The Effect of In Vitro Coinoculation on the Physiological Parameters of White Lupine Plants (Lupinus albus L.)

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Abstract: The aim of the study was to select microbiological inoculants for a specific plant species, i.e., white lupine (Lupinus albus L.), to increase the efficiency of the diazotroph process. The research involved an in vitro assessment of interactions between the symbiotic bacteria (Bradyrhizobium sp. isolated from Nitragina and Nitroflora commercial preparations dedicated to white lupine) and selected endophytes (Pseudomonas fluorescens or Bacillus subtilis) used for seed coinoculation. In addition, selected morphological traits of plants (the weight and length of aboveground and belowground parts) were examined after the inoculation/coinoculation. The degree of root colonisation by selected endophytes used as individual inoculants and in combination with bacteria of the Bradyrhizobium genus was determined. The diazotrophic parameters were also investigated (nitrogenase activity, the number, and weight of nodules). The results showed no antagonistic interactions have been demonstrated between bacterial strains of the genus Bradyrhizobium sp. isolated from Nitragina and Nitroflora, and the endophytes Pseudomonas fluorescens or Bacillus subtilis used for the study. The applied coinoculation in vitro had a stimulating effect on the weight of the stems and roots of white lupine causing an average increase of 13% and 28%, respectively. The level of nitrogenase activity in the coinoculation variants increased from 3.5 nMC2H4 plant−1 h−1 to an average of 32.34 nMC2H4 plant−1 h−1.

Keywords: Pseudomonas fluorescens; Bacillus subtilis; Bradyrhizobium sp.; diazotrophy; nodules; plants parameters

1. Introduction

In the era of climate change, one of the challenges for the modern world of science is to develop foundations which will enable the improvement of the physiological processes of plants, especially functional crops, and thus make better use of their biological potential. In recent years, there has been growing interest in legumes [1]. The greatest advantage of these crops is their ability to coexist with bacteria of the Rhizobium genus, which can fix atmospheric nitrogen [2]. Due to the requirements of modern agriculture, it is necessary to search for new technologies that support the development and yield of legumes because these crops contain large amounts of protein and have a strong stimulating effect in crop rotation. Microorganisms have developed various mechanisms which allow plants to adapt to the changing environment [3]. Researchers focus on the development of bioinoculants based on plant growth-promoting rhizobacteria (PGPR) [4]. The use of organic biofertilisers
and biopesticides containing PGPR isolates is an alternative strategy for reducing mineral fertilization [5,6].

The name PGPR bacteria is more and more often used to describe the bacteria which live in the rhizosphere and colonise plant tissues, thus affecting the growth, development, and productivity of plants. Inoculation with bacterial consortia ensures more balanced nutrition and improves the uptake of nutrients by plants [7,8]. Among the plant growth-promoting bacteria, Bacillus sp. and Pseudomonas sp. are important and dominant genera, which aggressively colonise the rhizosphere of various crops and exhibit a broad spectrum of antagonism against numerous pathogens [9]. Plant growth is directly stimulated in various ways, e.g., through the synthesis of phytohormones as well as the stimulation of the growth of the root system. Moreover, PGPR decompose organic compounds and thus provide essential nutrients to plants; they also reduce the level of harmful ethylene [10–12]. There are numerous reports in reference publications which indicate that the coinoculation (double inoculation) of plants with symbiotic bacteria and endophytes improves the physiological development of legumes [13,14]. In view of the challenges of modern agriculture, the search for methods supporting the development and yield of legumes, especially methods involving microbiological technologies, falls in line with the assumptions of the European Green Deal and the EU Biodiversity Strategy for 2030. These documents emphasise the significance of sustainable plant production, the need to develop agrobiology and environmentally friendly solutions, and they assume an increase in the area of plantations with crops grown in ecological production systems.

This study emphasises the positive effect of microbial consortia composed of Bradyrhizobium bacteria isolated from commercial preparations and selected endophytes on the biometric parameters of plants grown in a model experiment.

The aim of the study was to assess interactions between the symbiotic bacteria (Bradyrhizobium sp. isolated from commercial preparations Nitragina and Nitroflora) dedicated to white lupine and selected endophytes (Pseudomonas fluorescens or Bacillus subtilis) used for seed coinoculation. In addition, selected morphological traits of the plants were measured (the weight and length of the aboveground and belowground parts) after the applied inoculation/coinoculation. The degree of root colonisation by selected endophytes used as single inoculants and in interaction with bacteria of the Bradyrhizobium genus was also determined.

2. Materials and Methods

The experiment was conducted on white lupine (Lupinus albus L.) plants of the ‘Butan’ cultivar registered in 2000. The plants were acquired from HR Smolice Sp. z o.o., IHAR Group, Przędow Branch. According to the Central Research Centre for Crop Cultivars (COBORU), the ‘Butan’ cultivar has a high yield potential. In 2017, its average yield amounted to 2.73 t ha\(^{-1}\). It is the first thermoneutral cultivar [15] and it is also insensitive to delayed sowing. Its vegetation period is 2–14 days shorter than that of traditional cultivars. The cultivar is less sensitive to diseases caused by Fusarium fungi, it contains about 30–40% less alkaloids, and has a better feed value due to its content of protein (32–37%) and fat (10–12%).

