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Rhizobium tropici and Riboflavin Amendment Condition Arbuscular Mycorrhiza Colonization in *Phaseolus vulgaris* L.

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Abstract: *Phaseolus vulgaris* L. (Fabaceae) forms symbioses with arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing rhizobia (NFB). The tripartite relationship uses molecular signals to establish intracellular symbioses in roots. The goal of this study was to determine if *Rhizobium tropici* CIAT 899 and exogenous riboflavin (vitamin B2) have an effect on AMF species selection and root colonization of *P. vulgaris*. Using SSU rRNA fragment amplification of DNA extracted from *P. vulgaris* roots, we found that the presence of *R. tropici* altered the relative distribution of AMF species. *Dominikia bernensis* (Ohel) was the most abundant AMF species in *P. vulgaris* roots but when *R. tropici* was co-inoculated, *Glomus* species dominated. Rhizobacteria such as *R. tropici*, secrete riboflavin and could affect AMF symbiosis. Addition of 50 µM riboflavin to *P. vulgaris*, increased plant growth (28%), dry nodule weight (18%), AMF colonization (248%) and mycorrhizal vesicle frequency (56%) in bean roots. 3.12 and 12.5 µM riboflavin favored the presence of *Glomus macrocarpum* in *P. vulgaris* roots. This work provides the basis to further study of rhizobial and mycorrhizal co-inoculation of *Phaseolus vulgaris* bean.



Citation: Banuelos, J.; Martínez-Romero, E.; Montaña, N.M.; Camargo-Ricalde, S.L. *Rhizobium tropici* and Riboflavin Amendment Condition Arbuscular Mycorrhiza Colonization in *Phaseolus vulgaris* L. *Agronomy* **2023**, *13*, 876. <https://doi.org/10.3390/agronomy13030876>

Academic Editor: Jesús Montiel

Received: 16 January 2023

Revised: 4 March 2023

Accepted: 5 March 2023

Published: 16 March 2023



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Keywords: arbuscular mycorrhiza; species composition; riboflavin (vitamin B2); nitrogen fixing; vesicles; *Dominikia*; *Glomus macrocarpum*; *Rhizophagus*

1. Introduction

Phaseolus vulgaris L. (Fabaceae) is a widely used legume for human nutrition and can form symbiotic associations with arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing rhizobacteria (NFR) [1]. Among the bacterial species that form root nodules in *P. vulgaris*, *Rhizobium etli*, *Rhizobium phaseoli* and *Rhizobium tropici* have been extensively studied [2,3]. *R. tropici* has outstanding stress resistance [4] and has been successfully used as a *P. vulgaris* inoculant in South America [5]. Diverse AMFs have been used as inoculants in agriculture [6] to promote nutrient mobilization [7,8], mineral uptake [9] and general plant growth [10]. To some extent, AMF-inoculated plants had enhanced photosynthesis, and upregulate osmoprotectants [11]. Among arbuscular mycorrhizal fungi, the model species *Rhizophagus irregularis* and *Funneliformis mosseae* are among the most widely used species [11] that significantly increase yields in globally important crops [12,13], including *P. vulgaris* [14]. In five different legumes inoculated with *Funneliformis mosseae*, the highest spore number, nodule weight and percentage of AMF colonization were found in *Vigna radiata* L. (Wilczek), compared to other host plant such as *Glycine max* L. [15]. In different *Coffea arabica* cultivars, the combination of three selected AMF species was more efficient than inoculation with a single species, although the effect depended on the *C. arabica* cultivar [16]. Hosts may find their most suitable symbiont by selecting it from

a mycorrhizal mixture. Native AMF communities have been shown to interact positively with the plant host under stress conditions, in contrast to introduced AMF [17].

