

Article

Optimizing Nitrogen Fixation in *Vicia sativa*: The Role of Host Genetic Diversity

María Isabel López-Román ¹, Cristina Castaño-Herrero ¹, Lucía De la Rosa ² and Elena Ramírez-Parra ^{1,*}

¹ Centro de Biotecnología y Genómica de Plantas, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Consejo Superior de Investigaciones Científicas (CBGP, UPM-INIA/CSIC), Universidad Politécnica de Madrid, Campus de Montegancedo, 28223 Pozuelo de Alarcón, Spain; isabel.lopez@ictan.csic.es (M.I.L.-R.); ccastano.herrero@inia.csic.es (C.C.-H.)

² Centro de Recursos Fitogenéticos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Consejo Superior de Investigaciones Científicas (CRF-INIA/CSIC), 28805 Alcalá de Henares, Spain; lucia.delarosa@inia.csic.es

* Correspondence: ramirez.elena@inia.csic.es

Abstract: Common vetch (*Vicia sativa* L.) is a legume widely used both as a grain and as forage due to its high protein content, which provides considerable nutritional enrichment for livestock feed. As a cover crop, it has the potential to fix atmospheric nitrogen through symbiosis with rhizobia, contributing to sustainable agricultural systems by enhancing soil fertility and reducing the dependence on chemical fertilizers. Although much research has been focused on optimizing *Rhizobium* inoculants to enhance biological nitrogen fixation (BNF) in leguminous crops, the role of host plant genetic diversity in BNF has been underexplored. This study analyses a collection of *V. sativa* genotypes to evaluate their BNF by assaying their nodulation capacity, nodule nitrogenase activity, nitrogen fixation potential, and impact on biomass development. Our results reveal large variability in these parameters among the different genotypes, emphasizing the relevance of host legume diversity in the *Rhizobium* symbiosis. These findings show a direct relationship between nodule biomass development, nitrogen fixation capacity, shoot biomass production, and nitrogen content. However, no correlation was observed for other parameters such as the number of nodules, nitrogenase activity, and shoot nitrogen content. Taken together, these results suggest that selecting genotypes with high BNF capacity could be a promising strategy to improve nitrogen fixation in legume-based agricultural systems.

Keywords: legume; common vetch; *Vicia sativa* L.; symbiosis; genetic resources; nodulation; biological nitrogen fixation (BNF); rhizobium



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1. Introduction

Legumes (*Fabaceae*) are among the most agronomical and nutritionally important crop families due to their high protein content and ability to grow in diverse environments. As a primary source of plant-based protein for both human and animal consumption, legumes play a key role in global food security and sustainable agriculture [1]. Their nutritional value is largely attributable to the aptitude of these crops for nitrogen fixation, which allows them to assimilate atmospheric nitrogen and synthesize high-protein grains without relying on chemical fertilizers. This exceptional ability makes legumes valuable not only for their nutritional benefits but also for their environmental advantages. They are used as cover crops or in rotational practices, enhancing soil fertility and reducing agricultural reliance on synthetic fertilizers, whose excessive use has contributed to environmental issues such as eutrophication and greenhouse gas emissions [2–6].

Rhizobia are naturally present in many soils and infect the roots of legumes, forming nodules, where atmospheric nitrogen (N_2) is converted into biologically available forms in exchange for plant-derived carbon (C) resources from the soil [7]. BNF, as a natural and environmentally friendly process, is an attractive tool for low-impact farming. However, the establishment of the legume–rhizobium symbiosis is a process with high complexity due to the heterogeneity of environmental factors that influence the process, particularly soil-related constraints such as pH, salinity, drought, and nutrient availability, which can significantly limit nodule formation [8]. In addition, agronomic practices, especially the excessive application of mineral nitrogen fertilizers, can suppress nodulation by reducing the plant dependence on BNF [9]. The successful initiation of this symbiotic process also depends on the presence and activity of naturally occurring rhizobacteria in the soil, which are essential for effective nodule development and function [10]. Despite the ecological and agronomic relevance of this process, the efficiency of BNF varies widely depending on the species, the plant genotype, and the specific rhizobial strains present in the soil [11]. The interaction between legumes and rhizobia is highly specific, as each legume species associates with limited rhizobial strains. This specificity is mediated by molecular signaling, in which legumes release flavonoids that attract compatible rhizobia. In response, the bacteria produce Nod factors, which trigger nodule formation and establish the symbiotic relationship, enabling an efficient nitrogen-fixing partnership adapted to each legume species. It is well established that legumes selectively associate with specific rhizobial strains [12,13]; however the ecological and molecular mechanisms governing this specificity among different legume hosts or across species diversity remain underexplored. On the other hand, new bacterial strains with high nodulation capacity and high BNF efficiency have also been identified [13].

Vicia sativa (common vetch) is a model species for studying nitrogen-fixing legumes. Recent studies have linked nodule size, nitrogenase activity, and the carbon/nitrogen (C/N) balance to symbiotic nitrogen fixation efficiency [14,15]. Common vetch is a highly relevant crop, valued for its dual-purpose use as both a grain legume and forage, due to its high protein content and plays a key role in sustainable agricultural practices. Thus, it is broadly used in crop rotations, intercropping systems, and as a cover crop to improve soil health and quality [16]. In warmer areas, common vetch can add up to 179 kg N per hectare [17]. This amount of N is notably higher than that of other legumes used as cover crops, including various species of clover (*Trifolium* spp.), lupin (*Lupinus angustifolius*), or Austrian winter pea (*Pisum sativum*). These agronomic studies also suggest that the amount of fixed N not only depends on species but also may depend on the genotype [17]. While strategies to improve nodule formation and BNF in legumes often focus on selecting elite rhizobia strains to develop more efficient inoculants, the impact of host legume genotype remains scarcely analyzed. Despite its importance, to our knowledge, no commercial *V. sativa* varieties have been selected based on their nitrogen-fixing capacity, with most improvements focusing on agronomic yield traits rather than BNF efficiency.