The endophytic inoculants used in in vitro tests came from the Department of Soil Science and Microbiology, Poznań University of Life Sciences, Poland. They were isolated from the rhizosphere of legumes (white lupine) on media prepared according to Rodina [16] and King B [17] for Bacillus subtilis and Pseudomonas fluorescens, respectively. Next, they were identified genetically on the basis of a fragment of the 16S rRNA gene sequence.

The strains of rhizobia (Bradyrhizobium sp. (Lupinus)) forming a symbiotic relationship with lupine were isolated for in vitro tests from two commercial preparations, i.e., Nitragina (manufactured by the BIOFOOD company in Wałcz, Poland) and Nitroflora (manufactured by the Mykoflor company in Końskowola, Poland).
2.1. Isolation of Bradyrhizobium Bacteria from Commercial Preparations

In order to isolate nitrogen-fixing bacteria, 10 g of inoculated peat and 10 g of gel were weighed from the commercial preparations Nitragina and Nitroflora, respectively, and transferred into 100 mL of pyrophosphate. The resulting suspensions were shaken on a shaker at 150 rpm for 20 min and then diluted. 0.2 mL of the dilution of $10^8$ of each preparation was applied with a cell spreader on a YMA medium [18] and incubated at 28 °C for 7 days. A single colony was collected with a sterile inoculation loop from each of the Petri dishes and it was streaked onto the YMA medium again. The resulting isolates were then inoculated onto YMA slants and used for further laboratory investigations.

2.2. Interactions between Symbiotic Bacteria (Bradyrhizobium sp.) and Endophytes (Pseudomonas fluorescens or Bacillus subtilis)

In order to determine interactions between the rhizobia and endophytic bacteria, liquid cultures of all strains were prepared in the first stage. Three-day-old Bradyrhizobium bacterial cultures (isolated from the Nitragina or Nitroflora preparations) as well as Pseudomonas fluorescens and Bacillus subtilis were transferred several times on agar slants dedicated to each bacterial species—YMA [18] for Bradyrhizobium sp., agars for Bacillus subtilis bacteria according to Rodina [16] and King B [17] for Pseudomonas fluorescens, were used to make a suspension by adding 5 mL of saline into each test tube. Next, 0.1 mL of the bacterial suspension was used to inoculate 100 mL of the YMA liquid medium for Bradyrhizobium sp., the medium prepared according to Rodina for Bacillus subtilis, and King B for Pseudomonas fluorescens (five flasks for each variant).

The bacterial cultures were incubated at 28 °C and shaken on a shaker at 70 rpm for 96 h, 48 h, and 48 h for Bradyrhizobium sp., Bacillus subtilis, and Pseudomonas fluorescens, respectively.

The ring method was used for the analysis of interactions between the microorganisms used in the research (28) in eight experimental variants (five replicates in each):

1. Bradyrhizobium sp. (isolated from the Nitragina preparation) on Pseudomonas fluorescens;
2. Bradyrhizobium sp. (isolated from the Nitragina preparation) on Bacillus subtilis;
3. Pseudomonas fluorescens on Bradyrhizobium sp. (isolated from the Nitragina preparation);
4. Bacillus subtilis on Bradyrhizobium sp. (isolated from the Nitragina preparation);
5. Bradyrhizobium sp. (isolated from the Nitroflora preparation) on Pseudomonas fluorescens;
6. Bradyrhizobium sp. (isolated from the Nitroflora preparation) on Bacillus subtilis;
7. Pseudomonas fluorescens on Bradyrhizobium sp. (isolated from the Nitroflora preparation);
8. Bacillus subtilis on Bradyrhizobium sp. (isolated from the Nitroflora preparation).

A total of 5 mL of 2% agar was poured onto sterile Petri dishes for each of the abovementioned experimental variants. After it solidified, three sterile rings (diameter 10 mm) were applied with sterile tweezers into each Petri dish. Next, 10 mL of the mixture of the dissolved medium characteristic of a given bacterial species with their appropriate dilution ($10 \times 10^5$) was poured so that it would not get inside the rings. When it cooled down, the rings were removed from the dishes under sterile conditions. A total of 0.1 mL of the prepared bacterial suspension was applied into the places of two holes, while saline was placed in the third hole as the control variant. After one hour, the plates were placed in a thermostat at 28 °C and incubated for 72 h without inversion. After this time interactions between the bacteria were read.

2.3. Influence of Coinoculation on the Weight of Belowground and Aboveground Parts of Plants

The aim of the experiment was to examine the condition of the plants coinoculated in vitro. There were nine experimental variants (each with five replicates):

1. Uninoculated plant (control variant);
2. Plant inoculated with Bradyrhizobium sp. bacteria (isolated from the Nitragina preparation);
3. Plant inoculated with Bradyrhizobium sp. bacteria (isolated from the Nitroflora preparation);
4. Plant inoculated with Bacillus subtilis bacteria;
5. Plant inoculated with Pseudomonas fluorescens bacteria;
6. Plant coinoculated with *Bradyrhizobium* sp. (isolated from the Nitragina preparation) and *Bacillus subtilis* bacteria;
7. Plant coinoculated with *Bradyrhizobium* sp. (isolated from the Nitragina preparation) and *Pseudomonas fluorescens* bacteria;
8. Plant coinoculated with *Bradyrhizobium* sp. (isolated from the Nitroflora preparation) and *Bacillus subtilis* bacteria;
9. Plant coinoculated with *Bradyrhizobium* sp. (isolated from the Nitroflora preparation) and *Pseudomonas fluorescens* bacteria.