Inoculation with rhizobia has been practiced for more than a hundred years, while the coinoculation of rhizobia and mycorrhiza is more recent and leads to higher yields in compatible crops [18]. Coinoculation generally leads to increased AMF colonization, but depends on the AMF species [19]. In clover, dual inoculation of *Rhizophagus intraradices* and *Rhizobium trifolii* showed that the latter stimulated mycorrhizal colonization, and both had an additive effect on nitrogen (N) metabolites and N assimilation [20]. However, the presence of mycorrhizal and rhizobial symbionts in legume roots was not always beneficial for the plant [21]. The increased N assimilation could allow the plant to host other beneficial organisms, especially under unfavorable conditions [22]. The interaction between mycorrhizal fungi and rhizobial symbionts with the host could affect the efficiency or evolution of each symbiosis, and the challenge in a tripartite symbiosis is to understand how the taxonomic diversity of AMF interacts with symbiotic nitrogen-fixing bacteria and how it affects the host. It has been shown that AMF colonization in cowpea (*Vigna unguiculata* (L.) Walp.) AMF species composition in roots was altered when *Sinorhizobium meliloti* (Dangeard) was present [19].

To achieve the highest efficiency of AMF and NFR, the selection of appropriate AMF species compatible with NFR is essential [23]. Since there are more than 400 AMF species, the use of inocula with multiple strains could increase the interaction with the NFR to select the AMF species that is functional in the tripartite interaction. The response of AMF can be related to the rhizobial strains that are specifically associated with the plant. Nitrogen-fixing bacteria may stimulate and enhance mycorrhizal colonization even before nodule formation [24]. It is necessary to develop a sound strategy to increase the efficiency in the use of multispecies inoculants.

Mycorrhizal symbiosis is evolutionarily older than rhizobial symbiosis and it is considered the “mother of plant symbioses” [25,26]. Both rhizobia and mycorrhiza make use of a common genetic symbiotic pathway in legume plants [27]. This shared signaling pathway affects both symbionts; for example, AMF colonization may not occur in some non-nodulating legume mutants [28], as AMF may rely on the same signals as rhizobia to facilitate infection. Both mutualistic associations are present in the rhizosphere, and despite their apparent differences, several molecular signals are recruited by the plant cell for both symbioses [29]. Moreover, the Myc factor of mycorrhiza and the Nod factors of rhizobia have similar chemical structures [30] and the application of external Nod factors increases the mycorrhizal colonization [31].

Riboflavin (vitamin B2) is the precursor of the electron-carrying coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). These coenzymes are involved in metabolic processes including energy production and act as antioxidants. Riboflavin and lumichrome can increase shoot growth and CO₂ availability in roots [32]. Riboflavin is produced by some rhizobacteria [33] that require exogenous CO₂ for nodule growth [34]. Riboflavin overproducing *S. meliloti* strains exhibited 55% higher root colonization efficiency [35] and nanomolar amounts of riboflavin can stimulate alfalfa colonization by *S. meliloti* [36]. Riboflavin from rhizobia could potentially benefit not only plants, but also other symbionts such as AMF because riboflavin promotes hyphal growth in AMF [37] and stimulates rhizobial nodulation [38]. The extent to which riboflavin may affect AMF development during coinoculation with NFR has not yet been determined. The aim of this research was to explore if *Rhizobium tropici* CIAT 899 and exogenous riboflavin supplied to plants have an effect on the selection of AMF species (from a fungal mixture) by *Phaseolus vulgaris*.

2. Materials and Methods

2.1. Experimental Design

Two experiments were conducted under greenhouse conditions. In the first, a completely randomized experiment, with four treatments and four replicates was conducted.

P. vulgaris seedlings were inoculated with an AMF mixture and were irrigated weekly for 35 days with four concentrations of riboflavin (50, 25, 12.5 and 3.12 μM).

In the second experiment, a fixed three-factorial design was established: mycorrhized plants, *R. tropici* CIAT 899 and with or without riboflavin with eight replicates. Based on the results of the first experiment, a riboflavin concentration was selected that had a significant effect on AMF. Treatments without AMF or riboflavin were controls. Both experiments were conducted under greenhouse conditions (relative humidity average 78%, average temperature 20 °C) at the Faculty of Agricultural Sciences, Universidad Veracruzana (N 19° 30' 54"-W 96° 55' 05").