Plant genetic resources collections have become an essential tool in plant breeding, especially in the evaluation of cultivated landraces and their wild relatives. The National Spanish Plant Genetic Resources Center (CRF, INIA-CSIC) conserves in its genebank one of the largest world collections of common vetches, with over 1000 accessions. This collection includes landraces, wild populations, and commercial cultivars from Spain and other countries. Recent research has assessed the genetic variability of this collection through genotyping and the evaluation of key agronomic traits, such as yield, phenology, and drought tolerance [18]. Based on these analyses, a group of accessions that best represent the main genetic and phenotypic diversity of the entire collection has been selected [18]. However, there is currently no available data on the nodulation or nitrogen-fixing capacity

of these genotypes. In this study, we aim to explore the impact of plant host genotypes on BNF in *V. sativa* by using a representative selection of 19 accessions from this collection. Specifically, we address (1) the relationship between nodule size, nodule number, nitrogenase activity, and the plant C/N balance; (2) how the host's genotype influences nodule activity; and (3) the potential of host selection to enhance BNF efficiency. Despite the limited scope of these investigations, the results support the value of the genetic diversity present in *V. sativa* genebanks as a tool for selecting accessions with high BNF potential. By elucidating the mechanisms that regulate host–rhizobia specificity, this research advances the broader objective of optimizing legume-based nitrogen fixation for sustainable agriculture practices.

2. Materials and Methods

2.1. Plant Materials and Treatments

The selected international *V. sativa* accessions, including landraces, wild relatives, and commercial varieties, analyzed in this study are described in Table S1. Detailed passport data for each accession can be found in the Supplementary Materials, and additional information is available through the European Cooperative Programme for Plant Genetic Resources (ECPGR) database EURISCO website (<https://eurisco.ipk-gatersleben.de/>, accessed on 15 June 2025). Genotypic data using various types of molecular markers and the phenotypic characterization of this representative collection of 19 genotypes were previously described in other works from our laboratory, as well as complete phenotypic information, including morphological, production drought tolerance, and geographic passport data [18,19]. For all the analyses, plants were grown in a greenhouse under controlled conditions (a 16 h light/8 h dark photoperiod at 22 °C). For rhizobial symbiosis assays, seeds were surface-sterilized by immersion in 96% ethanol, followed by 12% bleach and water washes. Seeds were stratified and germinated on 1% agar plates. Inoculation experiments were conducted in sterilized Leonard units with N-deficient media, as described in [20]. Seedlings were inoculated with 1 mL of the early stationary-phase bacterial culture. *Rhizobium leguminosarum* strain V31 was employed as the inoculant, and after inoculation plants were grown in hydroponic N-deficient media in Leonard units until their analysis [20,21]. Non-inoculated control plants were supplied with the same nutrient solution supplemented with 10 mM NH_4NO_3 ; (N-fed plants) ten days after sowing; then they were grown in the same nutrient solution until their analysis [21]. Nodule formation and nitrogenase activity were assayed 32 ± 1 days after sowing. The experimental timeline is shown in Supplementary Figure S1. For the determination of the different parameters, at least four independent experiments were conducted, each with four plants per genotype, along with technical triplicates.

2.2. Sample and Data Collection of Shoot, Root, and Nodule Parameters

Shoot and root were separated, and fresh weight was measured at 32 ± 1 days after sowing. After these analyses, the aerial parts and roots were oven-dehydrated at 65 °C for 7 days for subsequent dry weight determination of the shoot and root biomass. At least four plants per genotype were measured in each assay, and four independent assays were performed. For nodule determination, nodules were manually detached one by one from each individual root, then immediately counted and weighed to measure their number and fresh biomass. Then, nodules were oven-dried at 65 °C for 7 days for subsequent dry weight determination.

Nodules were harvested from plants at 32 ± 1 days post-sowing and immediately processed for the quantification of nitrogenase activity. The nitrogen fixation activity of the nodules was estimated using the acetylene reduction assay (ARA), as previously described

by [21,22] using a Shimadzu GC-8 chromatograph, Kyoto, Japan. For the determination of nitrogen and carbon, shoots were oven-dried at 65 °C for 7 days, and dried tissues were then weighed, ground, homogenized, and then analyzed for the quantification of total nitrogen and carbon content using the Dumas method with a TruSpec C/N analyzer (Leco-St. Joseph, MI, USA) at the Ionomics Platform of CEBAS (CSIC, Murcia, Spain). Results were expressed as the percentage of C or N per dry weight (DW) of tissue.

2.3. Statistical Analysis and Additional Software

Data were statistically analyzed using the Real Statistics add-in for Excel. A two-way ANOVA was performed to determine differences in nodule number, size, nitrogenase activity, and C/N content among various accessions. Distinct letters indicate statistically significant differences (p -value < 0.05). Principal Component Analysis (PCA) and multivariable correlation analysis were conducted using the Statgraphics Centurion v18.1 software. For the final determination, at least four independent experiments were conducted, each with four plants, along with technical triplicates.

Grammarly, Inc.v2025 was used as a writing assistant software tool to review and polish the spelling, grammar, and style of the manuscript.

3. Results

3.1. *V. sativa* Genotype Impacts on Nodule Formation and Nodule Development

We analyzed the impact of the genotype on nodule formation and development. The results revealed that nodulation is strongly accession-dependent, and the genotype notably influences both nodule number (Figure 1A) and nodule biomass per plant (Figure 1B). Notably, accessions Aitana, 342, 381, and 515 exhibited a lower number of nodules compared to other genotypes, while accession 512 showed the highest nodule count. In fact, the mean number of nodules in the 512 genotype is 3.9-fold higher than that of the Aitana genotype. As expected, the corresponding controls with non-inoculated roots of the different genotypes did not show nodules. Interestingly, in this study the nodule number did not always correlate with the total nodule biomass. For example, accessions 434, 506, and 521 displayed the highest nodule biomass despite having moderate nodule numbers, indicating differences in nodule size or development efficiency among the different genotypes. Thus, the differences between the mean nodule biomass in genotype 434 is 3.7-fold higher than that of genotype 101, which is the one with the lowest mean nodule biomass. Figure 1C shows representative images of genotypes with different nodule sizes and nodule numbers. Regarding the nodule distribution on the root, no major differences were found among accessions. In all the genotypes nodules were located on the upper part of the root, both at primary and secondary roots in proximal location (Supplementary Figure S6). Overall, these results indicate important genotypic variation in nodulation attributes within the vetch collection, including the nodule number, mass, and size. Aiming for the selection of genotypes with stable and high production of nodules, these results may have direct implications for future breeding programs focused on enhancing symbiotic nitrogen fixation efficiency strategies.