In order to prepare a microbial culture, the seeds of the white lupine plants were sterilised in 5% sodium hypochlorite for 25 min on a shaker and then washed several times with sterile water. Next, the seeds were placed in sterile Petri dishes for 4 days to germinate at 24 °C. Sprouting lupine seeds were applied on slants with a nutrient for legumes according to selected Rothamsted biological papers 1926–1930. After 24 h, the rootlets were inoculated with a suspension of three-day-old bacterial strains at an amount of 0.1 mL per plant (10⁵ cfu) according to the variants listed above. The bacterial strains with which the plants were infected had been cultured on slants, on media dedicated to each of the bacterial genera: *Bradyrhizobium* sp., *Pseudomonas fluorescens*, and *Bacillus subtilis*. Next, the plants were transferred to a vegetation room with a temperature of 23 °C and a 16 h illumination period. After 21 days, the weight of the aboveground and belowground parts were measured with the gravimetric method.

### 2.4. Count of Endophytic Bacteria Colonising Root

The ability of *Bacillus subtilis* and *Pseudomonas fluorescens* endophytes to colonise white lupine roots was investigated by means of fluorescent in situ hybridisation (FISH). First, 5 mL of saline was poured on the test material (0.5 g of the plant root crushed in a mortar). Next, 0.05 mL of the resulting suspension was collected for further analysis by applying it to the surface of microscope slides using the breed chamber, and then it was fixed with a 4% PFA solution (paraformaldehyde). In order to clean the applied material from grease and proteins of the tested bacteria, the microscope slides with the biomaterial were rinsed in a PBS solution three times. A 0.5% Triton solution was then added and they were rinsed with the PBS solution three times again. Next, the microscope slides were placed in an alcohol series (70%, 80%, 96%), following which a 70% formamide solution was added, and a genetic probe at a concentration of 25 ng µL⁻¹, dedicated to *Bacillus subtilis* (5′/Cy3/TAC CGC CCT ATT CGA ACG GTA C-3′) (Posada et al., 2016) and a probe at a concentration of 0.5 ng µL⁻¹ dedicated to *Pseudomonas fluorescens* (5′-ACT ACC AGG CAG ATT CCT AGG CA-/Cy3/-3′) were applied [19]. The probes were labelled with fluorescent dyes, i.e., Cy5 for bacteria of the *Bacillus* genus and Cy3 for bacteria of the *Pseudomonas* genus, and suspended in a solution consisting of: 5M NaCl, 1M Tris/HCl, 10% SDS, and ddH₂O. After 24 h incubation, the presence of bacteria was determined on the basis of an image observed with a Zeiss Imager M1 fluorescence microscope.

The count of endophytic bacteria colonising the roots of white lupine was assessed with the Zeiss Imager M1 fluorescence microscope. After hybridisation, the number of bacterial cells labelled with the genetic probe was counted under the microscope in the field of view by making ten gradual shifts diagonally across the smear. Two smears were prepared for each experimental variant. The count of bacteria was determined according to the following formula:

\[
X = \frac{13,500 \times A}{20}
\]

where: \(X\)—the count of root-colonising bacteria; 13,500—the area of the field of view of the fluorescence microscope; \(A\)—the total number of cells in all fields of view; 20—10 shifts × 2 smears.

The value calculated with the formula above (\(X\)) was multiplied by 100. The result was the count of bacteria in 1 mL of the suspension obtained by grinding 0.5 g of the white lupine root.
2.5. Assessment of Diazotrophy Parameters

The following parameters were taken into account when assessing diazotrophy: the intensity of biological nitrogen fixation (BNF), the number of nodules, and the dry mass of nodules.

The intensity of biological nitrogen fixation was expressed as the level of nitrogenase activity.

The nitrogenase activity was determined after 8 weeks of vegetation. The number and colour of the nodules, the size of the plants, and nitrogenase activity were assumed as the activity index (ARA). A total of 10% of the gas phase volume of acetylene was injected into each tightly sealed test tube with the plant. After one hour, 1 mL of the gas phase was taken from the test tubes and analysed in a CHROM 5 gas chromatograph. Argon was used as a carrier. The nitrogenase activity was determined by virtue of the volume of acetylene reduced to ethylene (the average of five trials) and expressed as nmol C$_2$H$_4$ plant$^{-1}$ h$^{-1}$, applying the theoretical conversion factor N$_2$:C$_2$H$_4$ = 1:3.

The dry weight of the nodules was measured.