2.2. Soil

The soil used in the pots had the following characteristics: P 63.5 (%); available phosphorus (P), 40.52 mg kg⁻¹ (Olsen); ammonium (NH₄-N), 34.28 mg kg⁻¹ (salicylate method); nitrates (NO₃-N), 36.28 mg kg⁻¹; pyrophosphate-extractable iron (Fe) (PeF), 1016 mg kg⁻¹; organic matter (OM), 7.13% (Walkley-Black); organic carbon (OC), 4.14% (Walkley-Black); sodium (Na), 0.029 mol kg⁻¹ (atomic absorption spectrometry); calcium (Ca) 0.0173 mol kg⁻¹ (atomic absorption spectrometry) and magnesium (Mg) 0.0327 mol kg⁻¹ (atomic absorption spectrometry). The pH was of 6.7 and was obtained in a suspension of soil:deionized water (1:2 w/v). Soil analysis was performed by the Soil Analysis Laboratory of INECOL, Xalapa, Mexico. The soil used was sterilized by autoclaving to prevent competition from native microbes [11]. The soil was purchased from "Viversos El Olmo", a plant store located in Xalapa, Veracruz, Mexico.

2.3. Plant Material

Phaseolus vulgaris L. cv Negro Jamapa is a bean variety released in Veracruz, Mexico, sixty years ago [39]. Negro Jamapa bean seeds were surface-disinfected [40] by treating them with 70% ethanol for 1 min, followed by 1.2% sodium hypochlorite for 15 min, rinsing four times with sterilized water, once with 2% sodium thiosulfate and a final rinsing with water. Seeds were germinated in sterilized quartz sand at total water-holding capacity (42.7%) before being planted in 1 L pots (13 cm diameter) with autoclaved soil (120 °C for 40 min).

2.4. Riboflavin (Vitamin B2) Concentrations

A stock solution of 50 μM riboflavin from Sigma-Aldrich (BioReagent for cell culture) was prepared in deionized sterile water by stirring for 10 min in the dark and stored at 4 °C before to use. Dilutions of the stock solution were made with deionized sterile water to obtain 50, 25, 12.5 and 3.12 μM . 50 mL of riboflavin was added to the plant 2 cm from the stem at night to prevent riboflavin degradation. Five days after mycorrhizal inoculation and every five days until harvest. The control treatments were irrigated with sterile deionized water. Initially, these four riboflavin concentrations were tested and then only 50 μM was used in the presence of both symbionts.

2.5. Arbuscular Mycorrhizal Fungi

The AMF mixture was provided by the Laboratory of Beneficial Organisms, Faculty of Agricultural Sciences of Universidad Veracruzana, Mexico. The inoculant used has been tested as highly effective in pineapple [41], cover crops [42], avocado [43], and it is commercially available as Rhizofermic. The inoculant had only been identified morphologically and here, we identified its content for the first time by SSU rRNA sequence analysis and 14 species were identified; only the *Acaulospora* species could not be determined (Table 1).

Table 1. List of arbuscular mycorrhizal fungi (AMF) species contained in the inoculum identified by SSU rRNA sequence analysis.

Family	Species	Synonym
Archaeosporaceae	<i>Archaeospora trappei</i> (R.N. Ames and Linderman) J.B. Morton and D. Redecker	<i>Acaulospora trappei</i>
Acaulosporaceae	<i>Acaulospora</i> sp	
Gigasporaceae	<i>Dentiscutata savannicola</i> (R.A. Herrera and Ferrer) C. Walker and A. Schüßler <i>Gigaspora rosea</i> T.H. Nicolson and N.C. Schenck	<i>Gigaspora savannicola</i> ; <i>Fuscutata savannicola</i> ; <i>Scutellospora savannicola</i>
	<i>Cetraspora pellucida</i> (T.H. Nicolson and N.C. Schenck) Oehl, F.A. Souza and Sieverding	<i>Scutellospora pellucida</i>
Diversisporaceae	<i>Diversispora varaderana</i> J. Błaszowski, G. Chwat, Kovács and Góralska	
Claroideoglomeraceae	<i>Claroideoglomus etunicatum</i> (W.N. Becker and Gerd.) C. Walker and A. Schüßler	<i>Glomus etunicatum</i> ; <i>Entrophospora etunicata</i> [44]
Glomeraceae	<i>Dominikia bernensis</i> Oehl, Palenz., Sánchez-Castro, N.M.F. Sousa and G.A. Silva <i>Dominikia lithuanica</i> J. Błaszowski, G. Chwat, A. Góralska	
	<i>Rhizophagus aggregatus</i> (N.C. Schenck and G.S. Sm.) C. Walker	<i>Glomus aggregatum</i> ; <i>Rhizoglomus aggregatum</i>
	<i>Glomus macrocarpum</i> T.H. Nicolson and Gerd	<i>Endogone macrocarpa</i>
	<i>Funnelformis mosseae</i> T.H. Nicolson and Gerd	
	<i>Kamienskia bistrata</i> J. Błaszowski, G. Chwat and Kovács	
	<i>Rhizophagus irregularis</i> Błasz., Wubet, Renker and Buscot (C. Walker and A. Schüßler comb. Nov) <i>Rhizophagus neocaledonicus</i> D. Redecker, Crossay and Cilia	