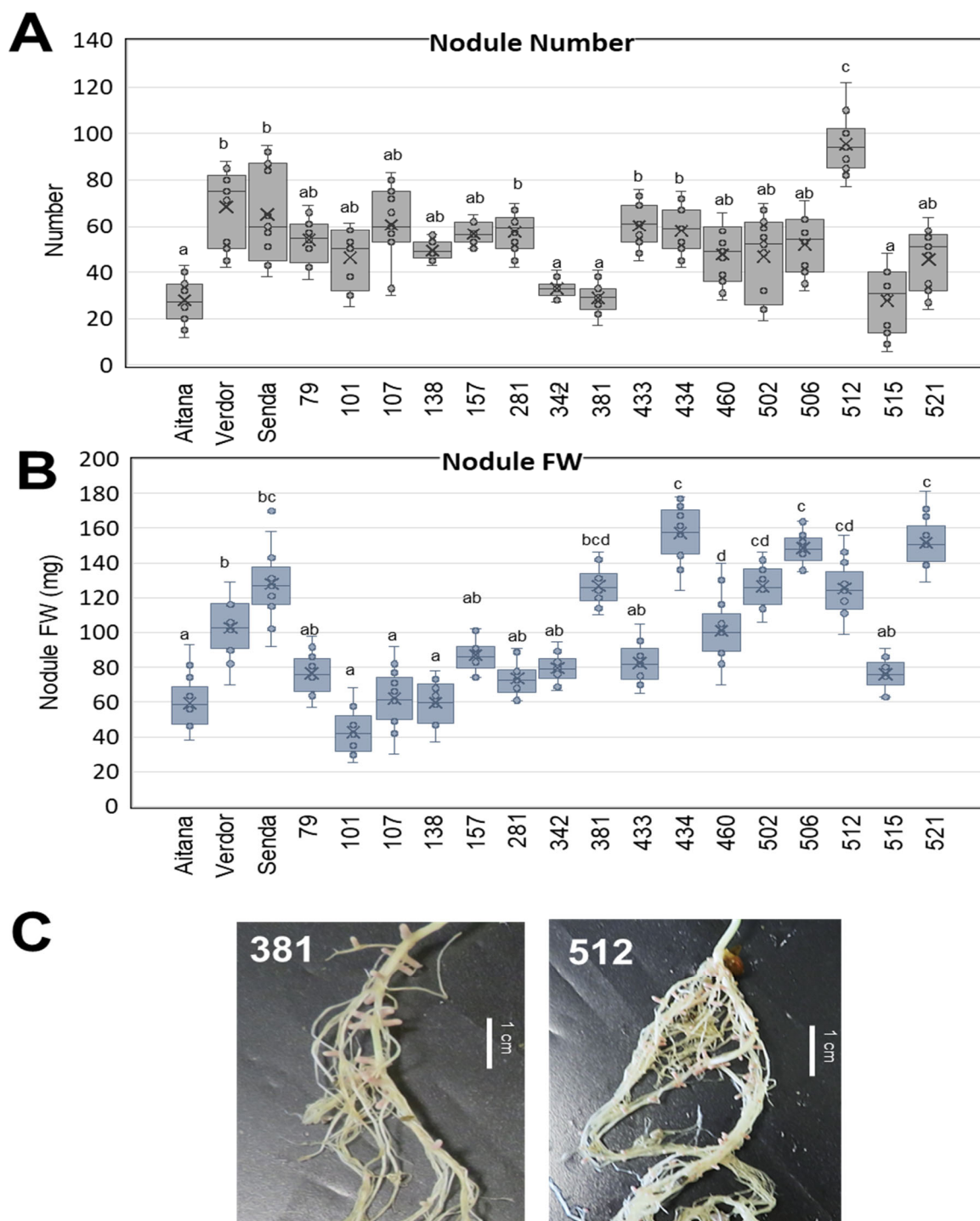


Figure 1. Nodule number (**A**) and nodule fresh weight (FW) per plant (**B**) of different common vetch inoculated genotypes. At least three independent experiments were conducted, with $n = 4$ plants each. Letters indicate significant differences (ANOVA and Tukey's HSD test; $p < 0.05$). (**C**) Representative images of nodules of genotypes with extreme values.

3.2. *V. sativa* Genotype Impacts on Nodule Nitrogenase Activity

Given the strong evidence that nodule number and nodule size do not always reflect their functional efficiency, we aimed to analyze their nitrogenase activity [21,22]. These assays also revealed a strong genotype-dependent effect on nitrogenase activity within the nodules. Among the evaluated genotypes, accessions 434, 460, and 502 exhibited the highest nitrogenase activity, presenting the high intravarietal variability of genotype 460.

In contrast, accessions Verdor, 281, 342, 381, and 433 showed the lowest levels of activity (Figure 2). The differences in mean nitrogenase activity are more than 15-fold between the lowest active genotype (342) and the most active (434). As expected, the corresponding controls with non-inoculated roots of the different genotypes showed no detectable activity.

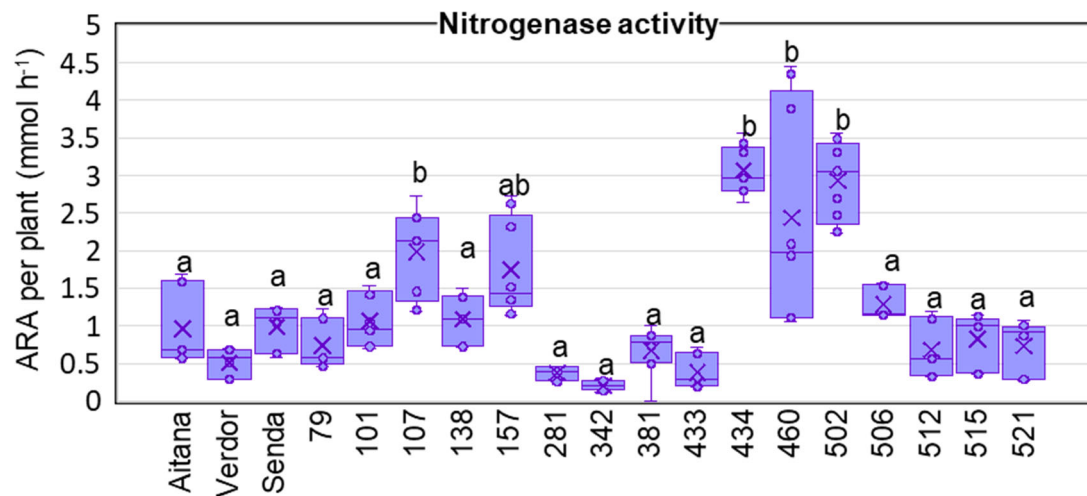


Figure 2. Nitrogenase activity measured by the acetylene reduction assay (ARA) per plant of different common vetch inoculated genotypes. At least three independent experiments were performed, with $n = 9$ assays each. Circles correspond to experimental replicates. Cross (x) refers to the mean (arithmetic average) of the data. The median line represents the median. Letters indicate significant differences (ANOVA and Tukey's HSD test; $p < 0.05$).

