 0.05$ (ANOVA, Tukey HSD).

Pea plant experiment also showed that, after inoculation with *R. leguminosarum* strains, less total nitrogen accumulated in plants which were grown in the soil compared to the plants grown in the sterilized vermiculite–sand mixture; only the control plants provided exceptions. The plant investigation in soil revealed that plants in all variants, including control, formed root nodules, but only 12 of 19 *R. leguminosarum* strains significantly increased total nitrogen accumulation in pea plants compared to the control. The highest total nitrogen accumulated in pea plants, where *R. leguminosarum* LIN06, *R. leguminosarum* EGLE07, *R. leguminosarum R.* EGLE10, and *R. leguminosarum* BAGOO07 strains where inoculated; total nitrogen was 5.1%, 4.8%, 4.4% and 4.4%, respectively, and was from 1.3-fold to 1.5-fold higher compared to the control. Five *R. leguminosarum* strains—*R. leguminosarum* LIN06, *R. leguminosarum* EGLE07, *R. leguminosarum* EGLE10, *R. leguminosarum* BAGOO07, and *R. leguminosarum* ASTR08—that enhanced total nitrogen accumulation in plants during the soil experiments were selected for competition analysis.

3.2. R. leguminosarum Strains' Biological Nitrogen Fixation Efficiency and Competition Analysis in Different Pea Genotypes

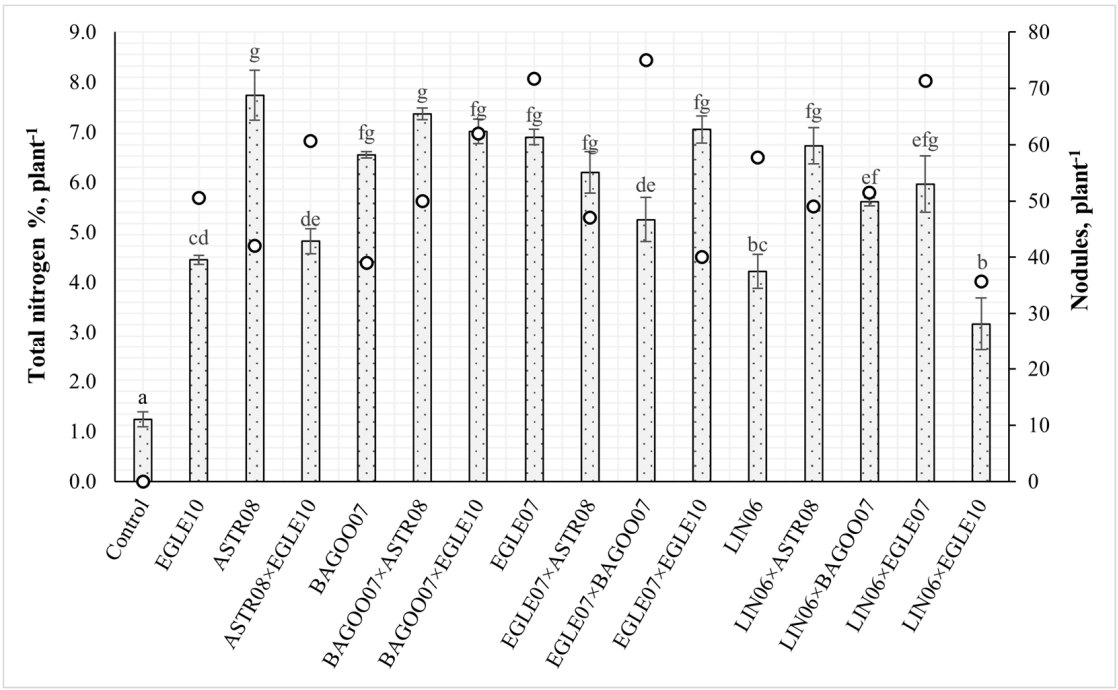
The total nitrogen accumulation in pea biomass and competition between *R. leguminosarum* isolates was analyzed in pea growth experiments in a sterile vermiculite–sand mixture. Investigation with pea genotypes "Egle DS" and "Respect", where *R. leguminosarum* strains were inoculated in different combinations or separately, showed that plants accumulated different amounts of total nitrogen (Figure 2). The total nitrogen accumulation results with "Egle DS" plants, where *R. leguminosarum* strains were inoculated separately, was similar compared to the results obtained in the sterile vermiculite–sand mixture, presented in Section 3.1 (Figure 2A), (Figure 1). *R. leguminosarum* EGLE07, BAGOO07 and ASTR08, strains were more efficient in terms of total nitrogen accumulation in the pea

plant biomass compared to *R. leguminosarum* EGLE10 and LIN06 strains. Total nitrogen accumulated in the plants where *R. leguminosarum* EGLE07, BAGOO07, and ASTR08, and EGLE10 and LIN06 strains were inoculated separately was 6.9%, 6.5%, 7.7%, and 4.4%, 4.2%, respectively. The total nitrogen accumulation increased by 5.7-fold with *R. leguminosarum* EGLE07 application, 5.4-fold with *R. leguminosarum* BAGOO07, and 6.4-fold with *R. leguminosarum* ASTR08 inoculation compared to the control.

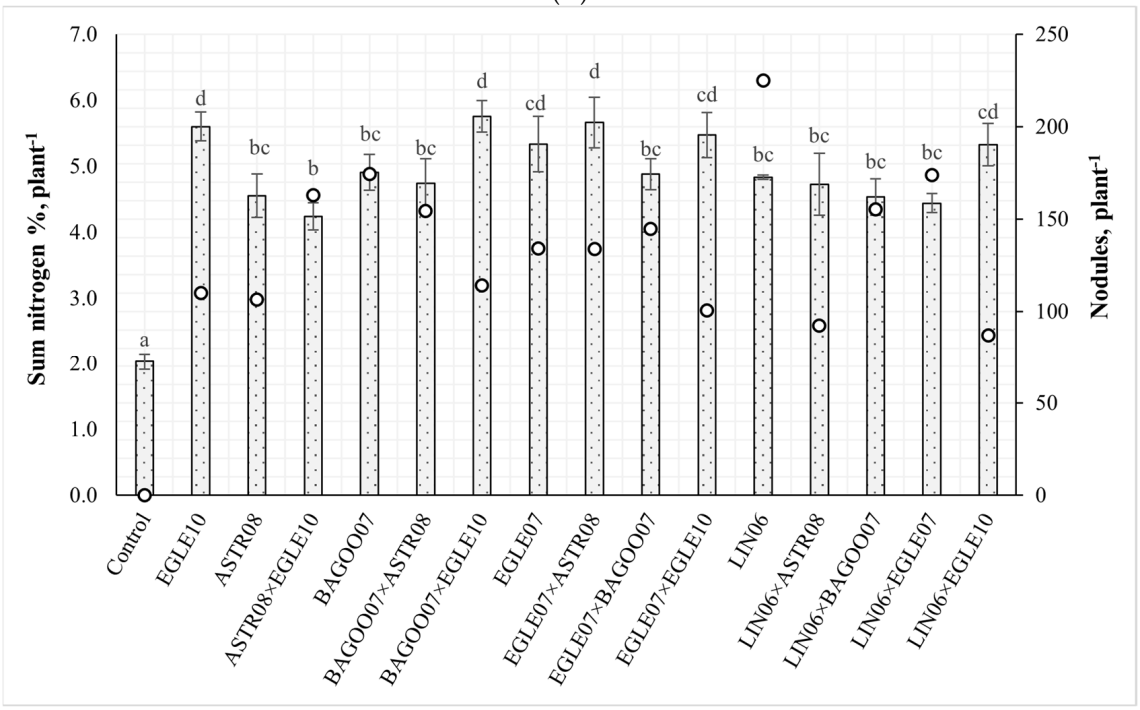
The total nitrogen accumulation results in "Egle DS" genotype plants inoculated with different *R. leguminosarum* strain combinations showed that total nitrogen was low in the plants which were inoculated with two low-efficiency *R. leguminosarum* strains. In combination with *R. leguminosarum* EGLE10 and *R. leguminosarum* LIN06, the total nitrogen accumulated in the plant biomass was only 3.2%. In combinations where one *R. leguminosarum* strain has high efficiency and another one has low efficiency, the total nitrogen varied from 4.8% to 7.1%. It was determined that the highest amount of total nitrogen was found in a variant where only one *R. leguminosarum* ASTR08 strain was inoculated; total nitrogen accumulated in the plant was, on average, 7.7%. It was also found that *R. leguminosarum* ASTR08 inoculation in combination with different *R. leguminosarum* strains increased total nitrogen accumulation in almost all pea plants. *R. leguminosarum* BAGOO07 and ASTR08, *R. leguminosarum* EGLE07 and EGLE10, and *R. leguminosarum* BAGOO07 and EGLE10 combinations have the highest influence on nitrogen accumulation in plant biomass compared to all analyzed *R. leguminosarum* combinations; the total nitrogen accumulated in pea plants was 7.4%, 7.1%, and 7.0%, respectively.

The nodule-counting results showed that inoculation of single *R. leguminosarum* strains or combinations of strains had different effects on plant nodulation (Figure 2A), (Supplementary Figure S1). The highest number of nodules was found in pea variants inoculated with the combination of *R. leguminosarum* EGLE07 and BAGOO7, *R. leguminosarum* EGLE07 as a single strain, and the combination of *R. leguminosarum* LIN06 and EGLE07. The average number of nodules in these variants varied from 71 to 75 units and made no significant difference. Nitrogen accumulation in these three variants was not very high, but the *R. leguminosarum* EGLE07 and BAGOO7 combination accumulated a significantly lower amount of total nitrogen compared to the *R. leguminosarum* EGLE07 variant. The *R. leguminosarum* EGLE07 strain was inoculated in all three variants where the highest number of nodules was observed. No nodules were found on control plants' roots. In other variants, the average number of nodules varied from 36 to 62 units per plant.