The inoculum was reproduced in *Brachiaria decumbens* Stapf (Poaceae) trap plants for 90 days, in quartz sand autoclaved and irrigated with sterile deionized water. Irrigation was suspended for 30 days to induce sporulation. The roots were analyzed for the percentage of AMF colonization obtaining 80% [45,46]. The sand contained 4658 spores per 100 g^{−1} and they were isolated by wet sieving and decantation followed by centrifugation in 40% sucrose solution [47]. Plants were inoculated with AMF five days after seed germination and before transplanting. We used a mixture of 10 g of roots and quartz sand with spores (1:9) for a total of 419 spores per germinated seed. For non-AMF-inoculated treatments, the inoculum was prepared as follows: 500 g of the inoculum was autoclaved for 45 min at 120 °C and 1.05 kg cm^{−2}.

2.6. Bacterial Strain and Preparation of Inoculum

Rhizobium tropici strain CIAT 899 [48] was obtained from the culture collection of the Center for Genomic Sciences, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México. The strain was cultured in sterilized PY broth (5 g peptone, 3 g yeast extract and 0.6 g CaCl₂ per liter) for 24 h at 30 °C with constant shaking (250 rpm). Cultures were centrifuged for 3 min at 4000 × g and the pellets were washed with MgSO₄ 10 mM [49]. The final concentration of bacteria was adjusted to 10⁸ cells mL^{−1} and 2 mL was inoculated to each germinated seed in each pot. A second inoculation was performed after one week.

2.7. Plant Analysis

All plants were harvested 35 days after inoculation. The soil was washed off from the roots and they were placed between 4 paper towels to remove the excess of water. The plants were weighted separately by roots and shoots. Subsamples of roots (200 mg) were collected for microscopic examination of AMF structures, and the remaining roots and shoots were separated and frozen at $-20\text{ }^{\circ}\text{C}$ for DNA extraction. A trypan blue solution was used to visualize the fungal structures in the root subsamples [46], and the percentage of AMF root colonization was determined [50].

2.8. Arbuscular Mycorrhizal Fungi (AMF) DNA Extraction and Amplification

Three random subsamples per root system were pooled by treatment, frozen at $-80\text{ }^{\circ}\text{C}$ with liquid N_2 and ground to a fine powder using a sterile mortar and pestle before DNA extraction [51,52]. DNA was extracted from the root samples using the Qiagen PowerSoil DNA extraction kit (Hilden, Germany), following the manufacturer's instructions, obtaining 50 μL of elution buffer containing total gDNA. The quantity and quality of the DNA extracts were assessed on 1.5% agarose gel before further processing.