3.3. *V. sativa* Genotype Impacts on Shoot and Root Biomass

Plant biomass is strongly influenced by the genotype, both in the shoot and the root, as observed in non-inoculated control plants supplemented with nitrogen (Supplementary Figure S2). To evaluate the specific impact of symbiosis and BNF on plant biomass, we analyzed the shoot and root biomass by fresh weight measurement in nodulated plants across different genotypes. Accessions 502, 506, 512, and 521 exhibited the highest shoot biomass (Figure 3A), while accessions 512 and 515 showed the greatest root biomass (Figure 3B). There are large differences between varieties. Thus, the mean aerial fresh biomass of genotype 502 is 2.9-fold higher than that of genotype 515, which has the lowest mean aerial fresh biomass (Figure 3A). Comparable trends were observed in the analysis of dry weight biomass (Supplementary Figure S3), supporting the influence of genotype on biomass accumulation under symbiotic conditions. Interestingly, accession 512 exhibited the highest shoot biomass under both inoculated and non-inoculated conditions. This effect is not solely attributable to the genotype itself, as nodulation specifically exerts a differential impact across the various genotypes. Thus, genotypes 502, 506, and 521, which display high shoot biomass production in nodulated plants, are less productive under non-inoculated conditions compared to the other genotypes under the same conditions (Figure 3A; Supplementary Figures S3 and S5). Regarding the root system, the different genotypes, both under inoculated and non-inoculated conditions, generally exhibit lower statistical differences compared to those observed in shoot biomass, with the exception of genotype 515. The mean root fresh biomass of genotype 515 was 3.1-fold higher than that of genotype Aitana, which had the lowest mean root biomass. The behavior of genotype 515 is particularly noteworthy; while it exhibits the highest root biomass under nodulated conditions (Figure 3A; Supplementary Figure S6), it shows the lowest root development in the absence of inoculation (Supplementary Figure S2B).

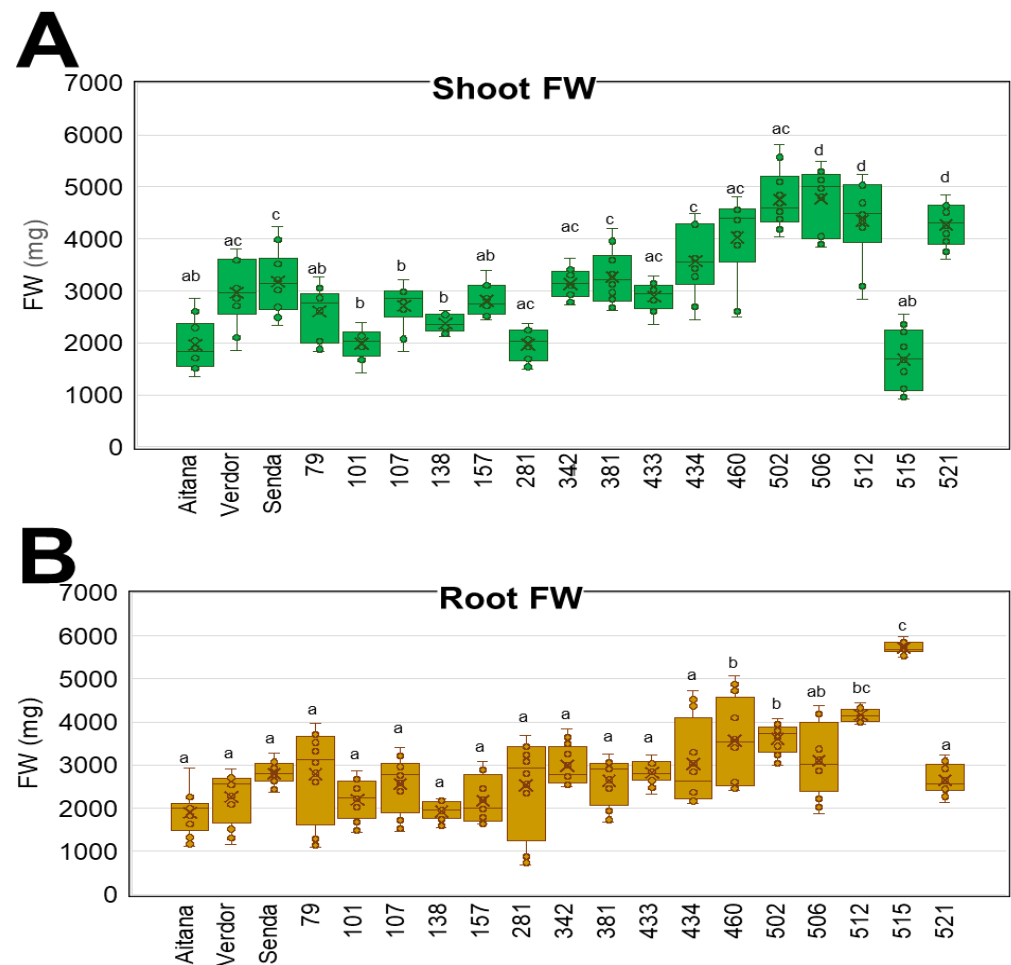


Figure 3. Fresh weight of the shoot (A) and root (B) biomass per plant of different common vetch inoculated genotypes. At least four independent experiments were conducted, with $n = 4$ plants each. Letters indicate significant differences (ANOVA and Tukey's HSD test; $p < 0.05$).