2.6. Statistical Analysis

A one-factor experiment under controlled conditions was set up. Plants were grown in test tubes on a substrate dedicated to legumes in nine variants, each in four replications. Factor levels were the type of inoculation with lupine seeds. The influence of the experimental factor (inoculation/coinoculation) on plant biometric parameters (mass and length of aboveground and underground parts of lupine) and diazotrophic parameters (level of nitrogenase activity, number of nodules, and nodule dry matter) were investigated using one-way ANOVA analysis.

The Statistica 12.0 package (StatSoft Inc., Cracow, Poland) was used for statistical analyses, which included one-way analysis of variance, where the method of inoculation of white lupine seeds was the differentiating factor (model 1).

\[ y_{k1} = \mu + \alpha_k \]  

where: \( \mu \)—overall average; \( \alpha_k \)—constant factor of the method of inoculation of white lupine seeds at the \( k \) level (\( k = 1, \ldots, 9 \)).

Homogeneous subsets of averages were identified with Tukey’s post hoc test at a significance level \( \alpha = 0.05 \).

Principal components analysis (PCA) was applied to estimate the cause-and-effect relationships between the parameters under analysis, including the weight of the aboveground and belowground parts.

3. Results

3.1. Interactions between Symbiotic Bacteria (Bradyrhizobium sp.) and Endophytes (Pseudomonas fluorescens or Bacillus subtilis)

The analysis of interactions between the strains of bacteria of the Bradyrhizobium genus isolated from the Nitragina preparation and the Pseudomonas fluorescens or Bacillus subtilis endophytes did not reveal any antagonistic interactions, as evidenced by the lack of brightening (halo) around the wells (Figure 1). There were similar results of the analysis of interactions between the strains of bacteria of the Bradyrhizobium genus isolated from the Nitroflora preparation and the Pseudomonas fluorescens or Bacillus subtilis endophytes (Table 1).
3. Results

3.1. Interactions between Symbiotic Bacteria (Bradyrhizobium sp.) and Endophytes (Pseudomonas fluorescens or Bacillus subtilis).

<table>
<thead>
<tr>
<th>Bradyrhizobium sp. (Isolated from Nitragina Preparation)</th>
<th>Bradyrhizobium sp. (Isolated from Nitroflora Preparation)</th>
<th>Pseudomonas fluorescens</th>
<th>Bacillus subtilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradyrhizobium sp. (isolated from Nitragina preparation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sp. (isolated from Nitroflora preparation)</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td></td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td></td>
<td>-</td>
<td>X</td>
</tr>
</tbody>
</table>

Explanation: ‘-’ no antagonistic interactions (no halo) between the microorganisms.

3.2. Influence of Coinoculation on Weight of Belowground and Aboveground of Plants and Degree of Root Colonisation by Endophytic Bacteria—Laboratory Test

The analyses showed that different variants of the inoculation of lupine plants significantly influenced the weight and length of their aboveground and belowground parts (Table 2). Both the inoculation (single inoculation) and coinoculation (simultaneous inoculation with symbiotic bacteria and endophytes) stimulated the growth of the aboveground and belowground parts of the plants, which were longer and heavier than the control plants. The length of the lupine stems ranged from 17.5 to 20.3 cm. The longest stem was found in variant nine, where the plants had been coinoculated with bacteria of the Bradyrhizobium genus isolated from the Nitroflora preparation and the Bacillus subtilis endophyte. The weight of the stems ranged from 1.88–2.84 g. Coinoculation also resulted in the heaviest weight of the stems. In variant seven, where the plants had been coinoculated with bacteria of the Bradyrhizobium genus isolated from the Nitragina preparation and the Bacillus subtilis, the endophyte weight was 2.84 g. The length of roots ranged from 12.6 to 20.9 cm (Table 2). The highest values were noted after the coinoculation of seeds with the Bradyrhizobium bacteria isolated from the Nitroflora preparation and the Pseudomonas fluorescens endophyte (variant eight). The analysis of the weight of the belowground parts revealed the highest values in variant five (inoculation with the Bacillus subtilis endophyte only)—1.01 g, in variant eight (coinoculation with the Bradyrhizobium bacteria isolated from the Nitroflora preparation and the Pseudomonas fluorescens endophyte)—0.97 g, and in variant two (inoculation with bacteria of the Bradyrhizobium genus isolated from the Nitragina preparation)—0.96 g.

Figure 1. An example showing the effect of Pseudomonas fluorescens bacteria on bacteria of the Bradyrhizobium genus isolated from the Nitragina preparation. Saline was used as the control variant.
Table 2. The influence of the inoculation variant on the length and weight of the aboveground and belowground parts of white lupine. ANOVA and Tukey’s post hoc test for inoculation variant.