PCR, cloning and sequencing were performed by MACROGEN, Korea. PCR amplifications were performed individually from the DNA extracted from roots using primer pairs NS1 (GTAAGTCATATGCTTGTCTC) and NS41 (CCCGTGTGAGTCAAATTA) to amplify a 1.2 kb fragment [53]. The PCR products were used as templates in a subsequent nested PCR. The primer set for the second round used AM1 (ATCACTTTC-GATGGTAGGATAGA) and AM2 (GAACCCAAACACTTTGGTTTCC) primers [54], yielding a product of 800 bp targeting the variable regions of the nuclear 18S rRNA gene (SSU). Amplicon libraries were then prepared using the Nextera XT DNA library preparation kit and sequenced as single end reads using Illumina platform [55]. For the first round of PCR, the conditions were as follows: $94\text{ }^{\circ}\text{C}$ for 3 min; 25 cycles of $94\text{ }^{\circ}\text{C}$, 3 min; $60\text{ }^{\circ}\text{C}$, 1 min; $72\text{ }^{\circ}\text{C}$, 1 min; final extension at $72\text{ }^{\circ}\text{C}$ for 7 min. For the second round, the conditions were $94\text{ }^{\circ}\text{C}$ for 3 min; 32 cycles of $94\text{ }^{\circ}\text{C}$, 1 min; $55\text{ }^{\circ}\text{C}$, 1 min; $72\text{ }^{\circ}\text{C}$, 1 min and final extension at $72\text{ }^{\circ}\text{C}$ for 10 min. Raw data were uploaded to SRA database.

2.9. Bioinformatic Analysis

The quality of the raw sequences was checked using FASTQC v0.11.8 [56] and raw demultiplexed raw sequences were processed using QIIME2 package version 2021.8 [57]. Sequences were denoised, dereplicated and filtered for chimeras using the DADA2 plugin [58]. Sequences were trimmed to include only bases with quality values > 20 . The first 21–24 nucleotides were trimmed. ASVs with frequency less than 0.1% were removed. For taxa comparisons, we used the QIIME2 q2-feature-classifier plugin and the naive Bayes classifier trained on the MaarjAM database for QIIME 2 [59] and for ambiguous sequences a BLAST was performed with close sequences from GenBank and UNITE.

2.10. Statistical Analysis

One-way ANOVA and *t*-test comparing treatments were performed for growth variables. Results were expressed as means, $\pm$ standard deviation. Significant differences between treatment means, were analyzed using Tukey post hoc analysis (HSD $p \leq 0.05$) or Student's *t*-test. Normality and homogeneity of variances were tested using Shapiro–Wilk's and Levene's test. Analysis was performed in Minitab 21.1.0.

3. Results

3.1. Riboflavin Stimulates Plant Growth

The addition of riboflavin to *P. vulgaris* increased plant weight (ANOVA $p \leq 0.000$; $F_{(2,17)} = 6.63$; HSD $p \leq 0.05$) (Figure 1). Riboflavin treatments increased root and shoot weight (Figure 1). Riboflavin together with *R. tropici* and the AMF mixture further increased plant growth (Figure 1).