The total nitrogen accumulation results in the "Respect" genotype plants, where *R. leguminosarum* strains were inoculated separately, showed that *R. leguminosarum* EGLE10 strain has the highest influence on the total nitrogen accumulation in the plant biomass (Figure 2B). Nitrogen accumulated in plant biomass inoculated with *R. leguminosarum* EGLE10 strain was, on average, 5.6%, and 2.8-fold higher compared to the control. *R. leguminosarum* BAGOO07, *R. leguminosarum* ASTR08, and *R. leguminosarum* LIN06 strains showed similar, statistically similar results; the average nitrogen accumulated in plant biomass varied from 4.5% to 4.9% and was from 2.2-fold to 2.4-fold higher compared to the control. Total nitrogen accumulated in the pea biomass after *R. leguminosarum* EGLE07 inoculation was, on average, 5.3%, around 2.6-fold higher compared to the control, with no statistical difference compared to *R. leguminosarum* BAGOO07 and *R. leguminosarum* EGLE10.



(**A**)



(**B**)

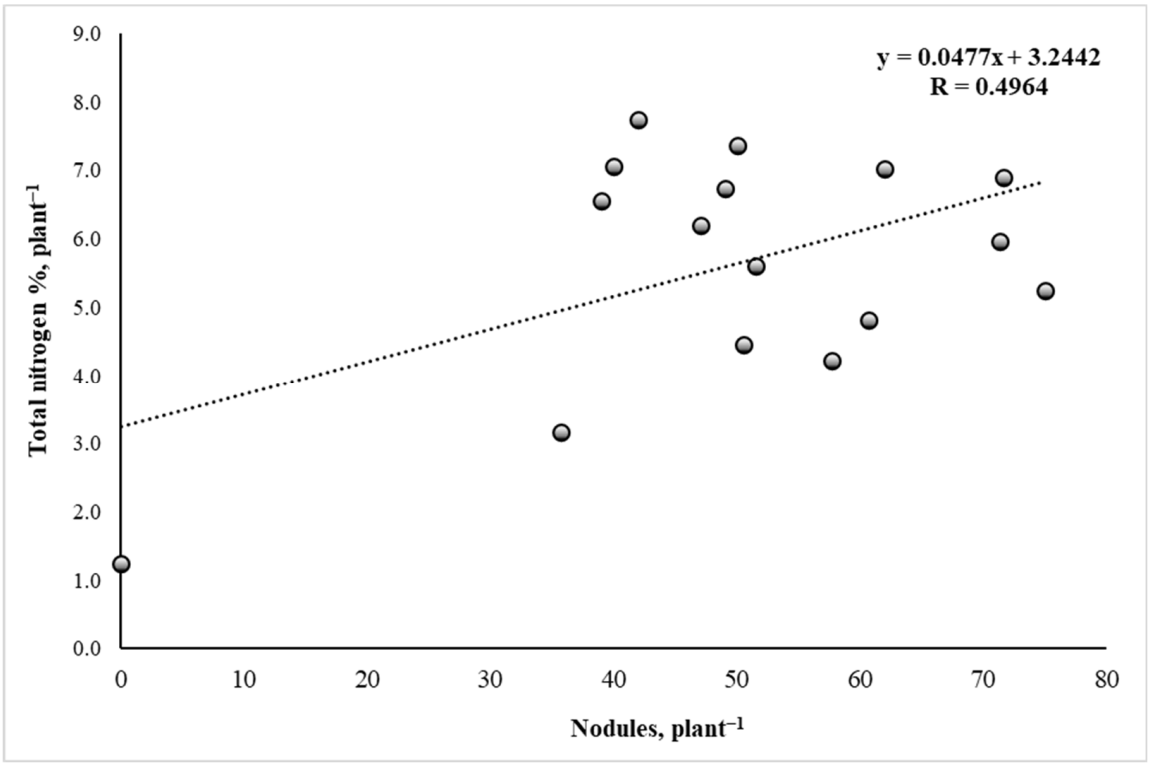
Figure 2. (**A**) The averages of total nitrogen accumulated in the "Egle DS" pea genotype biomass (columns), as well as the number of nodules on roots (ovals) after inoculation with different rhizobia isolate combinations. Error bars indicate the standard deviations within four biological replications at each treatment. Values marked with the same letter are not significantly different at $p \geq 0.05$ (ANOVA, Tukey HSD). (**B**) The averages of total nitrogen accumulated in "Respect" pea genotype biomass (columns) and the number of nodules on roots (ovals) after inoculation with different rhizobia isolate combinations. Error bars indicate the standard deviations within four biological replications at each treatment. Values marked with the same letter are not significantly different at $p \geq 0.05$ (ANOVA, Tukey HSD).

The total nitrogen accumulation results in "Respect" genotype plants inoculated with different *R. leguminosarum* strain combinations demonstrated that *R. leguminosarum* EGLE10 strain inoculation increased total nitrogen averages in almost all combinations; the only exception was the combination with *R. leguminosarum* ASTR08. *R. leguminosarum* EGLE10 stain insertion, in combination with *R. leguminosarum* BAGOO07, significantly increased total nitrogen accumulation in the plant biomass compared to single-strain inoculation with the *R. leguminosarum* BAGOO07 strain. The average nitrogen accumulated in the pea biomass was 5.8%, when the combination of *R. leguminosarum* EGLE10 and *R. leguminosarum* BAGOO07 was applied, which was 2.9-fold higher compared to the control.

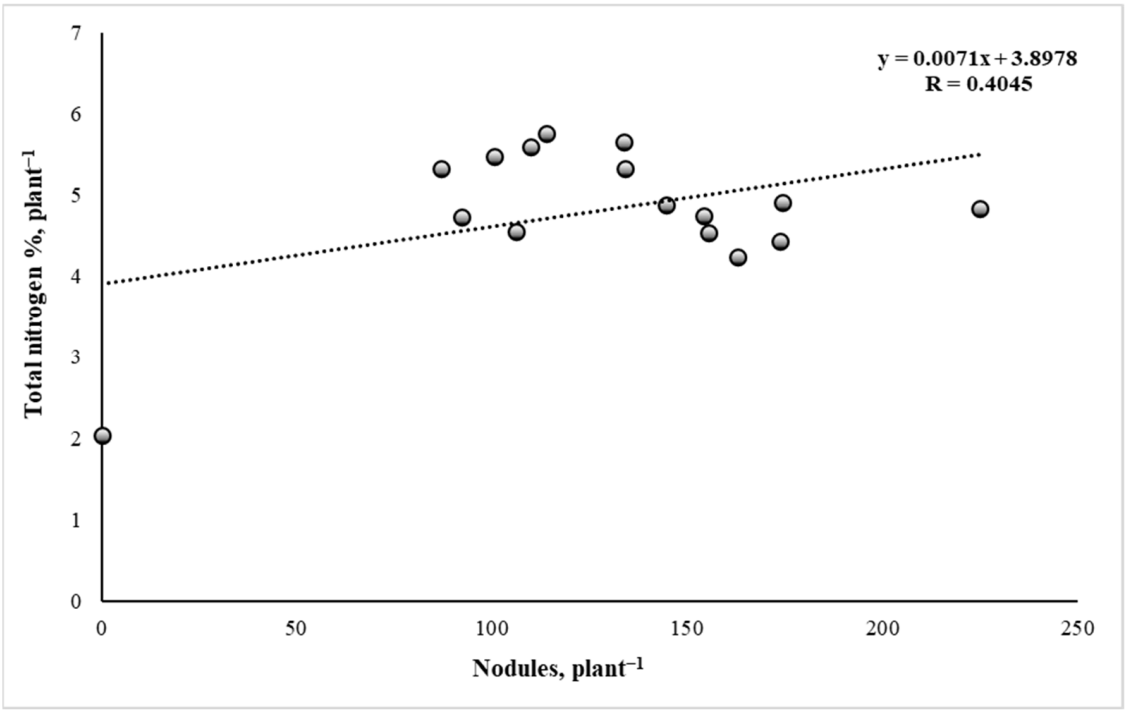
The nodule-counting results showed that the use of single *R. leguminosarum* strains or inoculation with different combinations had different effects on "Respect" genotype pea nodulation (Figure 2B), (Supplementary Figure S2). The highest number of nodules was found in pea variants where a single *R. leguminosarum* LIN06 strain was inoculated. The average number of nodules in this variant was 225 units, which was significantly higher compared to other variants inoculated with *R. leguminosarum* combinations or single strains. However, nitrogen accumulation in plants inoculated with the single *R. leguminosarum* LIN06 strain was not very high; the average total nitrogen was 4.8%. No nodules were found on control plants' roots. In other variants, the average number of nodules varied from 87 to 174 units per plant.

A correlation analysis was carried out to investigate the relationship between the number of nodules and total nitrogen in dry plant biomass in both pea genotypes (Figure 3). The results indicated a positive and significant correlation ($p < 0.05$) between the number of nodules and nitrogen accumulated in plant biomass with pea genotype "Egle DS" (Figure 3A). The strength of the correlation was moderately positive, with a correlation coefficient of R = 0.4964. A correlation analysis with pea genotype "Respect" also showed a positive and significant correlation ($p < 0.05$) between the number of nodules and nitrogen accumulated in plant biomass (Figure 3B). The strength of the correlation was moderately positive, with a correlation coefficient of R = 0.4045.

A competition investigation and competition index of each *R. leguminosarum* strain were determined in experiments with "Egle DS" and "Respect" pea genotypes (Figure 4). The competition analysis between *R. leguminosarum* strains showed that the *R. leguminosarum* LIN06 strain was the most competitive compared to all strains analyzed with the "Egle DS" genotype (Figure 4B). The competitiveness index of the *R. leguminosarum* LIN06 strain varied from 3.02 to 8.52 units and was significantly higher compared to other *R. leguminosarum* strains; however, when compared with the *R. leguminosarum* BAGOO07, the competition index of the *R. leguminosarum* LIN06 strain showed no significant difference in the *R. leguminosarum* EGLE07 competition index. The highest competitiveness index in the *R. leguminosarum* LIN06 strain was achieved against the *R. leguminosarum* EGLE10 and *R. leguminosarum* EGLE07 strains, at 6.20 and 8.52, respectively. The results also showed that *R. leguminosarum* EGLE10 strain competition index was usually significantly lower in combination with other investigated strains. The competitiveness index of the *R. leguminosarum* EGLE10 strain when in competition with the *R. leguminosarum* EGLE07, *R. leguminosarum* BAGOO07, *R. leguminosarum* ASTR08, and *R. leguminosarum* LIN06 strains was only 0.19, 1.18, 0.75, and 0.27 units, respectively. The competitiveness index of other *R. leguminosarum* strains was similar; in some variants, *R. leguminosarum* EGLE07 showed significantly improved competitiveness results.