<table>
<thead>
<tr>
<th>Experiment Variant</th>
<th>Aboveground Parts</th>
<th>Belowground Parts</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length [cm]</td>
<td>Weight [g]</td>
<td>Length [cm]</td>
</tr>
<tr>
<td>F-statistic</td>
<td>2.39</td>
<td>6.31</td>
<td>6.91</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1 Uninoculated seeds (control variant)</td>
<td>17.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 Seeds inoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from Nitragina preparation</td>
<td>18.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.59&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 Seeds inoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from Nitroflora preparation</td>
<td>19.4&lt;sup&gt;a–c&lt;/sup&gt;</td>
<td>2.67&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>19.0&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 Seeds inoculated with <em>Pseudomonas fluorescens</em></td>
<td>18.1&lt;sup&gt;a–c&lt;/sup&gt;</td>
<td>2.34&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>12.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 Seeds inoculated with <em>Bacillus subtilis</em></td>
<td>19.0&lt;sup&gt;a–c&lt;/sup&gt;</td>
<td>2.63&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6 Seeds coinoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from Nitragina preparation and <em>Pseudomonas fluorescens</em> bacteria</td>
<td>18.9&lt;sup&gt;a–c&lt;/sup&gt;</td>
<td>2.64&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7 Seeds coinoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from Nitroflora preparation and <em>Bacillus subtilis</em> bacteria</td>
<td>19.1&lt;sup&gt;a–c&lt;/sup&gt;</td>
<td>2.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>8 Seeds coinoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from the Nitroflora preparation and <em>Pseudomonas fluorescens</em> bacteria</td>
<td>20.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.71&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>9 Seeds coinoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from Nitroflora preparation and <em>Bacillus subtilis</em> bacteria.</td>
<td>20.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.75&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviation: The same letters next to the mean values denote the groups of means which do not differ significantly from one another. The statistical significance level was $\alpha = 0.05$.

Principal components analysis (PCA) was applied to find dependences between the applied variants of the experiment and the length and weight of the aboveground and belowground parts of the plants (Figure 2). The first principal component explained 70.80% of the variation, whereas the second principal component explained 16.70% of the variation. Both components explained 87.50% of the total variation.

The principal components analysis (PCA) showed the strongest relation between the length of the aboveground parts of the plants and their weight. There was a similar relation between the weight of the aboveground parts of the plants and the weight of their belowground parts. There were no such relations between the length of the belowground parts of the plants and the other parameters under analysis.

The strongest influence on the plant parameters under analysis was observed in the coinoculation variants (variants seven, eight, and nine). Most of the parameters, i.e., the length and weight of the aboveground parts of the plants as well as the weight of their belowground parts, were the most strongly influenced by the coinoculation of white lupine seeds with bacteria of the *Bradyrhizobium* genus isolated from the Nitroflora preparation and the *Bacillus subtilis* endophyte (variant nine) (Figure 2).

There were no relationships between the biometric parameters observed in the control variant, where no seed inoculation had been applied.
Bacillus subtilis was a higher count of with rhizobia of the Bradyrhizobium genus and the respective endophyte (variant nine) (Figure 2).

There were no relationships between the biometric parameters observed in the controls. The principal component analysis (PCA) of the basic physiological parameters (the length and weight of belowground and aboveground parts of the plants) for individual variants of the experiment. Explanation: 1. Uninoculated plant (control variant); 2. Plant inoculated with Bradyrhizobium sp. bacteria (isolated from the Nitragina preparation); 3. Plant inoculated with Bradyrhizobium sp. bacteria (isolated from the Nitroflora preparation); 4. Plant inoculated with Bacillus subtilis bacteria; 5. Plant inoculated with Pseudomonas fluorescens bacteria; 6. Plant coinoculated with Bradyrhizobium sp. (isolated from the Nitragina preparation) and Bacillus subtilis bacteria; 7. Plant coinoculated with Bradyrhizobium sp. (isolated from the Nitragina preparation) and Pseudomonas fluorescens bacteria; 8. Plant coinoculated with Bradyrhizobium sp. (isolated from the Nitroflora preparation) and Bacillus subtilis bacteria; 9. Plant coinoculated with Bradyrhizobium sp. (isolated from the Nitroflora preparation) and Pseudomonas fluorescens bacteria.

3.3. Count of Endophytic Bacteria Colonising Roots

Fluorescence in situ hybridisation (FISH) was applied to assess the degree of colonisation of the white lupine roots by the endophytic bacteria: Pseudomonas fluorescens and Bacillus subtilis. The presence of the endophytes was detected in all variants of the experiment. This may have resulted from the fact that these microorganisms are part of the indigenous microbiome of white lupine seeds. In comparison with the control variant (variant one), there were high counts of endophytic bacteria in the variants where the seeds had been inoculated with the Bradyrhizobium sp. bacteria isolated from the Nitragina preparation. On the other hand, there was a higher count of Bacillus subtilis bacteria when Bradyrhizobium bacteria isolated from the Nitragina preparation had been used for coinoculation. On the other hand, there was a higher count of Pseudomonas fluorescens bacterial cells in the interaction with Bradyrhizobium bacteria isolated from the Nitroflora preparation.