3.4. *V. sativa* Genotype Impacts on Biological Nitrogen Fixation

Inoculated plants are expected to exhibit increased nitrogenase activity and enhanced biomass development, resulting in a measurable impact on the total nitrogen content, especially in the shoot. To evaluate this effect, we analyzed total shoot nitrogen across the different genotypes of the nodulated plants (Figure 4A). Notable differences among genotypes on total nitrogen percentages were observed, despite the relatively small overall variation of this parameter. Thus, accessions 502, 506, 512, and 521 exhibited the highest nitrogen percentages in shoot tissue, with up to a 38% increase observed between the genotype with the lowest nitrogen percentage (genotype 79) and the one with the highest (genotype 502). Nitrogen percentage and total shoot biomass were used to estimate the total content of shoot nitrogen (Figure 4B). We observed that the genotype had also a substantial impact on total nitrogen accumulation per plant, with the highest values associated with genotypes 502 and 506. Thus, the differences in mean nitrogenase activity are more than 4-fold between the genotype with the lowest total N content (Aitana) and the genotype with the highest (506).

Notably, genotype 502, which also has the highest nitrogenase value and shoot biomass, accumulated more shoot nitrogen than any other genotype, emphasizing its superior performance under symbiotic conditions. Total carbon levels were also determined in different genotypes; however, only accession 502 showed major differences compared to the other genotypes (Supplementary Figure S4).

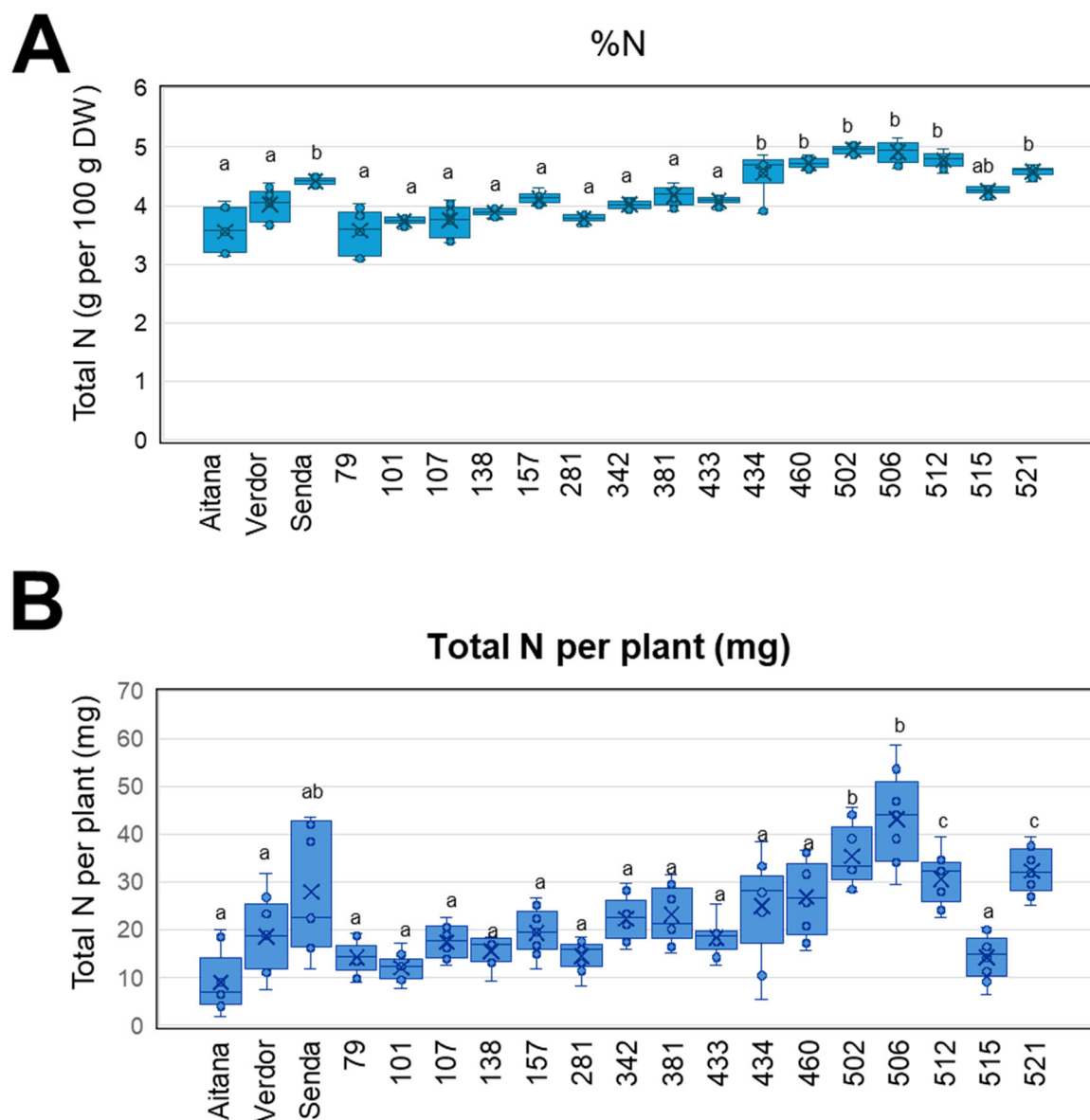


Figure 4. Total nitrogen content. Box plots showing the nitrogen content as the percentage of dry weight (A) or nitrogen content per plant (B) in the shoot parts of different common vetch inoculated genotypes. At least three independent experiments were conducted, with $n = 9$ assays each. Letters indicate significant differences (ANOVA and Tukey's HSD test; $p < 0.05$).

3.5. Parameter Correlation

In this study, over 10 parameters related to BNF and plant development were analyzed, including root and shoot biomass, nitrogenase activity, nodule-associated traits, and carbon/nitrogen (C/N) content. These measurements were conducted across 19 different accessions. Due to the size and complexity of the generated dataset, multivariate analysis and Principal Component Analysis (PCA) were employed to explore correlations among the various parameter analyzed (Figure 5). Interestingly, nodule biomass, but not nodule number, is directly correlated with nitrogenase activity, total nitrogen percentage, and total nitrogen content per plant. A relevant correlation was also observed among nodule biomass and total carbon content. As expected, fresh weight- and dry weight-related parameters were correlated (Figure 5A,B; Supplementary Table S2). PCA indicates that PC1 (Principal Component 1) and PC2 (Principal Component 2) explained

cumulatively more than 66% of the total variance. PC1 explains 43.0% of the variance, and PC2 explains 23.3% of the variance.