(**A**)



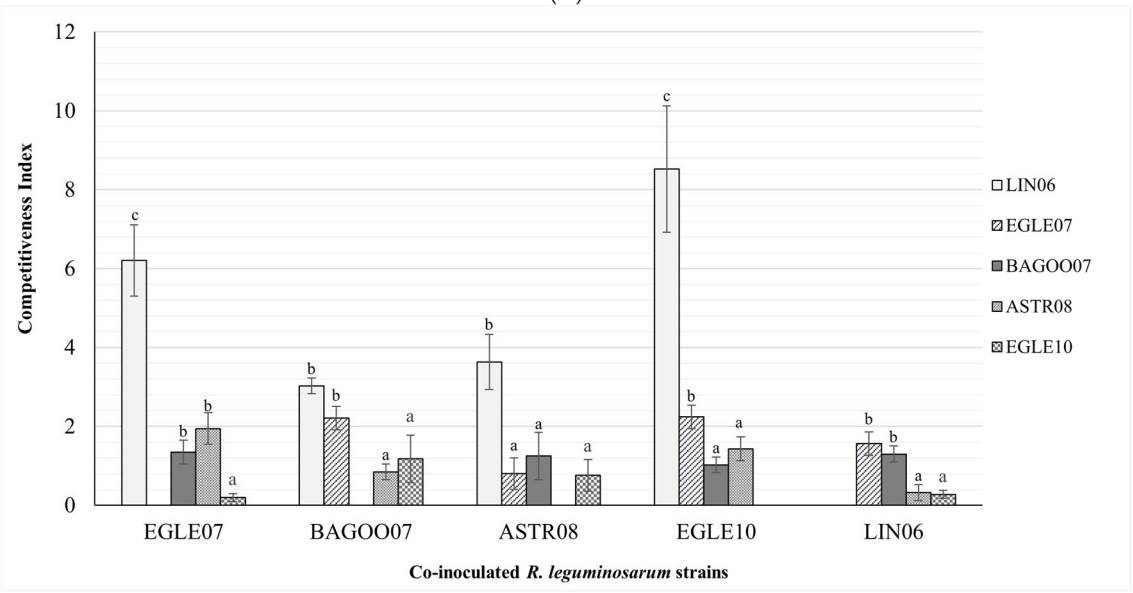
(**B**)

Figure 3. (**A**) Correlation between total nitrogen accumulated in "Egle DS" genotype plant biomass and number of nodules on roots, $p < 0.05$. (**B**) Correlation between total nitrogen accumulated in "Respect" genotype plant biomass and number of nodules on roots, $p < 0.05$.

A competition analysis of *R. leguminosarum* strains in combination with the "Respect" pea genotype showed that the *R. leguminosarum* EGLE10 strain was more competitive than *R. leguminosarum* EGLE07, *R. leguminosarum* BAGOO07, and *R. leguminosarum* ASTR08 strains (Figure 4C). The competitiveness index against these rhizobia was 2.87, 2.06, and

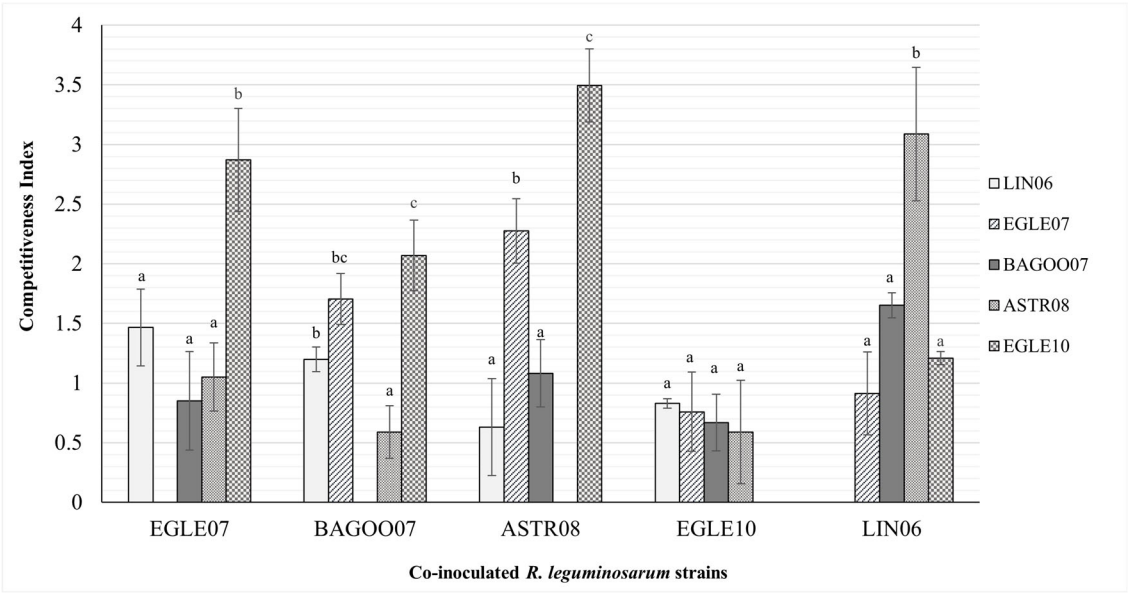
3.49, respectively. The *R. leguminosarum* EGLE10 competitiveness index was significantly higher in competition with *R. leguminosarum* EGLE07 and *R. leguminosarum* ASTR08 strains compared to other *R. leguminosarum* strains analyzed. *R. leguminosarum* ASTR08 was the most competitive strain in competition with the *R. leguminosarum* LIN06 strain. The competitiveness index of the *R. leguminosarum* ASTR08 strain was 3.09 and was significantly higher compared to other strains examined.

(**A**)



(**B**)

Figure 4. *Cont*.



(**C**)

Figure 4. Competition assays between *R. leguminosarum* strains. (**A**) Pea root system inoculated with *gusA* [pOPS0263] tagged strain (blues nodules) and strain without any marker (white nodules) after staining with X-GlcA. (**B**) The competitiveness index between *R. leguminosarum* isolates inoculated in "Egle DS" genotype pea plants. Error bars indicate the standard deviations within four biological replications at each treatment. Values marked with the same letter are not significantly different at $p \geq 0.05$ (ANOVA, Tukey HSD). (**C**) The competitiveness index between *R. leguminosarum* isolates inoculated in "Respect" genotype pea plants. Error bars indicate the standard deviations within four biological replications at each treatment. Values marked with the same letter are not significantly different at $p \geq 0.05$ (ANOVA, Tukey HSD).

The total nitrogen accumulation in pea biomass and the competitiveness results with the different *R. leguminosarum* strains investigated in this research showed that these strains acted differently in different pea genotypes (Figure 5). Rhizobia strains' nitrogen fixation efficiency on different pea genotypes differed by up to 1.7 times; the highest differences were found in variants where a single *R. leguminosarum* ASTR08 strain and a combination of *R. leguminosarum* EGLE10 and LIN06 were inoculated. *R. leguminosarum* ASTR08 strain was 1.7-fold more effective in pea genotype "Egle DS" compared to the "Respect" genotype, and the combination of *R. leguminosarum* EGLE10 and LIN06 was 1.7-fold more effective in pea genotype "Respect" compared to the "Egle DS" genotype. Nitrogen accumulation in control plants of the "Egle DS" genotype was lower compared to the "Respect" genotype; however, nitrogen accumulation in the plants with bacteria application was higher in the "Egle DS" genotype. Some combinations, such as *R. leguminosarum* EGLE07 with *R. leguminosarum* EGLE10 or *R. leguminosarum* BAGOO07 with *R. leguminosarum* EGLE10, were highly efficient in terms of nitrogen fixation in both pea genotypes. The *R. leguminosarum* EGLE07 and *R. leguminosarum* EGLE10 combination significantly increased nitrogen accumulation by 5.9-fold compared to the control in the "Egle DS" genotype and 2.7-fold compared to the control in the "Respect" genotype. The *R. leguminosarum* BAGOO07 with *R. leguminosarum* EGLE10 combination significantly increased nitrogen accumulation by 6.1-fold compared to control in the "Egle DS" genotype and 2.3-fold compared to control in the "Respect" genotype. The number of nodules formed in the "Respect" genotype was higher compared to "Egle DS". Nodulation results in both genotypes showed that the effect of a high number of nodules on *R. leguminosarum* strains' efficiency regarding total nitrogen accumulation was lower or significantly not different compared to the other strains investigated (Figure 5 and Figure 2).

"EGLE DS"								"RESPECT"					
Competitiveness index of *R. leguminosarum*					Nodules, plant^{-1}	Total nitrogen accumulated, %	Combinations	Total nitrogen accumulated, %	Nodules, plant^{-1}	Competitiveness index of *R. leguminosarum*				
LIN06	EGLE07	BAGOO07	ASTR08	EGLE10						LIN06	EGLE07	BAGOO07	ASTR08	EGLE10
					0	1.2	Control	2.0	0					
					51	4.4	EGLE10	5.6	110					
					42	7.7	ASTR08	4.6	106					
			1.43	0.76	61	4.8	ASTR08×EGLE10	4.2	163				0.59	3.49
					39	6.5	BAGOO07	4.9	174					
		1.25	0.85		50	7.4	BAGOO07×ASTR08	4.7	154			1.08	0.59	
		1.02		1.18	62	7.0	BAGOO07×EGLE10	5.8	114			0.67		2.07
					72	6.9	EGLE07	5.3	134					
	0.80		1.95		47	6.2	EGLE07×ASTR08	5.7	134		2.27		1.05	
	2.21	1.35			75	5.3	EGLE07×BAGOO07	4.9	145		1.71	0.85		
	2.24			0.20	40	7.1	EGLE07×EGLE10	5.5	101		0.76			2.87
					58	4.2	LIN06	4.8	225					
3.63			0.32		49	6.7	LIN06×ASTR08	4.7	92	0.63			3.09	
3.03		1.30			52	5.6	LIN06×BAGOO07	4.5	155	1.20		1.65		
6.21	1.56				71	6.0	LIN06×EGLE07	4.4	174	1.47	0.91			
8.52				0.27	36	3.2	LIN06×EGLE10	5.3	87	0.83				1.21

Figure 5. Heat map of *R. leguminosarum* strains' nitrogen fixation efficiency (blue), nodulation (bronze) and competitiveness index (yellow and green) with different pea genotypes. The heat map color scale represents relative values: brighter shades indicate higher values and lighter shades indicate lower values. Colors illustrate the comparison between the two genotypes.