**Figure 2.** The principal component analysis (PCA) of the basic physiological parameters (the length and weight of belowground and aboveground parts of the plants) for individual variants of the experiment. Explanation: 1. Uninoculated plant (control variant); 2. Plant inoculated with Bradyrhizobium sp. bacteria (isolated from the Nitragina preparation); 3. Plant inoculated with Bradyrhizobium sp. bacteria (isolated from the Nitroflora preparation); 4. Plant inoculated with Bacillus subtilis bacteria; 5. Plant inoculated with Pseudomonas fluorescens bacteria; 6. Plant coinoculated with Bradyrhizobium sp. (isolated from the Nitragina preparation) and Bacillus subtilis bacteria; 7. Plant coinoculated with Bradyrhizobium sp. (isolated from the Nitragina preparation) and Pseudomonas fluorescens bacteria; 8. Plant coinoculated with Bradyrhizobium sp. (isolated from the Nitroflora preparation) and Bacillus subtilis bacteria; 9. Plant coinoculated with Bradyrhizobium sp. (isolated from the Nitroflora preparation) and Pseudomonas fluorescens bacteria.
3.4. Assessment of Diazotrophy Parameters

The lowest values of the parameters characterising the diazotrophy level in the white lupine plants were noted in the control variant, where the seeds had not been inoculated (Table 3).

The intensity of biological nitrogen fixation (BNF), expressed as the nitrogenase activity level, was the highest after the coinoculation of plants with bacteria of the *Bradyrhizobium* sp. genus isolated from the Nitragina preparation in combination with *Bacillus subtilis* (variant nine). Moreover, the coinoculation of white lupine seeds with rhizobia isolated from the Nitragina preparation in combination with *Pseudomonas fluorescens* (variant six), as well as with *Bacillus subtilis* (variant seven) also resulted in a statistically significant increase in biological nitrogen fixation.

Another parameter characterising the diazotrophy level was the number of nodules. The largest number of nodules was noted after the coinoculation of white lupine seeds with *Bradyrhizobium* bacteria isolated from the Nitroflora preparation in combination with *Bacillus subtilis* (variant nine) (Table 3). The number of nodules was also high in the other variants (six–eight), where coinoculation was applied, regardless of the consortium of rhizobia and endophytic bacteria.

The statistical analysis of the effect of different variants of inoculation of lupine seeds on the dry weight of the nodules also showed that the strongest nodulation, expressed as the dry weight of the lupine root nodules, was observed in the variants in which the seeds had been inoculated with bacteria of the *Bradyrhizobium* genus isolated from the Nitroflora preparation in combination with *Bacillus subtilis* (variant nine). The dry weight of the nodules was 0.027 g greater than in the control variant (Table 3).
Table 3. The influence of different variants of inoculation of white lupine seeds on selected diazotrophy parameters. ANOVA and Tukey’s post hoc test for inoculation variant.

<table>
<thead>
<tr>
<th>Variant Description</th>
<th>BNF nMC&lt;sub&gt;2H&lt;/sub&gt;&lt;sub&gt;4&lt;/sub&gt; Plant&lt;sup&gt;−1&lt;/sup&gt; h&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>Number of Root Nodules</th>
<th>Dry Weight of Root Nodules (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Uninoculated seeds (control variant)</td>
<td>2366.22</td>
<td>7.87</td>
<td>2.75</td>
</tr>
<tr>
<td>2. Seeds inoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from Nitragina preparation</td>
<td>19.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.31&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.020&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>3. Seeds inoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from Nitroflora preparation</td>
<td>17.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.13&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.025&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>4. Seeds inoculated with <em>Pseudomonas fluorescens</em></td>
<td>12.8&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.06&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.027&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>5. Seeds inoculated with <em>Bacillus subtilis</em></td>
<td>14.3&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.16&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.029&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>6. Seeds coinoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from Nitragina preparation and <em>Pseudomonas fluorescens</em> bacteria</td>
<td>36.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.51&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>7. Seeds coinoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from Nitragina preparation and <em>Bacillus subtilis</em> bacteria</td>
<td>33.50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.52&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.024&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>8. Seeds coinoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from the Nitroflora preparation and <em>Pseudomonas fluorescens</em> bacteria</td>
<td>24.33&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.54&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.031&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>9. Seeds coinoculated with bacteria of <em>Bradyrhizobium</em> genus isolated from Nitroflora preparation and <em>Bacillus subtilis</em> bacteria.</td>
<td>39.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.041&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviation: The same letters next to the mean values denote the groups of means which do not differ significantly from one another. The statistical significance level was α = 0.05.

4. Discussion

Microorganisms may significantly increase plants’ resistance to abiotic and biotic stresses if their unique tolerance to extreme environmental conditions, ubiquity, genetic diversity, and interactions with crops are properly taken advantage of, and if methods of their successful application in agricultural production are developed. The use of individual species of microorganisms or microbial consortia as biopreparations or biofertilisers may influence the yield of crops and thus provide new methods for the application of microorganisms in agriculture, including the cultivation of legumes. A key factor influencing the beneficial effects of bacterial consortia is the interaction between their members to guarantee a stable long-term co-existence [20]. The communication relies on the production, detection, and response to extracellular signalling molecules that regulate and shape the bacterial population in the consortium, where only compatible microbes are involved in altering the plant defence response affecting overall plant health and growth [21]. Consortium communication is highly dependent on molecular signals; among them, quorum sensing plays a significant role in bacterial compatibility in consortium formulations [22]. Quorum sensing allows bacteria to switch between two distinct gene expression programmes: (i) one at low cell density for individual and asocial behaviour, and (ii) another at high cell density for social and group behaviours, which are preferential for consortia [23].