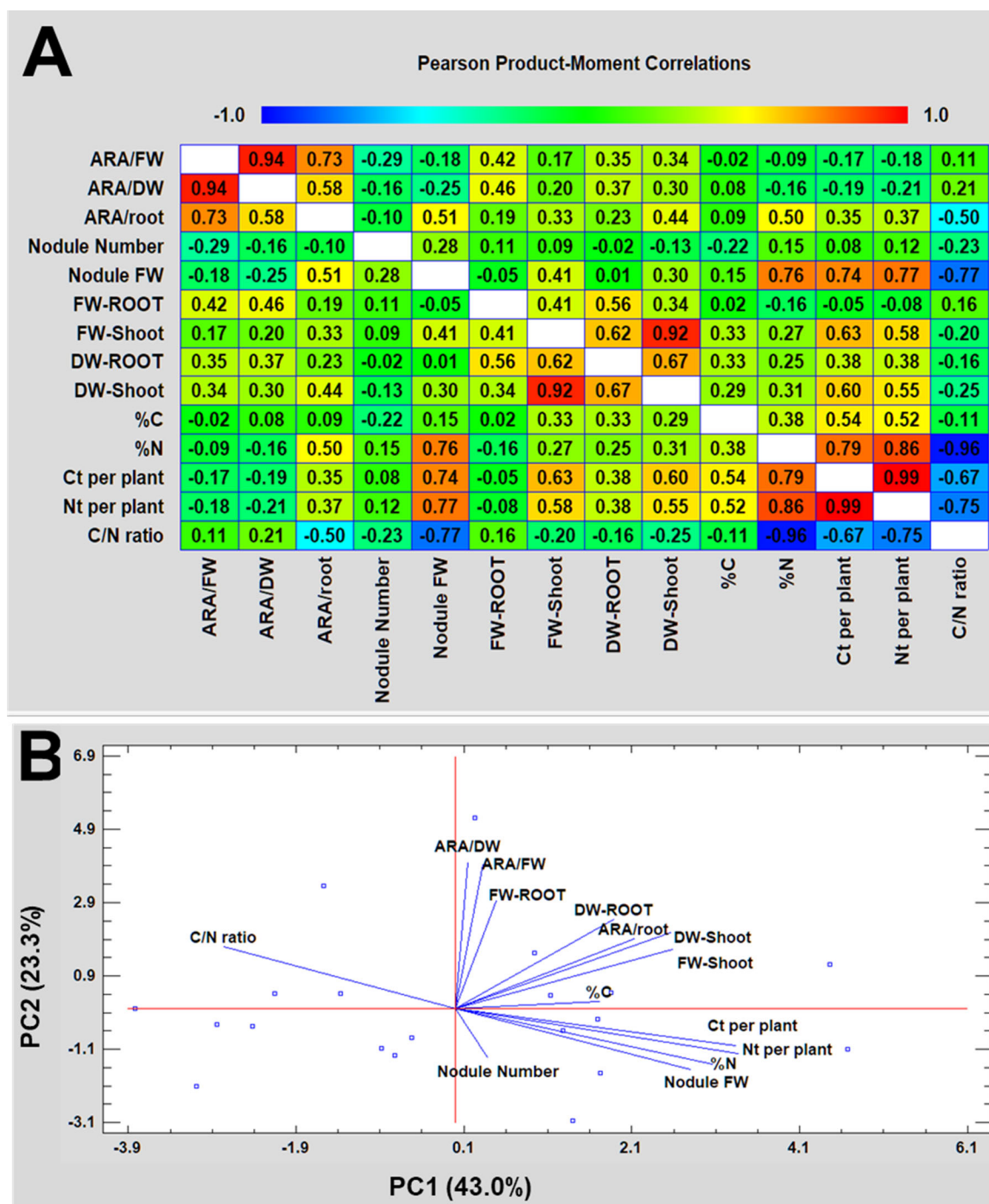


Figure 5. Multivariate analysis and Principal Component Analysis (PCA). **(A)** Pearson's Product Moment correlations among the different parameters analyzed in this work. **(B)** Principal Component Analysis of indicated parameters. Blue-points indicate different analyzed genotypes. ARA/FW: nitrogenase activity normalized per mg of nodule fresh weight; ARA/DW: nitrogenase activity normalized per mg of nodule dry weight; ARA/root: nitrogenase activity normalized per root unit; Ct: total C content per plant; and Nt: total N content per plant.

4. Discussion

Legumes are known for their ability to establish a mutualistic symbiotic relationship with *Rhizobium* bacteria, stimulating BNF and thus reducing dependence on synthetic

fertilizers [23–26]. Optimizing BNF in legumes such as *V. sativa* is therefore a critical strategy for promoting more environmental farming systems. While most research to date has focused on improving rhizobial strains to enhance nitrogen fixation [13,27], the influence of the host plant genotype has received less attention. In this study, we aimed to explore the impact of host genetic diversity on nodulation and nitrogen fixation in common vetch, a legume of both agronomic and ecological relevance. Our results revealed important variability among different *V. sativa* genotypes in nodulation capacity, nitrogenase activity, nitrogen fixation potential, and biomass development. These differences suggest that host genetic factors play an essential role in symbiotic efficiency of this species, reinforcing the relevance of selecting optimal host genotypes as a strategy to enhance BNF performance. Our results are in agreement with studies in other legumes such as *Medicago truncatula*, soybean, and lentil where the host genotype has been shown to influence both nodulation and nitrogen fixation [28–30].