Competition analysis of *R. leguminosarum* strains demonstrated that the same strains' competition index was different in different pea genotypes (Figure 5), (Figure 4). In pea genotype "Egle DS" *R. leguminosarum* LIN06 strain competition index was higher compared to all strains analyzed, but strain nitrogen fixation efficiency was poor compared to the results obtained for single strains and inoculation with different combinations. It was also determined that nitrogen accumulation tended to decrease in all combined variants where the *R. leguminosarum* LIN06 strain was included; however, in combination with the *R. leguminosarum* EGLE10 strain, total nitrogen was significantly reduced to around 27% compared to total nitrogen accumulated in plant inoculated with *R. leguminosarum* EGLE10 single strain. In the plant experiment with the "Respect" genotype, the *R. leguminosarum* LIN06 strain showed the highest nodulation efficiency, with significant differences (Figure 5) (Supplementary Figure S2); however, this strain's nitrogen efficiency and competition index were low compared to all examined strains. In the "Respect" genotype, the *R. leguminosarum* EGLE10 strain showed a high competition index against *R. leguminosarum* EGLE07, *R. leguminosarum* BAGO07, and *R. leguminosarum* ASTR08 strains and demonstrated the highest efficiency regarding nitrogen fixation for the plant, although this strain's nodulation efficiency was average. The nitrogen accumulation has a tendency to increase and nodule number has a tendency to decrease in variants where consortiums with *R. leguminosarum* EGLE10 strain were applied. In pea genotype "Egle DS", the *R. leguminosarum* EGLE10 strain showed reduced nitrogen fixation and competition.

None of the analyzed *R. leguminosarum* strains with the "Egle DS" pea genotype showed both high biological nitrogen fixation and high competitiveness (Table 4). Only one *R. leguminosarum* ASTR08 strain showed high biological nitrogen fixation with low competition, and only one *R. leguminosarum* LIN06 demonstrated high competition, but nitrogen fixation was low. Experiments with the "Respect" pea genotype demonstrated that only *R. leguminosarum* EGLE10 presented both high biological nitrogen fixation and high competitiveness. *R. leguminosarum* EGLE07 was highly effective in terms of biological nitrogen fixation, but showed average results regarding its competitiveness. *R. leguminosarum* ASTR08 and BAGOO07 strains obtained average results for both and *R. leguminosarum* LIN06 was not effective regarding biological nitrogen fixation and not competitive.

Table 4. *R. leguminosarum* strains' nitrogen fixation efficiency and competition with different pea genotypes. Low (LN), average (AN), or high (HN) biological nitrogen fixation; low (LC), average (AC), or high (HC) competitiveness.

Pea Genotype	*R. leguminosarum* Strains				
	LIN06	**EGLE07**	**BAGOO07**	**ASTR08**	**EGLE10**
"Egle DS"	LN/HC	AN/AC	AN/LC	HN/LC	LN/LC
"Respect"	LN/LC	HN/AC	AN/AC	AN/AC	HN/HC

A two-way analysis of variance (ANOVA) was applied to investigate strain, genotype, and the interaction between them, and how these influenced total nitrogen accumulation in the plant (Table 5). The results showed that each factor—strain ($F = 10.55$, $p < 0.0001$), genotype ($F = 23.42$, $p < 0.0001$), and the interaction between them ($F = 5.63$, $p < 0.0001$)—were significant in terms of nitrogen accumulation in pea plants at the probability level 99.9%.

Table 5. Two-way ANOVA of total nitrogen accumulated in two pea genotypes "Egle DS" and "Respect" after inoculation with sixteen different *R. leguminosarum* strain combinations.

	df	**MS**	**SS**	**F**
Strain	1	6.32583889	94.88758333	10.55 ***
Genotype	15	14.04540000	14.04540000	23.42 ***
G × S	15	3.37420000	50.61300000	5.63 ***

df: degrees of freedom; MS: mean square; SS: sum of squares; F: F value; ***: $p < 0.001$.

4. Discussion

Our results showed that *R. leguminosarum* strains' influence on nitrogen accumulation in the pea plants was different in different substrates. Pea plants grown in sterilized vermiculite–sand mixture accumulated higher amounts of total nitrogen compared to the plants that were grown in unsterilized soil. The lower total nitrogen accumulation in the pea plants grown in the unsterilized soil suggests that there were some factors which inhibited rhizobia efficiency and nitrogen fixation for these plants. Some studies show that high levels of nitrogen availability in the soil and soil acidity can negatively affect symbiotic nitrogen fixation in legumes [11,59,60]. The excessive nitrogen in the soil, especially during the period between seed inoculation and germination, can reduce the reliance of plants on nitrogen fixation and limit root nodule development [61,62]. Based on a report from the Saskatchewan Ministry of Agriculture, soil nitrogen levels exceeding 40 kg ha^{-1} inhibit nodulation and BNF in pea plants. Additionally, nitrogen levels surpassing 50 kg ha^{-1} prevent nodulation and BNF in pea plants [63]. Different research evaluating rhizobia and legume plant symbiosis efficiency at different soil pH levels show that pH 6.0–7.0 is optimal for rhizobia growth and nodulation, and soil pH levels lower than 5.5 can negatively impact legume plants' nodulation and nitrogen fixation abilities [64–66]. In our study, mineral nitrogen content in the soil was 10.42 mg kg^{-1}, at around 27 kg ha^{-1}, and soil pH was 6.7. This amount of nitrogen and soil pH should have no negative impact on pea nodulation or biological nitrogen fixation. Another reason why pea plants in unsterilized soil accumulated lower amounts of nitrogen compared to the pea plants grown in a sterile vermiculite–sand mixture could be a 'competition problem' with native strains. In our research, control plants in unsterilized soil formed nodules on the roots; this shows that native strains were in the soil. Native rhizobia strains often have low symbiotic effectiveness in nitrogen fixation with legume plants because of their long-established adaptation to prevailing agroclimatic conditions and horizontal gene transfer mechanisms that promote their competitiveness and survival [67–69]. There are some scientific studies showing that

rhizobia inoculants that contain extremely efficient nitrogen fixing rhizobia strains failed to compete against highly competitive native strains in agricultural fields [70–72]. Indigenous rhizobia strains are often better adapted to soil properties such as soil's granulometric and chemical composition, pH, and salinity, and are better distributed into the soil and have higher resistance to the herbicides or other chemical products applied to the soil as part of agricultural practice [73,74]. It could be that native strains in the soil used for experiment were weak in terms of biological nitrogen fixation but were strong regarding competition against inoculated strains, and, as a result, formed the majority of the root nodules. Additionally, our investigated *R. leguminosarum* strains' adaptation possibilities in soil were not evaluated in previous studies. It could be that reduced nitrogen fixation in the soil is also related to soil properties and chemical composition. Therefore, highly competitive, easily adaptable, and efficient nitrogen fixing rhizobia strains need to be selected for the development of biostimulants.

However, the choice of pea genotype is also an important factor in achieving effective symbiosis and high yields. According to the scientific literature, the pea genotype has extremely high influence on *R. leguminosarum* strains' nodulation and nitrogen fixation [75–78]. Our two-way ANOVA analysis of rhizobia strain, pea genotype, and the interaction between strain and genotype's influence on nitrogen accumulation in plant biomass confirmed all these factors were significantly important for nitrogen accumulation. This means that nitrogen accumulated in pea plants depends on pea genotype, *R. leguminosarum* strain, and the interaction between genotype and strain. Genetic differences between pea genotypes' physiology, and differences in root and nodule morphogenesis, may result in variations in root exudation and signal exchange between the rhizobia and legume plants [79]. Some research shows that rhizosphere microbial community structure varies in relation to root location, and that these variations are related to qualitative and quantitative changes in root exudation [80,81]. These plant metabolism changes can be related to nutrient availability, humidity, and other environmental factors [82], and may lead to a differential efficiency regarding their interaction with different rhizobia genotypes. The rhizobia genotype may consequently influence nodule physiology and biological nitrogen fixation efficiency. Genetic differences between rhizobia genotypes can differently affect symbiosis efficiency, because different genes are responsible for adaptation to survive in the soil, signaling molecules' recognition, and nodulation [12,83]. Both pea genotype and rhizobia strain should be compatible to form a successful symbiotic relationship. Different scientific investigations demonstrated that some *R. leguminosarum* strains' nitrogen fixation efficiencies significantly differed by more than two times in different pea genotypes [84,85]. In our research, rhizobia strains' nitrogen fixation efficiencies on different pea genotypes differed by up to 1.7 times. We also found that, although nitrogen accumulation in control plants of the "Egle DS" genotype was lower compared to the "Respect" genotype, the nitrogen accumulation in the plants with bacterial application was higher in the "Egle DS" genotype. These nitrogen accumulation results were not related to nodulation, because the number of nodules formed in the "Respect" genotype was higher compared to "Egle DS". Nevertheless, the nodulation results in both genotypes showed that the effects of intensive nodulation regarding *R. leguminosarum* strains' efficiency in total nitrogen accumulation was lower or significantly not different compared to the other strains investigated. Correlation analysis between nodule number and nitrogen accumulated per plant in each genotype showed that the relationship between these two parameters was significantly positive and moderate in both genotypes ($r = 0.4964$ in "Egle DS" and $r = 0.4045$ in "Respect"). These results suggest that while nitrogen accumulation in the plant biomass tends to increase with the number of nodules, there are more determinants that are significantly important for nitrogen accumulation in the plant, such as soil composition, strain nitrogen fixation

efficiency, climate, and the plant's genetic characteristics [86,87]. These nodulation results confirmed other scientists' findings that nodule number is an important parameter demonstrating rhizobia's nodulation ability and, in the presence of other strains, determines their nodulation competitiveness; however, a high nodule number does not guarantee efficient biological nitrogen fixation [71,88]. Some research shows that abundant nodule formation due to plant genotype or rhizobia strain can have a negative impact on host plants. Nodule formation and maintenance are demanding processes in terms of energy and resources. If nodulation becomes excessive and host plants invest more resources than they receive in return, the relationship can shift from mutualist to parasitism and plant growth can be reduced [89–91]. This means that nodule-counting alone is not a suitable method for the selection of *R. leguminosarum* strains with highly efficient nitrogen fixation abilities. Since both pea genotypes were inoculated with the same rhizobia strains and were grown under the same experimental conditions, these results show that most *R. leguminosarum* strains showed more effective nitrogen fixation abilities in the "Egle DS" genotype compared to the "Respect" genotype, even in the presence of a lower number of nodules.