The results of the in vitro investigations revealed some interactions which may occur between the endophytic bacteria of the *Bacillus* and *Pseudomonas* genera and the symbiotic bacteria of the *Bradyrhizobium* sp. genus, which were selected for the research. Reference
publications provide a lot of information about the properties of bacteria belonging to the PGPR group and their influence on plants. However, there have been few studies analysing the direct interactions between endophytic bacteria and rhizobia. Niewiadomska and Swędryńska [24] conducted research on interactions between the strains of Sinorhizobium meliloti (rhizobia) and Herbaspirillum frisingense (endophytic bacteria). The authors observed that H. frisingense stimulated the growth of S. meliloti in all variants of the experiment (48 h culture, sediment, supernatant). When the same bacterial strains were used for inoculation in the reverse order, S. meliloti had a positive effect on H. frisingense in the sediment obtained after centrifugation, but there were antagonistic relations observed in the other cases. The negative effects were explained by the fact that during incubation, secondary metabolites produced by H. frisingense, which were toxic to S. meliloti, had accumulated in the medium.

The analysis of the influence of the endophytic strains on the symbiotic bacteria used in our experiment showed the mutualistic effect of the endophytic bacteria selected for coinoculation. This was manifested by increased microbial growth around the wells. It is impossible to definitely determine what stimulated the growth of the endophytic bacteria. However, it is very likely that this effect of Bacillus subtilis and Pseudomonas fluorescens on the strains of rhizobia was caused by the production of phytohormones, such as indoleacetic acid (IAA), gibberellins, and cytokinins [12,25], or by the production of vitamins by bacteria of the Bacillus genus [26]. By contrast, the bacteria of the Bradyrhizobium genus used in the experiment were neutral to the endophytic bacteria. Such an ambivalent interaction between bacterial strains towards each other was also observed by Martins et al. [27]. According to Niewiadomska [28], it is necessary to study interactions between selected strains to create possible consortia composed of various species of bacteria because the secondary metabolites or endotoxins produced by microbial strains in the consortium may adhere to the outer wall of the bacterial cell, and thus inhibit the growth and development of the other components of the inoculation consortium. The antagonistic interaction of bacterial strains in pure cultures may suggest a similar effect if such a consortium is created in the same environment with the plant. Pseudomonas fluorescens secretes secondary metabolites, such as hydrocyanic acid and antibiotics, which are antagonistic to other microorganisms. The former has fungistatic properties, but it may also limit the growth of other bacteria [29]. A similar effect can be expected from the antibiotics secreted by these bacteria, such as 2,4-diacetylphloroglucinol and phenazine [30].

Laboratory experiments involving the coinoculation of legumes with endophytic bacteria and rhizobia [14,24,27,28], which were conducted without the influence of additional biotic and abiotic factors, showed various effects of this treatment on the biometric parameters of the plants, i.e., the shoot length, the weight of the aboveground and belowground parts of the plants, the degree of root colonisation by bacteria, and the nitrogenase activity. In a study conducted by Marek-Kozaczuk et al. [31], the coinoculation had either no influence or an antagonistic effect on the selected parameters of plants and the physiological activity of bacteria. On the other hand, studies conducted by Chebotar et al. [32], Niewiadomska and Swędryńska [24], and Grover et al. [33] showed that coinoculation increased the length of the shoots of legumes, the weight of the aboveground and belowground parts of the plants, and the number of nodules. Our in vitro model experiment showed that the treatment of white lupine plants with selected bacterial strains of the Bradyrhizobium sp. genus isolated from the Nitragina and Nitroflora preparations as well as Pseudomonas fluorescens and Bacillus subtilis endophytic bacteria had a synergistic effect on the biometric parameters of the plants and the degree of root colonisation by the endophytic bacteria, as well as diazotrophy parameters (nitrogenase activity, the number and weight of nodules).

It is important to note that the effects of inoculation and coinoculation were considerably diversified. The strongest mutualistic effect on the plant parameters was observed in the variants where the white lupine seeds had been coinoculated with bacteria of the Bradyrhizobium genus isolated from the Nitroflora preparation and the Bacillus subtilis endophyte (variant nine). The coinoculation of the seeds with bacteria of the Bradyrhizobium
genus isolated from the Nitroflora preparation and the *Pseudomonas fluorescens* endophyte had a slightly weaker effect (variant eight). As a result of the coinoculation with the *Bradyrhizobium* sp. isolates from the Nitragina preparation and *Pseudomonas fluorescens*, the length and weight of the aboveground parts of the plants were 6.6% and 4% lower, respectively, than the values of these parameters in the best variant nine (*Bradyrhizobium* sp. from the Nitroflora preparation and *Bacillus subtilis*). Similar effects were observed for the diazotrophy parameters.

Studies on legumes have shown that synergism between *Bacillus* and *Bradyrhizobium* bacteria in the rhizosphere increased the nodulation (the number and dry weight of nodules) and biomass of plants [34–38]. Some studies found that the coinoculation of legume seeds with bacteria of the *Pseudomonas* sp. genus and *Rhizobium* also improved the condition of the nodules [39].