Nodulation, a critical step in BNF, varied considerably among the genotypes. While accession 512 produced the highest number of nodules, others like 434, 506, and 521 showed the highest nodule biomass, and accessions 434, 460, and 502 showed the highest nitrogenase activity. These results indicate that the nodule number does not necessarily correlate with nodule biomass or activity. In fact, genotypes such as 434 and 506 exhibited great nodule biomass without having the highest nodule counts, suggesting that nodule size and function, rather than quantity, are more critical determinants of BNF efficiency. Although some studies have observed a connection between nodule number and nodule biomass, the relationship is not consistently strong, reinforcing the idea that nodule count alone is not a reliable indicator of symbiotic effectiveness or total nodule biomass [31]. Our measurements were intentionally conducted at 32 days after inoculation, a time point chosen to ensure that nodules were fully established and mature in all genotypes, but without entering the phase of nodule senescence or experiencing any loss of nitrogenase activity. This timing was selected to capture optimal nodule function and avoid the confounding effects associated with both early symbiotic development and the onset of nodule degradation. One of the most notable insights in this work is the strong correlation between nodule biomass and both nitrogen fixation capacity and shoot biomass. This suggests that selecting genotypes with increased nodule size or weight could be a useful strategy to enhance nitrogen acquisition and, consequently, plant productivity. These results are consistent with those by Kurdali et al. (1997) [32], who demonstrated that lentil genotypes with greater nodule biomass also had higher nitrogen fixation rates and produced more shoot biomass under greenhouse conditions. Our results support previous research emphasizing that total BNF in legumes is determined by the combined effects of nodule number, size, and activity [33]. Similar observations have been reported in other legume species, where the quality of nodules and the nitrogenase efficiency are often more critical than the total nodule count [14]. For example, in common bean and pea, a higher number of nodules does not necessarily translate into greater nodule biomass or BNF efficiency [34,35]. The variations observed in those studies were primarily attributed to differences in *Rhizobium* strains used for inoculation rather than to natural genetic variation among plant genotypes. However, the process of BNF through the establishment of symbiosis is a highly complex process that can be altered by a variety of factors. Thus, genotypes 434, 460, and 502 had the highest nitrogenase activity, but none of them had the highest total nitrogen content. In contrast, 281, 342, 381, and 433 had low nitrogenase activity, yet the nodule number and nodule weight were not the lowest among all the genotypes tested. This apparent discrepancy may be due to the multiple and complex factors that affect the process of BNF through the establishment of symbiosis. This may be primarily due to the existence of non-functional or senescent nodules that are inactive

for BNF as previously described in alfalfa [36], but also to potential differences in nitrogen utilization efficiency (NUE) due to differences among genotypes that may be less efficient at transporting or assimilating fixed nitrogen from nodules into plant tissues, leading to lower total nitrogen content despite high nitrogenase activity or the fact that nitrogenase activity at the time of measurement does not necessarily indicate sustained high activity throughout the plant's development. Total N content integrates N accumulation over time, so accessions with late or short-lived peaks in nitrogenase activity may accumulate less total nitrogen [37].

Our data emphasize the value of considering host genetic variation as a key determinant of symbiotic performance. Both genetic and environmental factors influence nodule development, and metrics such as nodule mass and metabolic activity offer a more accurate assessment of nitrogen-fixing capacity. Furthermore, we observed a genotype-dependent correlation between nodule fresh weight and shoot biomass. This relationship is consistent with previous results which showed that shoot biomass is more strongly correlated with the biomass of symbiotic nodules than with their number [31,35]. The observed differences among inoculated genotypes in shoot and root biomass suggest that genetic factors affect plant growth and development, even under controlled environmental conditions. Accessions with the greatest shoot biomass (502, 506, 512, and 521) and root biomass (512 and 515) may have enhanced nutrient uptake efficiency or photosynthetic capacity, which could be further explored in future studies. Root biomass was significantly higher in non-inoculated plants supplied with NH_4NO_3 compared to nodulated plants grown in nitrogen-free conditions. This difference is likely due to the high energetic cost of maintaining BNF, which can limit root development in the absence of exogenous nitrogen, as analyzed in previous studies [38].

The analysis of BNF and nitrogen content in the shoot biomass supports the notion that specific vetch genotypes are more efficient in nitrogen accumulation. Notably, genotypes 434 and 460 exhibited higher nitrogen accumulation and nitrogenase activity, marking them as promising candidates for effective nitrogen fixation. The variation in nitrogen content among these genotypes may be due to differences in nodule activity or capacity of the plant to assimilate nitrogen into its tissues [12]. Our data are also coherent with previous studies that suggested that different host genotypes may exhibit different rhizobial compatibility, affecting both nodule formation and nitrogen fixation efficiency [11,13]. These differences in nitrogen uptake and storage and the selection of outstanding genotypes more effective in these aspects have important implications for optimizing *V. sativa* as a nitrogen-fixing cover crop. Increased nitrogen fixation enhances soil fertility and reduces the reliance on synthetic fertilizers, making agricultural systems more sustainable.

These results emphasize the complex interaction between host genotype, rhizobial strain, and environmental factors in regulating BNF. Importantly, our results suggest that certain genotypes, such as accessions 434 and 502, may produce fewer but more functionally efficient nodules, leading to enhanced nitrogen fixation rates, a trait potentially more beneficial for agronomic practices. Nitrogenase activity, a direct measure of BNF, varied markedly among the genotypes. Accessions 434, 460, and 502 exhibited the highest nitrogenase activity, indicating their superior ability to convert atmospheric nitrogen into plant-available forms, mainly ammonium and nitrates. Finally, the total nitrogen content in plant tissues is used as the most integrative measure of BNF success. The variability found in shoot nitrogen content further confirms that some genotypes are more efficient at incorporating fixed nitrogen into their biomass. Selecting such genotypes could be particularly valuable in low-input or organic systems, where synthetic nitrogen availability is limited. Our results showed that the genotype substantially influenced the total nitrogen per plant, with genotypes 502 and 506 accumulating more shoot N than any other genotypes. In

contrast, commercial cultivars used in this work such as Aitana, Senda, and Verdor showed minimal nitrogenase activity and nitrogen accumulation, indicating that BNF traits may not have been prioritized in their selection or breeding. This emphasizes the potential value of the genotypes identified in these studies. Interestingly, genotype 502 not only showed high levels of nitrogen content, nitrogenase activity, and shoot biomass but also accumulated the highest total carbon (C) compared to other genotypes (Figure S4). Our observations suggest that genotype 502 is especially effective at nitrogen acquisition and utilization, which probably plays a key role in promoting better plant growth. The high total carbon content further supports this idea, as it could indicate greater accumulation of structural biomass or a stronger photosynthetic capacity, both signs of increased productivity.