Competitiveness in rhizobia refers to the relative ability of a given strain to infect a legume plant and cause nodule formation in the presence of the other strains [92]. Plant symbiosis with highly efficient rhizobia strains leads to an increase in biological nitrogen fixation in the plant, and symbiosis with partially efficient or inefficient rhizobia strains can reduce legume plants' productivity, so rhizobia strains' efficiency and competitiveness determine the final nitrogen supply to the plant [93–95]. A competition analysis of our *R. leguminosarum* strains showed that it is extremely important to select not only highly competitive rhizobia strains with effective biological nitrogen fixation abilities, but also to consider into their efficiency for different pea genotypes. In pea genotype "Egle DS", the *R. leguminosarum* LIN06 strain was highly competitive, but strain nitrogen fixation efficiency was low; nitrogen accumulation was also reduced in all combinations where the *R. leguminosarum* LIN06 strain was included. In plant experiments with the "Respect" genotype, the same *R. leguminosarum* LIN06 strain was low regarding nitrogen fixation ability and had low competition analysis results, but the *R. leguminosarum* EGLE10 strain demonstrated a high competition index and the highest efficiency regarding nitrogen fixation. However, in pea genotype "Egle DS", the *R. leguminosarum* EGLE10 strain was not so very effective regarding nitrogen fixation and had low competition analysis results. These competition and nitrogen fixation efficiency differences between the *R. leguminosarum* EGLE07 and *R. leguminosarum* EGLE10 strains in the "Egle DS" and "Respect" genotypes could be related not only to the pea genotypes but also to the strains' genetic differences in terms of *nodC* genes. Based on our previous studies (Table 1) *R. leguminosarum* LIN06 and *R. leguminosarum* EGLE10 strains were assigned to different clusters according to *nodC* gene phylogenetic analysis. *Nod* genes are responsible for the attraction and recognition of symbiotic bacteria in leguminous plants [96–98]. So, the differences between *R. leguminosarum* LIN06 and *R. leguminosarum* EGLE10 *nodC* genes may have been significant in the production and recognition of plant and bacterial signaling compounds.

Some experiments suggest that the host plants prioritize beneficial and more effective nitrogen fixing stains versus ineffective strains. The same effectiveness among strain combinations was observed when a highly efficient strain was mixed with an ineffective strain or with another highly effective strain [99,100].However, other research shows that when plants were inoculated with two high-efficacy nitrogen fixing strains, the prevalence of coinfected nodules was significantly higher compared to treatments inoculated with one or two ineffective strains [55,101]. Our research results using the "Egle DS" genotype demonstrated that there was no prioritization of beneficial and more effective nitrogen fixing stains. The most competitive strain, *R. leguminosarum* LIN06, has the lowest nitrogen

fixation efficiency and significantly reduced nitrogen accumulation in all consortia variants. The results with the "Respect" genotype were the opposite; a higher competition index was found with strains that were more effective in terms of nitrogen fixation, such as *R. leguminosarum* EGLE10 and EGLE07. The number of nodules did not increase in variants where highly efficient strain combinations were inoculated. These results show that some pea genotypes can prioritize and form symbiosis with more effective nitrogen fixing strains and some genotypes cannot.

Different results are published in scientific papers about the influence of inoculation with single-strain and multi-strain rhizobia combinations on nitrogen accumulation in the plant. There is some information showing that host plants inoculated with two or more strains receive significantly less benefit from rhizobia strains than expected based on the clonal inoculations with a single strain. In A. Rahman and colleagues' research, nitrogen fixation efficiency was significantly reduced in ~50% of strain combinations examined, and a lower number of nodules were found in combinations compared to single inoculation [101]. W. R. Roper and colleagues' results were the opposite. He and his colleagues demonstrated that no significant differences were observed in terms of nitrogen fixation after the inoculation of *R. leguminosarum* single-strain and multi-strain combinations [102]. Our research results after inoculating single strain and multi-strain combinations into "Egle DS" and "Respect" genotypes demonstrated that by using high–efficiency biological nitrogen fixing strains in multi-strain combinations with ineffective biological nitrogen fixing strains, it is possible to increase nitrogen fixation for the plant; however, the nitrogen accumulated in the plants with the rhizobia combination was lower compared to nitrogen accumulated with a single effective inoculated strain. Nevertheless, in this investigation, multi-strain combinations were prepared from only two different *R. leguminosarum* strains, so in natural soil with a high diversity of indigenous *R. leguminosarum* strains, the results can differ. These differences were also obtained in our own analysis of *R. leguminosarum* strains in non-sterile soil and a sterile vermiculite–sand mixture. The rhizobia nitrogen fixation efficiency obtained under sterile conditions was reduced in soil experiments due to the influence of indigenous strains. The reason why the best nitrogen accumulation in plants grown in soil was achieved with *R. leguminosarum* LIN06 strain is that this strain was the most competitive. It was confirmed through competition analysis that *R. leguminosarum* LIN06 had the highest competitiveness index amongst all strains examined. To summarize, even *R. leguminosarum* LIN06 strain nitrogen fixation efficiency was low compared to other analyzed strains; in a soil experiment with Egle DS" genotype, this strain significantly increased nitrogen accumulation in pea plant biomass by 1.6-fold compared to the uninoculated control. Aleksander Westphal Muniz and colleagues' investigation results with different *R. leguminosarum* strains demonstrated that after inoculation with the most efficient *R. leguminosarum* EEL 6802 strain, total nitrogen accumulated in pea biomass was 1.5-fold higher compared to the uninoculated control, and this led to increased pea yield [18]. Anteneh Argaw and Abere Mnalku's results showed that the most efficient *R. leguminosarum* NSFPR8 strain of all investigated strains increased total nitrogen in plants by 1.3-fold, compared to the uninoculated control [17]. Fesenko and colleagues observed a 1.3-fold increase in nitrogen accumulation in pea after inoculation with *R. leguminosarum* strains compared to the control [103]. These other authors' results suggest that our *R. leguminosarum* LIN06 strain has high potential as a biostimulator. Since competition analysis revealed that the most competitive strain demonstrated the best performance in terms of nitrogen accumulation in soil investigations, it can be assumed that the *R. leguminosarum* EGLE10 strain in the "Respect" genotype, which was the most competitive and efficient in terms of nitrogen fixation, would also be the most valuable regarding nitrogen fixation in soil experiments; however, this needs to be analyzed in further investigations. In

further investigations, *R. leguminosarum* EGLE07 with *R. leguminosarum* EGLE10 and *R. leguminosarum* BAGOO07 with *R. leguminosarum* EGLE10 combinations should be examined because these combinations showed high efficiency regarding nitrogen fixation in both pea genotypes, "Egle DS" and "Respect". It is also important to investigate these strains' adaptation possibilities and resistance to different environmental conditions. In general, the effectiveness of the *R. leguminosarum* strains must be studied in soil containing other indigenous strains, under natural conditions, and with different pea genotypes. So, additional field trials are necessary to validate the findings obtained in this research. The efficacy studies of biostimulants under sterile conditions show only the potential of the product; the obtained results do not reflect reality.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture15161784/s1, Figure S1: The average number of nodules on "Egle DS" pea genotype roots after inoculation with different *R. leguminosarum* strain combinations; Figure S2: The average number of nodules on "Respect" pea genotype roots after inoculation with different *R. leguminosarum* strain combinations.