There were also significant differences in the degree of white lupine root colonisation by the endophytes as well as in the coinoculation with the rhizobia isolated from the Nitroflora and Nitragina preparations. After the inoculation of lupine seeds with the endophytes, the count of bacterial cells of the *Pseudomonas* genus colonising the white lupine roots was greater than the count of *Bacillus* cells. Additionally, the coinoculation with rhizobia isolated from the Nitroflora preparation and bacteria of the *Pseudomonas* genus resulted in a larger number of cells colonising the white lupine roots than the coinoculation with the *Bradyrhizobium* sp. bacteria isolated from the Nitragina preparation. These differences in the degree of root colonisation by the endophytes applied in different variants of the experiment may have been caused by certain allelopathy resulting from the plant-microorganism and microorganism–microorganism interactions.

During the development of plants, a certain proportion of the substances they produce are released into the environment in a controlled manner. This is a species-specific ‘chemical profile’ which includes phenolic compounds—molecules formed as a result of transformations of aromatic amino acids (mainly phenylalanine and, to a lesser extent, tyrosine) or their direct precursors. As results from the data provided in reference publications show, secondary metabolites, especially phenolic compounds and alkaloids, are used by other organisms inhabiting the phytosphere as a chemical signal indicating the presence of an appropriate interaction partner. Flavonoids, which are secreted by the roots of legumes, significantly influence the symbiosis between these plants and rhizobia [40,41]. These compounds play an important role in the interaction of partners participating in the symbiosis. They mostly affect the expression of Nod genes in symbiotic bacteria. As a result, these bacteria produce the Nod factor, which is a lipochitooligosaccharide responsible for nodulation. Apart from inducing the expression of Nod genes, flavonoids also affect the chemotaxis of microorganisms and thus help to find symbiotic partners. As far as bacteria of the *Bradyrhizobium* genus are concerned, isoflavones (strong activators of Nod genes) do not induce a chemotactic response, whereas weak inducers of Nod genes (hydroxycinnamic acids, coniferyl alcohol) are chemoattractants [42].

Besides flavonoids, a wide spectrum of polysaccharides (exopolysaccharides—EPS), which are present on the surface of rhizobia cells and have a species-specific composition, also play an important role in establishing a symbiotic relationship between legumes and symbiotic bacteria [43]. For example, succinoglycans are exopolysaccharides (EPS) of particular importance in the formation of symbiotic systems with legumes. They are a necessary factor conditioning the elongation of the infectious strand in *Sinorhizobium meliloti*. The increased synthesis of these exopolysaccharides increases the efficiency of nodulation in *Meliloti truncatula* [44]. In 2015, the membrane receptor (EPR3) for bacterial exopolysaccharides was identified in *Lotus japonicas*. Its interaction with EPS depends on their structure. Moreover, researchers also observed that EPR3 synthesis is conditioned by the presence of specific Nod factors. This means that the infection of the host plant by rhizobia can be controlled by two independent sets of receptors receiving both Nod and EPS signals [45,46].
Phenolic compounds are believed to significantly affect root colonisation not only by rhizobia but also by endophytic bacteria [47]. It is presumed that certain genotypes of these bacteria are highly adapted to colonisation of the rhizosphere of their respective plants and that as in the case of rhizobia, it largely depends on the secretion of bioactive phenolic compounds by the roots [48]. The appearance of these compounds in the environment is recognised and used by bacteria, e.g., from the Pseudomonas and Azospirillum genera, as a chemical signal responsible for the initiation of rhizosphere interactions and indicating the presence of a plant which is an appropriate host for a particular group of microorganisms [48,49]. According to Schelud’ko et al. [50], legumes mainly secrete lecithin, which influences the mobility of PGPR bacteria and the colonisation of the side roots of these plants. All these dependencies resulting from the presence of various chemical compounds secreted by the white lupine roots may have influenced the degree of their colonisation by specific endophytes used in the model experiment conducted in our study. Interestingly, Zgadzaj and colleagues [51] have shown that Mesorhizobium loti induces infection threads that can selectively guide non-rhizobial endophytic bacteria towards nodule primordia. These endophytes are then able to migrate and colonize the root nodule together with rhizobia, benefiting from the increased carbon concentrations found within the nodule.

5. Conclusions

The study did not reveal any antagonistic interactions between bacterial strains of the Bradyrhizobium sp. genus isolated from the Nitragina and Nitroflora preparations and the Pseudomonas fluorescens or Bacillus subtilis endophytes. This means that they can be used to form bacterial consortia composed of rhizobia and endophytes for the inoculation of white lupine.

In vitro coinoculation increased the weight of white lupine stems and roots and as well as the values of diazotrophic parameters (the nitrogenase activity and nodulation).

However, it is recommended to conduct soil experiments to fully investigate the effect of coinoculation on the cultivation of white lupine as well as other legumes. Soil and weather conditions are additional factors which significantly affect the diazotrophy process and the yield of legumes.

The cost of the preparation of inoculants composed of rhizobia and endophytic bacteria and their possible application to the soil also needs to be taken into consideration. It is necessary to remember that not every endophyte selected for coinoculation, or even used for individual inoculation in the field, will stimulate the growth of plants.


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