Recent studies have emphasized an additional ecological benefit of these symbiotic systems by conferring tolerance to abiotic stresses such as drought [21]. *V. sativa* and *Pisum sativum* *Rhizobium* symbiosis conferred drought tolerance compared to nitrogen-supplemented controls. Nodulated plants survived better under water-limited conditions, retained more water, and showed lower levels of stress and oxidation markers. Transcriptomic analyses revealed that these plants activate unique molecular responses to drought, involving both general and symbiosis-specific pathways [21]. Similarly, other legumes, such as faba beans, further support the role of rhizobial interactions in improving resilience to abiotic stress [39,40]. These results highlight the growing relevance of *Rhizobium*–legume symbiosis, not only as a sustainable source of nitrogen but also as a biological strategy to enhance plant resilience under water-limited conditions. As droughts become more frequent and intense due to climate change, the ability of symbiotic systems to confer drought tolerance adds considerable value to their agronomic and ecological relevance.

Selecting genotypes with high nodulation capacity, nitrogenase activity, and nitrogen accumulation can enhance BNF efficiency and reduce the need for synthetic fertilizers. This strategy aligns with the goals of ecological intensification, which focus on improving crop production while minimizing environmental impacts. The diversity of *V. sativa* in cover cropping systems can enhance soil fertility and promote sustainable agricultural practices. Our study emphasizes the importance of integrating genetic diversity into future breeding strategies. Beyond traditional agronomic traits like yield and drought tolerance [16], it is important to also consider biological nitrogen fixation (BNF) efficiency as a key selection criterion. The characterization and exploitation of the genetic resources available in genebanks, such as the one maintained by CRF, INIA-CSIC, may allow us to identify or develop new cultivars with enhanced nitrogen fixation capabilities, thereby improving the sustainability of legume-based cropping systems.

Future Directions

Our results offer novel perspectives into the role of the host genotype on symbiotic interaction and BNF efficiency. However, several important questions remain to be explored. Thus, future research could explore the genetic factors and molecular mechanisms underlying the genotype-dependent differences in nodulation and nitrogen fixation. Transcriptomic analysis, quantitative trait loci (QTL) mapping, and genome-wide association studies (GWAS) could be used to identify candidate genes involved in these processes. Further studies are needed to investigate the specificity of the interaction between the host genotype and the rhizobial strain in determining BNF efficiency. Understanding the molecular mechanisms behind host–rhizobia specificity is essential to developing more effective inoculation strategies. This can help optimize legume-based nitrogen fixation, supporting long-term soil health and reducing reliance on chemical inputs. Examining how environmental factors, like soil composition and climatic conditions, interact with the host genotype to influence BNF will provide a more comprehensive understanding of the

factors driving the efficiency of nitrogen fixation in vetch. This study serves as an initial exploratory analysis of the role of BNF among a diverse set of *V. sativa* genotypes under controlled symbiotic conditions. By employing a single, well-characterized *Rhizobium* strain, we were able to focus specifically on the impact of host plant genetic diversity, minimizing the complex effects from microbial variability. While our findings highlight the importance of the host genotype in BNF, we must be aware that future research should incorporate multiple rhizobial strains to further elucidate host–bacteria specificity and optimize legume–rhizobium combinations. Our work has been carried out under controlled experimental conditions, considering the enormous difficulty of performing this work under field conditions. Translating these results to agronomic practice will require field trials that consider the complexity of natural environments, including competition among native rhizobia, soil composition, and climatic factors. Addressing these challenges will be essential for fully realizing the potential of genetic diversity to improve nitrogen fixation in legume-based agricultural systems. In any case, we strongly believe that our work aims to contribute an additional factor to be considered in breeding programs: the selection of accessions with good BNF ability for optimizing symbiotic interactions.

5. Conclusions

Our research emphasizes how exploring genetic variation in hosts can be a valuable approach to improving the effectiveness of symbiotic nitrogen fixation. By integrating this variability into legume improvement programs, we can support both productive and environmentally sustainable agricultural practices. Nevertheless, the complexity of symbiotic systems requires us to consider the role of different bacterial strains, as well as other environmental factors not addressed in this study, when interpreting these results. Further research is necessary to elucidate these factors and limitations and to improve our understanding of the complexity of symbiotic nitrogen fixation systems.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy15061479/s1>. Supplementary Figure S1. Illustration and timeline of the assays performed for inoculation treatment. Supplementary Figure S2. Fresh (A,B) and dry weights (C,D) of shoot (A,C) and root (B,D) biomass per plant of different common vetch genotypes that were not inoculated (NI; N-fed supplemented with 10 mM NH_4NO_3). At least three independent experiments were performed, with $n = 4$ plants each. Letters indicate significant differences (ANOVA and Tukey's HSD test; $p < 0.05$). Supplementary Figure S3. Dry weight of shoot (A) and root (B) biomass per plant of different common vetch inoculated genotypes. Supplementary Figure S4. Total carbon content of different common vetch inoculated genotypes. Supplementary Figure S5. Multivariate analysis and Principal Component Analysis (PCA). (A) Pearson's Product Moment correlations among biomass parameters analyzed in this work on inoculated and non-inoculated plants. (B) Principal Component Analysis of indicated parameters. Supplementary Figure S6. Representative phenotypic images from a standard assay showing (A) whole-plant appearance and (B) root morphology and nodule images from the analyzed genotypes. Images correspond to inoculated plants at 32 dai. Supplementary Table S1. Accession numbers and passport data of the common vetch varieties that have been analyzed in this work. Supplementary Table S2. Table of The StatAdvisor component weights for the different parameters, showing the main contributions. Bold indicates the highest values.

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Data Availability Statement: Details regarding the data supporting reported results can be found in the Supplementary Materials and in the publicly CRF repository: <https://eurisco.ipk-gatersleben.de/>, accessed on 15 June 2025.

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Abbreviations

The following abbreviations are used in this manuscript:

ARA	Acetylene Reduction Assay
BNF	Biological Nitrogen Fixation
C/N	Carbon/Nitrogen
CRF	National Spanish Plant Genetic Resources Center
GWAS	Genome-Wide Association Studies
NUE	Nitrogen Utilization Efficiency
PCA	Principal Component Analysis
PC	Principal Component
QTL	Quantitative Trait Loci

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