Author Contributions: Conceptualization, J.K. and S.S.; methodology, J.K. and S.S.; software, J.K.; validation, J.K. and S.S.; formal analysis—investigation, J.K., S.S., A.G., K.R., R.M., and L.L.; resources, J.K., A.G., and K.R.; data curation, J.K., S.S., and L.L.; writing—original draft preparation, J.K.; writing—review and editing, J.K., S.S., A.G., K.R., R.M., and L.L.; visualization, J.K.; supervision, J.K.; project administration, J.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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

References

1. Farshi, P.; Mirmohammadali, S.N.; Rajpurohit, B.; Smith, J.S.; Li, Y. Pea protein and starch: Functional properties and applications in edible films. *J. Agric. Food Res.* **2024**, *15*, 100927. [CrossRef]
2. Wu, D.T.; Li, W.X.; Wan, J.J.; Hu, Y.C.; Gan, R.Y.; Zou, L. A Comprehensive Review of Pea (*Pisum sativum* L.): Chemical Composition, Processing, Health Benefits, and Food Applications. *Foods* **2023**, *12*, 2527. [CrossRef]
3. Rubiales, D.; Annicchiarico, P.; Vaz Patto, M.C.; Julier, B. Legume Breeding for the Agroecological Transition of Global Agri-Food Systems: A European Perspective. *Front. Plant Sci.* **2021**, *12*, 782574. [CrossRef] [PubMed]
4. Lu, Z.X.; He, J.F.; Zhang, Y.C.; Bing, D.J. Composition, physicochemical properties of pea protein and its application in functional foods. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 2593–2605. [CrossRef] [PubMed]
5. Kumari, T.; Deka, S.C. Potential health benefits of garden pea seeds and pods: A review. *Legume Sci.* **2021**, *3*, e82. [CrossRef]
6. Skoufogianni, E.; Giannoulis, K.; Bartzialis, D.; Danalatos, N. Relations between crop rotation with pea and soil structure. *Bulg. J. Agric. Sci.* **2019**, *25*, 1205–1210.
7. Kebede, E. Contribution, Utilization, and Improvement of Legumes-Driven Biological Nitrogen Fixation in Agricultural Systems. *Front. Sustain. Food Syst.* **2021**, *5*, 7. [CrossRef]
8. Chen, C.; Neill, K.; Burgess, M.; Bekkerman, A. Agronomic benefit and economic potential of introducing fall-seeded pea and lentil into conventional wheat-based crop rotations. *Agron. J.* **2012**, *104*, 215–224. [CrossRef]
9. Lupwayi, N.Z.; Lafond, G.P.; May, W.E.; Holzapfel, C.B.; Lemke, R.L. Intensification of field pea production: Impact on soil microbiology. *Agron. J.* **2012**, *104*, 1189–1196. [CrossRef]
10. Domagała-Świątkiewicz, I.; Siwek, P. Effect of field pea (Pisum sativum subsp. arvense (L.) Asch.) and pea-oat (Avena sativa L.) biculture cover crops on high tunnel vegetable under organic production system. *Org. Agric.* **2022**, *12*, 91–106. [CrossRef]
11. Abd-Alla, M.H.; Al-Amri, S.M.; El-Enany, A.W.E. Enhancing Rhizobium–Legume Symbiosis and Reducing Nitrogen Fertilizer Use Are Potential Options for Mitigating Climate Change. *Agriculture* **2023**, *13*, 2092. [CrossRef]
12. Wang, Q.; Liu, J.; Zhu, H. Genetic and molecular mechanisms underlying symbiotic specificity in legume-rhizobium interactions. *Front. Plant Sci.* **2018**, *9*, 313. [CrossRef] [PubMed]

13. Mabrouk, Y.; Hemissi, I.; Salem IBen Mejri, S.; Saidi, M.; Belhadj, O. Potential of Rhizobia in Improving Nitrogen Fixation and Yields of Legumes. *Symbiosis* **2018**, *107*, 1–16.
14. Lindström, K.; Mousavi, S.A. Effectiveness of nitrogen fixation in rhizobia. *Microb. Biotechnol.* **2020**, *13*, 1314–1335. [CrossRef]
15. Heder Asdrubal Montañez Valencia HFAJMNDSA. Rhizobia inoculation increases pea grain yield: An overview and challenges. *Biosci. J.* **2024**, *40*, 1981–3163. [CrossRef]
16. Rapčan, I.; Milaković, Z.; Šarić, G.K.; Šeput, J.; Subašić, D.G. Reaction of garden pea (Pisum sativum L.) to inoculation and nitrogen fertilization in Eastern Croatia. *J. Cent. Eur. Agric.* **2017**, *18*, 889–901. [CrossRef]
17. Argaw, A.; Mnalku, A. Symbiotic effectiveness of Rhizobium leguminosarum bv. vicieae isolated from major highland pulses on field pea(Pisum sativum L.) in soil with abundant rhizobial population. *Ann. Agrar. Sci.* **2017**, *15*, 410–419. [CrossRef]
18. Aleksander, W.M.; Murilo, D.C.; Enilson LSde, S.; Ana Edemar, B. Symbiotic efficiency of pea (Pisum sativum) rhizobia association under field conditions. *Afr. J. Agric. Res.* **2017**, *12*, 2582–2585. [CrossRef]
19. Flores-Félix, J.D.; Carro, L.; Cerda-Castillo, E.; Squartini, A.; Rivas, R.; Velázquez, E. Analysis of the interaction between pisum sativum l. And rhizobium laguerreae strains nodulating this legume in Northwest Spain. *Plants* **2020**, *9*, 1755. [CrossRef]
20. Tripolskaja, L.; Kazlauskaite-Jadzevice, A.; Razukas, A. Organic Carbon, Nitrogen Accumulation and Nitrogen Leaching as Affected by Legume Crop Residues on Sandy Loam in the Eastern Baltic Region. *Plants* **2023**, *12*, 2478. [CrossRef]
21. Li, G.; Tang, X.; Hou, Q.; Li, T.; Xie, H.; Lu, Z.; Zhang, T.; Liao, Y.; Wen, X. Response of soil organic carbon fractions to legume incorporation into cropping system and the factors affecting it: A global meta-analysis. *Agric. Ecosyst. Environ.* **2023**, *342*, 108231. [CrossRef]
22. Yeboah, S.; Zhang, R.; Cai, L.; Li, L.; Xie, J.; Luo, Z.; Liu, J.; Wu, J. Tillage effect on soil organic carbon, microbial biomass carbon and crop yield in spring wheat-field pea rotation. *Plant Soil. Environ.* **2016**, *62*, 279–285. [CrossRef]
23. Wysokinski, A.; Lozak, I.; Kuziemska, B. Sources of nitrogen for winter triticale (Triticosecale wittm. ex a.camus) succeeding pea (*Pisum sativum* L.). *Agronomy* **2021**, *11*, 527. [CrossRef]
24. Kaziūnienė, J.; Pini, F.; Shamshitov, A.; Razbadauskienė, K.; Frercks, B.; Gegeckas, A.; Mažylytė, R.; Lapinskienė, L.; Supronienė, S. Genetic Characterization of Rhizobium spp. Strains in an Organic Field Pea (*Pisum sativum* L.). *Field Lithuania. Plants* **2024**, *13*, 1888. [CrossRef] [PubMed]
25. Sainju, U.M.; Lenssen, A.; Caesar-Thonthat, T.; Waddell, J. Carbon Sequestration in Dryland Soils and Plant Residue as Influenced by Tillage and Crop Rotation. *J. Environ. Qual.* **2006**, *35*, 1341–1347. [CrossRef] [PubMed]
26. Naseer, I.; Ahmad, M.; Nadeem, S.M.; Ahmad, I.; Najm-ul-Seher Zahir, Z.A. Rhizobial Inoculants for Sustainable Agriculture: Prospects and Applications. In *Biofertilizers for Sustainable Agriculture and Environment*; Springer: Cham, Switzerland, 2019.
27. Ma, Y.; Suo, Y.; Qi, H.; Tang, F.; Wang, M. Effects of Rhizobium Inoculation on Rhizosphere Soil Microbial Communities, Physicochemical Properties, and Enzyme Activities in Caucasian Clover Under Field Conditions. *Agronomy* **2024**, *14*, 2880. [CrossRef]
28. Barros-Rodríguez, A.; Rangseekaew, P.; Lasudee, K.; Pathom-aree, W.; Manzanera, M. Regulatory risks associated with bacteria as biostimulants and biofertilizers in the frame of the European Regulation (EU) 2019/1009. *Sci. Total Environ.* **2020**, *740*, 140239. [CrossRef] [PubMed]
29. Buntić, A.V.; Stajković-Srbinović, O.S.; Knežević, M.M.; Kuzmanović, D.; Rasulić, N.V.; Delić, D.I. Development of liquid rhizobial inoculants and pre-inoculation of alfalfa seeds. *Arch. Biol. Sci.* **2019**, *71*, 379–387. [CrossRef]
30. Arora, N.K.; Verma, M.; Mishra, J. Rhizobial Bioformulations: Past, Present and Future. In *Microorganisms for Sustainability*; Springer: Singapore, 2017.
31. Allito, B.; Nana, E.M.; Alemneh, A. Rhizobia Strain and Legume Genome Interaction Effects on Nitrogen Fixation and Yield of Grain Legume: A Review. *Mol. Soil Biol.* **2015**, *6*. [CrossRef]
32. Goyal, R.K.; Mattoo, A.K.; Schmidt, M.A. Rhizobial–host interactions and symbiotic nitrogen fixation in legume crops toward agriculture sustainability. *Front. Microbiol.* **2021**, *12*, 669404. [CrossRef]
33. Oparah, I.A.; Hartley, J.C.; Deaker, R.; Gemell, G.; Hartley, E.; Kaiser, B.N. Symbiotic effectiveness, abiotic stress tolerance and phosphate solubilizing ability of new chickpea root-nodule bacteria from soils in Kununurra Western Australia and Narrabri New South Wales Australia. *Plant Soil* **2024**, *495*, 371–389. [CrossRef]
34. Janczarek, M.; Kozieł, M.; Adamczyk, P.; Buczek, K.; Kalita, M.; Gromada, A.; Mordzińska-Rak, A.; Polakowski, C.; Bieganowski, A. Symbiotic efficiency of Rhizobium leguminosarum sv. trifolii strains originating from the subpolar and temperate climate regions. *Sci. Rep.* **2024**, *14*, 6264. [CrossRef] [PubMed]
35. Stephens, J.H.G.; Rask, H.M. Inoculant production and formulation. *Field Crops Res.* **2000**, *65*, 249–258. [CrossRef]
36. Somasegaran, P.; Hoben, H.J. Analyzing Nodule Occupancy Using Antibiotic-Resistant Markers. In *Handbook for Rhizobia*; Springer: New York, NY, USA, 1994.
37. Ramos, H.J.O.; Souza, E.M.; Soares-Ramos, J.R.L.; Pedrosa, F.O. Determination of bean nodule occupancy by Rhizobium tropici using the double gfp and gusA genetic markers constitutively expressed from a new broad-host-range vector. *World J. Microbiol. Biotechnol.* **2007**, *23*, 713–717. [CrossRef]

38. Schneider, M.; De Bruijn, F.J. Rep-PCR mediated genomic fingerprinting of rhizobia and computer-assisted phylogenetic pattern analysis. *World J. Microbiol. Biotechnol.* **1996**, *12*, 163–174. [CrossRef]
39. Howieson, J.G.; Dilworth, M.J. *Working with Rhizobia*; Australian Centre for International Agricultural Research: Canberra, ACT, Australia, 2016.
40. Sessitsch, A.; Hardarson, G.; De Vos, W.M.; Wilson, K.J. Use of marker genes in competition studies of Rhizobium. *Plant Soil* **1998**, *204*, 35–45. [CrossRef]
41. Xiong, A.-S.; Peng, R.-H.; Cheng, Z.-M.; Li, Y.; Liu, J.-G.; Zhuang, J.; Gao, F.; Xu, F.; Qiao, Y.-S.; Zhang, Z.; et al. Concurrent mutations in six amino acids in β-glucuronidase improve its thermostability. *Protein Eng. Des. Sel.* **2007**, *20*, 319–325. [CrossRef]
42. Xiong, A.-S.; Peng, R.-H.; Zhuang, J.; Liu, J.-G.; Xu, F.; Cai, B.; Guo, Z.-K.; Qiao, Y.-S.; Chen, J.-M.; Zhang, Z.; et al. Directed evolution of beta-galactosidase from Escherichia coli into beta-glucuronidase. *J. Biochem. Mol. Biol.* **2007**, *40*, 419–425. [CrossRef]
43. Yeom, S.-J.; Han, G.H.; Kim, M.; Kwon, K.K.; Fu, Y.; Kim, H.; Lee, H.; Lee, D.-H.; Jung, H.; Lee, S.-G.; et al. Controlled aggregation and increased stability of β-glucuronidase by cellulose binding domain fusion. *PLoS ONE* **2017**, *12*, e0170398. [CrossRef]
44. Callanan, M.J.; Russell, W.M.; Klaenhammer, T.R. Modification of Lactobacillus β-glucuronidase activity by random mutagenesis. *Gene* **2007**, *389*, 122–127. [CrossRef]
45. Wang, Y.C.; Yu, M.; Shih, P.Y.; Wu, H.Y.; Lai, E.M. Stable pH Suppresses Defense Signaling and is the Key to Enhance Agrobacterium-Mediated Transient Expression in Arabidopsis Seedlings. *Sci. Rep.* **2018**, *8*, 17071. [CrossRef] [PubMed]
46. Ullah, Z.; Iqbal, J.; Abbasi, B.A.; Akhtar, W.; Kanwal, S.; Ali, I.; Chalgham, W.; El-Sheikh, M.A.; Mahmood, T. Assessment of Gus Expression Induced by Anti-Sense OsPPO Gene Promoter and Antioxidant Enzymatic Assays in Response to Drought and Heavy Metal Stress in Transgenic Arabidopsis thaliana. *Sustainability* **2023**, *15*, 12783. [CrossRef]
47. Li, G.; Li, B.; Dong, G.; Feng, X.; Kronzucker, H.J.; Shi, W. Ammonium-induced shoot ethylene production is associated with the inhibition of lateral root formation in Arabidopsis. *J. Exp. Bot.* **2013**, *64*, 1413–1425. [CrossRef]
48. Shtratnikova, V.Y.; Kulaeva, O.N. Cytokinin-dependent expression of the ARR5::GUS construct during transgenic arabidopsis growth. *Russ. J. Plant Physiol.* **2008**, *55*, 756–764. [CrossRef]
49. Wang, Y.; Yang, Z.; Kong, Y.; Li, X.; Li, W.; Du, H.; Zhang, C. GmPAP12 Is Required for Nodule Development and Nitrogen Fixation Under Phosphorus Starvation in Soybean. *Front. Plant Sci.* **2020**, *11*, 450. [CrossRef]
50. Žydrė, K.; Monika, T.; Kristyna, R.; Lina, Š.; Irena, D.; Skaidrė, S.; Roma, S.; Aušra, A. Selection of New Field Pea Varieties for the Organic and Conventional Farming Systems in the Nemoral Climatic Zone. *Agriculture* **2025**, *15*, 687. [CrossRef]
51. Ramachandran, V.K.; East, A.K.; Karunakaran, R.; Downie, J.A.; Poole, P.S. Adaptation of Rhizobium leguminosarum to pea, alfalfa and sugar beet rhizospheres investigated by comparative transcriptomics. *Genome Biol.* **2011**, *12*, R106. [CrossRef]
52. Beringer, J.E. R factor transfer in Rhizobium leguminosarum. *J. Gen. Microbiol.* **1974**, *84*, 188–198. [CrossRef]
53. Suproniene, S.; Decorosi, F.; Pini, F.; Bellabarba, A.; Calamai, L.; Giovannetti, L.; Bussotti, F.; Kadziuliene, Z.; Razbadauskiene, K.; Toleikiene, M.; et al. Selection of Rhizobium strains for inoculation of Lithuanian Pisum sativum breeding lines. *Symbiosis* **2021**, *83*, 193–208. [CrossRef]
54. Couchoud, M.; Salon, C.; Girodet, S.; Jeudy, C.; Vernoud, V.; Prudent, M. Pea Efficiency of Post-drought Recovery Relies on the Strategy to Fine-Tune Nitrogen Nutrition. *Front. Plant Sci.* **2020**, *11*, 204. [CrossRef]
55. Mendoza-Suárez, M.A.; Geddes, B.A.; Sánchez-Cañizares, C.; Ramírez-González, R.H.; Kirchhelle, C.; Jorrin, B.; Poole, P.S. Optimizing Rhizobium-legume symbioses by simultaneous measurement of rhizobial competitiveness and N2 fixation in nodules. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 9822–9831. [CrossRef]
56. Mwenda, G.M.; Hill, Y.J.; O'Hara, G.W.; Reeve, W.G.; Howieson, J.G.; Terpolilli, J.J. Competition in the Phaseolus vulgaris-Rhizobium symbiosis and the role of resident soil rhizobia in determining the outcomes of inoculation. *Plant Soil* **2023**, *487*, 61–77. [CrossRef] [PubMed]
57. Nelson, D.W.; Sommers, L.E. Total Nitrogen Analysis of Soil and Plant Tissues. *J. AOAC Int.* **1980**, *63*, 770–778. [CrossRef]
58. SAS Institute Inc. *SAS Software 9.4*; SAS Institute Inc.: Cary, NC, USA, 2014.
59. Abd-Alla, M.H.; Nafady, N.A.; Bashandy, S.R.; Hassan, A.A. Mitigation of effect of salt stress on the nodulation, nitrogen fixation and growth of chickpea (Cicer arietinum L.) by triple microbial inoculation. *Rhizosphere* **2019**, *10*, 100148. [CrossRef]
60. Abd-Alla, M.H.; Bagy, M.K.; El-enany, A.W.E.S.; Bashandy, S.R. Activation of Rhizobium tibeticum with flavonoids enhances nodulation, nitrogen fixation, and growth of fenugreek (Trigonella foenum-graecum L.) grown in cobalt-polluted soil. *Arch. Environ. Contam. Toxicol.* **2014**, *66*, 303–315. [CrossRef]
61. Herliana, O.; Harjoso, T.; Anwar, A.H.S.; Fauzi, A. The Effect of Rhizobium and N Fertilizer on Growth and Yield of Black Soybean (Glycine max (L) Merril). In *IOP Conference Series: Earth and Environmental Science*; IOP: London, UK, 2019.
62. Brito-Santana, P.; Duque-Pedraza, J.J.; Bernabéu-Roda, L.M.; Carvia-Hermoso, C.; Cuéllar, V.; Fuentes-Romero, F.; Acosta-Jurado, S.; Vinardell, J.M.; Soto, M.J. Sinorhizobium meliloti DnaJ Is Required for Surface Motility, Stress Tolerance, and for Efficient Nodulation and Symbiotic Nitrogen Fixation. *Int. J. Mol. Sci.* **2023**, *24*, 5848. [CrossRef]
63. Yang, C.; Bueckert, R.; Schoenau, J.; Diederichsen, A.; Zakeri, H.; Warkentin, T.D. Evaluation of growth and nitrogen fixation of pea nodulation mutants in western Canada. *Can. J. Plant Sci.* **2017**, *97*, 1121–1129. [CrossRef]

64. Paulino, V.T.; Olivares, J.; Bedmar, E.J. Nodulation and nitrogenase activity of pea nodules as affected by low pH and aluminium. *Plant Soil* **1987**, *101*, 299–302. [CrossRef]
65. Abera, T.; Abebe, Z. Effects of Fertilizer, Rhizobium Inoculation and Lime Rate on Growth and Yields Field Pea in Horro and Gedo Highlands. *Adv. Crop Sci. Technol.* **2018**, *6*, 397. [CrossRef]
66. Zavalin, A.A.; Sokolov, O.A.; Shmyreva NYa Lukin, S.V. Legume reaction to soil acidity. *Amazon. Investig.* **2019**, *8*, 162–170.
67. Bogino, P.; Nievas, F.; Banchio, E.; Giordano, W. Increased competitiveness and efficiency of biological nitrogen fixation in peanut via in-furrow inoculation of rhizobia. *Eur. J. Soil. Biol.* **2011**, *47*, 188–193. [CrossRef]
68. Zahran, H.H. Rhizobium -Legume Symbiosis and Nitrogen Fixation under Severe Conditions and in an Arid Climate. *Microbiol. Mol. Biol. Rev.* **1999**, *63*, 968–989. [CrossRef] [PubMed]
69. Liu, S.; Jiao, J.; Tian, C.F. Adaptive Evolution of Rhizobial Symbiosis beyond Horizontal Gene Transfer: From Genome Innovation to Regulation Reconstruction. *Genes* **2023**, *14*, 274. [CrossRef] [PubMed]
70. Batstone, R.T.; Ibrahim, A.; MacLean, L.T. Microbiomes: Getting to the root of the rhizobial competition problem in agriculture. *Curr. Biol.* **2023**, *33*, R777–R780. [CrossRef]
71. Mendoza-Suárez, M.; Andersen, S.U.; Poole, P.S.; Sánchez-Cañizares, C. Competition, Nodule Occupancy, and Persistence of Inoculant Strains: Key Factors in the Rhizobium-Legume Symbioses. *Front. Plant Sci.* **2021**, *12*, 1–16. [CrossRef]
72. Pastor-Bueis, R.; Sánchez-Cañizares, C.; James, E.K.; González-Andrés, F. Formulation of a Highly Effective Inoculant for Common Bean Based on an Autochthonous Elite Strain of Rhizobium leguminosarum bv. phaseoli, and Genomic-Based Insights Into Its Agronomic Performance. *Front. Microbiol.* **2019**, *10*, 2724. [CrossRef]
73. Albareda, M.; Dardanelli, M.S.; Sousa, C.; Megías, M.; Temprano, F.; Rodríguez-Navarro, D.N. Factors affecting the attachment of rhizospheric bacteria to bean and soybean roots. *FEMS Microbiol. Lett.* **2006**, *259*, 67–73. [CrossRef]
74. Iturralde, E.T.; Covelli, J.M.; Alvarez, F.; Pérez-Giménez, J.; Arrese-Igor, C.; Lodeiro, A.R. Soybean-nodulating strains with low intrinsic competitiveness for nodulation, good symbiotic performance, and stress-tolerance isolated from soybean-cropped soils in Argentina. *Front. Microbiol.* **2019**, *10*, 1061. [CrossRef]
75. Horácio, E.H.; Gavilanes, F.E.Z.; Feliciano, M.V.; de Moraes, J.G.; Zucareli, C.; Andrade, D.S.; Maddela, N.R.; Prasad, R. Exploring the interaction effects between common bean cultivars and rhizobia inoculation on plant growth and yield. *J. Agric. Food Res.* **2024**, *15*, 100926. [CrossRef]
76. Burghardt, L.T.; Epstein, B.; Hoge, M.; Trujillo, D.I.; Tiffin, P. Host-Associated Rhizobial Fitness: Dependence on Nitrogen, Density, Community Complexity, and Legume Genotype. *Appl. Environ. Microbiol.* **2022**, *88*, e0052622. [CrossRef]
77. Koutroubas, S.D.; Damalas, C.A.; Fotiadis, S.; Markopoulos, T. Species, Cultivar and Seasonal Effects on Nodulation and Nitrogen Utilization of Spring Mediterranean Grain Legumes. *J. Soil. Sci. Plant Nutr.* **2023**, *23*, 4463–4473. [CrossRef]
78. Hossain, Z.; Wang, X.; Hamel, C.; Gan, Y. Nodulation and nitrogen accumulation in pulses vary with species, cultivars, growth stages, and environments. *Can. J. Plant Sci.* **2017**, *98*, 527–542. [CrossRef]
79. Bourion, V.; Heulin-Gotty, K.; Aubert, V.; Tisseyre, P.; Chabert-Martinello, M.; Pervent, M.; Delaitre, C.; Vile, D.; Siol, M.; Duc, G.; et al. Co-inoculation of a pea core-collection with diverse rhizobial strains shows competitiveness for nodulation and efficiency of nitrogen fixation are distinct traits in the interaction. *Front. Plant Sci.* **2018**, *8*, 2249. [CrossRef] [PubMed]
80. Baudoin, E.; Benizri, E.; Guckert, A. Metabolic fingerprint of microbial communities from distinct maize rhizosphere compartments. *Eur. J. Soil. Biol.* **2001**, *37*, 85–93. [CrossRef]
81. Yang, C.H.; Crowley, D.E. Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Appl. Environ. Microbiol.* **2000**, *66*, 345–351. [CrossRef]
82. Yeremko, L.; Czopek, K.; Staniak, M.; Marenych, M.; Hanhur, V. Role of Environmental Factors in Legume-Rhizobium Symbiosis: A Review. *Biomolecules* **2025**, *15*, 118. [CrossRef]
83. Rodrigo da-Silva, J.; Alexandre, A.; Brígido, C.; Oliveira, S. Can stress response genes be used to improve the symbiotic performance of rhizobia? *AIMS Microbiol.* **2017**, *3*, 365–382. [CrossRef]
84. Yang, C.; Bueckert, R.; Schoenau, J.; Diederichsen, A.; Zakeri, H.; Warkentin, T. Symbiosis of selected Rhizobium leguminosarum bv. viciae strains with diverse pea genotypes: Effects on biological nitrogen fixation. *Can. J. Microbiol.* **2017**, *63*, 909–919. [CrossRef]
85. Abi-Ghanem, R.; Carpenter-Boggs, L.; Smith, J.L. Cultivar effects on nitrogen fixation in peas and lentils. *Biol Fertil Soils.* **2011**, *47*, 115–120. [CrossRef]
86. Lagunas, B.; Richards, L.; Sergaki, C.; Burgess, J.; Pardal, A.J.; Hussain, R.M.F.; Richmond, B.L.; Baxter, L.; Roy, P.; Pakidi, A.; et al. Rhizobial nitrogen fixation efficiency shapes endosphere bacterial communities and Medicago truncatula host growth. *Microbiome* **2023**, *11*, 146. [CrossRef]
87. Khaitov, B.; Kurbonov, A.; Abdiev, A.; Adilov, M. Effect of chickpea in association with Rhizobium to crop productivity and soil fertility. *Eurasian J. Soil. Sci.* **2016**, *5*, 105–112. [CrossRef]
88. Voisin, A.S.; Prudent, M.; Duc, G.; Salon, C. Pea nodule gradients explain N nutrition and limited symbiotic fixation in hypernodulating mutants. *Agron. Sustain. Dev.* **2015**, *35*, 1529–1540. [CrossRef]

89. Lau, J.A.; Bowling, E.J.; Gentry, L.E.; Glasser, P.A.; Monarch, E.A.; Olesen, W.M.; Waxmonsky, J.; Young, R.T. Direct and interactive effects of light and nutrients on the legume-rhizobia mutualism. *Acta Oecologica* **2012**, *39*, 80–86. [CrossRef]
90. Regus, J.U.; Gano, K.A.; Hollowell, A.C.; Sofish, V.; Sachs, J.L. Lotus hosts delimit the mutualism-parasitism continuum of Bradyrhizobium. *J. Evol. Biol.* **2015**, *28*, 447–456. [CrossRef]
91. Calvert, M.B.; Hoque, M.; Wood, C.W. Genotypic variation in resource exchange, use, and production traits in the legume–rhizobia mutualism. *Ecol. Evol.* **2024**, *14*, e70245. [CrossRef]
92. Raposeiras, R.; Marriel, I.E.; Scotti Muzzi, M.R.; Paiva, E.; Pereira Filho, I.A.; Costa Carvalhais, L.; Passos, R.V.M.; Pinto, P.P.; de Sá, N.M.H. Rhizobium strains competitiveness on bean nodulation in Cerrado soils. *Pesqui. Agropecu. Bras.* **2006**, *41*, 439–447. [CrossRef]
93. Morel Revetria, M.A.; Berais-Rubio, A.; Giménez, M.; Sanjuán, J.; Signorelli, S.; Monza, J. Competitiveness and Phylogenetic Relationship of Rhizobial Strains with Different Symbiotic Efficiency in Trifolium repens: Conversion of Parasitic into Non-Parasitic Rhizobia by Natural Symbiotic Gene Transfer. *Biology* **2023**, *12*, 243. [CrossRef]
94. Den Herder, G.; Parniske, M. The unbearable naivety of legumes in symbiosis. *Curr. Opin. Plant Biol.* **2009**, *12*, 491–499. [CrossRef]
95. Irisarri, P.; Cardozo, G.; Tartaglia, C.; Reyno, R.; Gutiérrez, P.; Lattanzi, F.A.; Rebuffo, M.; Monza, J. Selection of competitive and efficient rhizobia strains for white clover. *Front. Microbiol.* **2019**, *10*, 768. [CrossRef]
96. Abdel-Lateif, K.; Bogusz, D.; Hocher, V. The role of flavonoids in the establishment of plant roots endosymbioses with arbuscular mycorrhiza fungi, rhizobia and Frankia bacteria. *Plant Signal. Behav.* **2012**, *7*, 636–641. [CrossRef]
97. Kidaj, D.; Krysa, M.; Susniak, K.; Matys, J.; Komaniecka, I.; Sroka-Bartnicka, A. Biological activity of Nod factors. *Acta Biochim. Pol.* **2020**, *67*, 435–440. [CrossRef]
98. Geurts, R.; Fedorova, E.; Bisseling, T. Nod factor signaling genes and their function in the early stages of Rhizobium infection. *Curr. Opin. Plant Biol.* **2005**, *8*, 346–352. [CrossRef] [PubMed]
99. Blanco, A.R.; Sicardi, M.; Frioni, L. Competition for nodule occupancy between introduced and native strains of Rhizobium leguminosarum biovar trifolii. *Biol. Fertil. Soils* **2010**, *46*, 419–425. [CrossRef]
100. Gano-Cohen, K.A.; Wendlandt, C.E.; Stokes, P.J.; Blanton, M.A.; Quides, K.W.; Zomorrodian, A.; Adinata, E.S.; Sachs, J.L.; Johnson, N. Interspecific conflict and the evolution of ineffective rhizobia. *Ecol. Lett.* **2019**, *22*, 914–924. [CrossRef] [PubMed]
101. Rahman, A.; Manci, M.; Nadon, C.; Perez, I.A.; Farsamin, W.F.; Lampe, M.T.; Le, T.H.; Torres Martínez, L.; Weisberg, A.J.; Chang, J.H.; et al. Competitive interference among rhizobia reduces benefits to hosts. *Curr. Biol.* **2023**, *33*, 2988–3001.e4. [CrossRef]
102. Roper, W.R.; Duckworth, O.W.; Grossman, J.M.; Israel, D.W. Rhizobium leguminosarum strain combination effects on nodulation and biological nitrogen fixation with Vicia villosa. *Appl. Soil. Ecol.* **2020**, *156*, 103703. [CrossRef]
103. Fesenko, A.N.; Provorov, N.A.; Orlova, I.F.; Orlov, V.P.; Simarov, B.V. Selection of Rhizobium leguminosarum bv. viceae strains for inoculation of Pisum sativum L. cultivars: Analysis of symbiotic efficiency and nodulation competitiveness. *Plant Soil* **1995**, *172*, 189–198. [CrossRef]