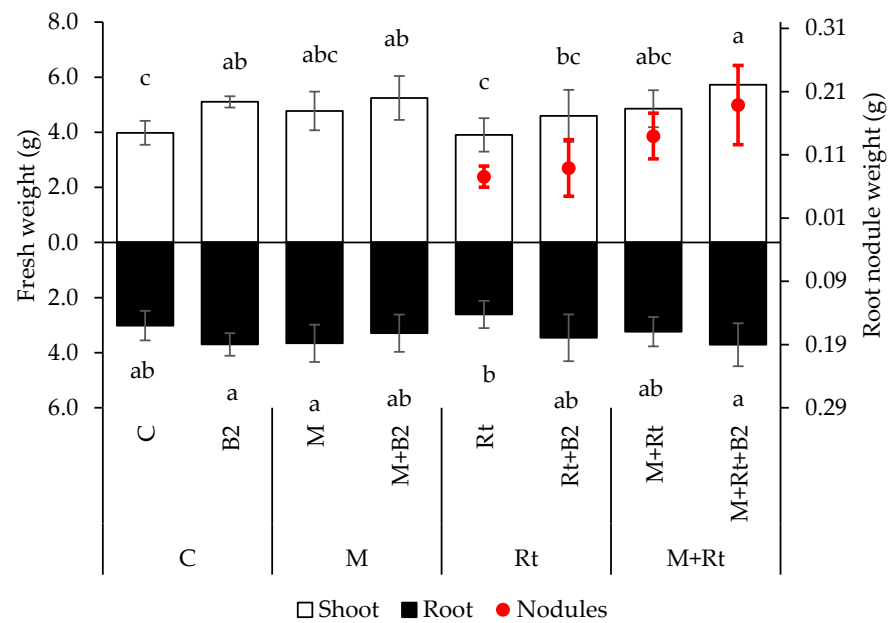


Figure 1. Total fresh weight in *Phaseolus vulgaris* (common bean) 35 days after being co-inoculated with arbuscular mycorrhizal fungi (AMF) inoculum and *Rhizobium tropici* CIAT 899. $n = 8$. Bars represent the mean $\pm$ standard deviation. Different letters indicate significant differences among treatments of same tissue, Tukey HSD ($p \leq 0.05$). M, arbuscular mycorrhizal fungi inoculated; B2, irrigated with 50 μ M riboflavin; Rt, *Rhizobium tropici* CIAT 899 inoculation; C, control without inoculation.

3.2. Riboflavin Stimulates Arbuscular Mycorrhizal Fungi (AMF) Colonization

Riboflavin increased AMF colonization significantly at 12.5 μ M (HSD $p \leq 0.05$). Riboflavin at 25 μ M had the highest AMF root colonization, but there were no significant differences among the various riboflavin concentrations (Figure 2a). Notably the distribution of the structures changed with the addition of riboflavin, as vesicles increased and arbuscules and hyphae were reduced. The different riboflavin concentrations had a differential effect on root colonization, with a higher increase of vesicles at 25 μ M (Figure 2b).

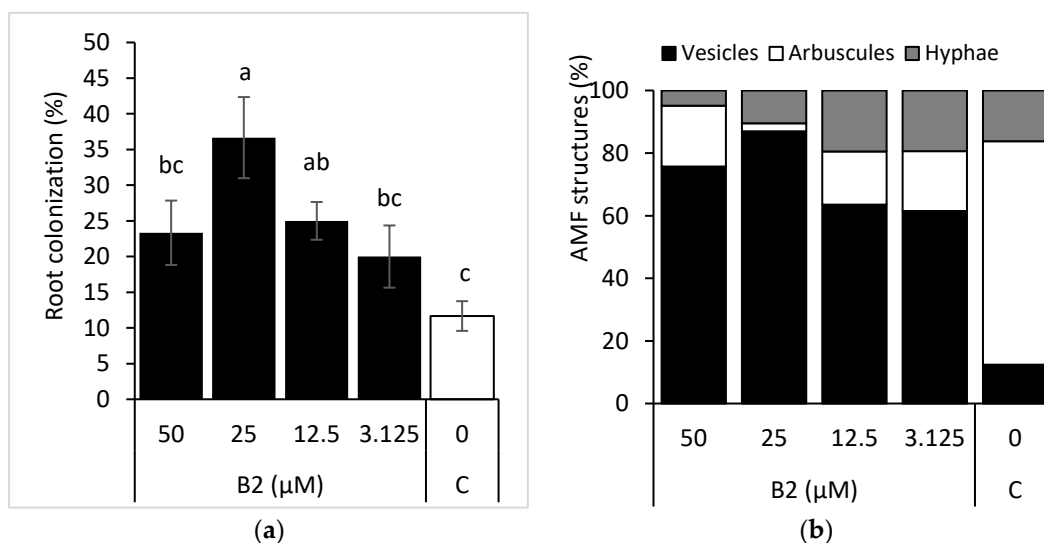


Figure 2. Riboflavin stimulates arbuscular mycorrhizal fungi (AMF) colonization. (a) Root colonization percentage by AMF, and (b) proportion of AMF structures in roots with different riboflavin concentrations 35 days after inoculation. $n = 4$. Bars represent the mean $\pm$ standard deviation. Different letters indicate significant differences between treatments, Tukey HSD ($p \leq 0.05$).

3.3. Effect of the Co-Inoculation of *R. tropici* and Riboflavin

The co-inoculation with *R. tropici* CIAT 899 increased the AMF colonization by 31.4% compared to only mycorrhized plants, but there was no difference when riboflavin was added to co-inoculated bean plants (Figure 3a). The proportion of vesicles increased by the addition of riboflavin, whereas arbuscules and hyphae were reduced as compared with only mycorrhized plants, as in the first experiment (Figure 2b). A similar effect was observed in the co-inoculated bean roots (Figure 3b). *R. tropici* and riboflavin affected the root length and AMF colonization.

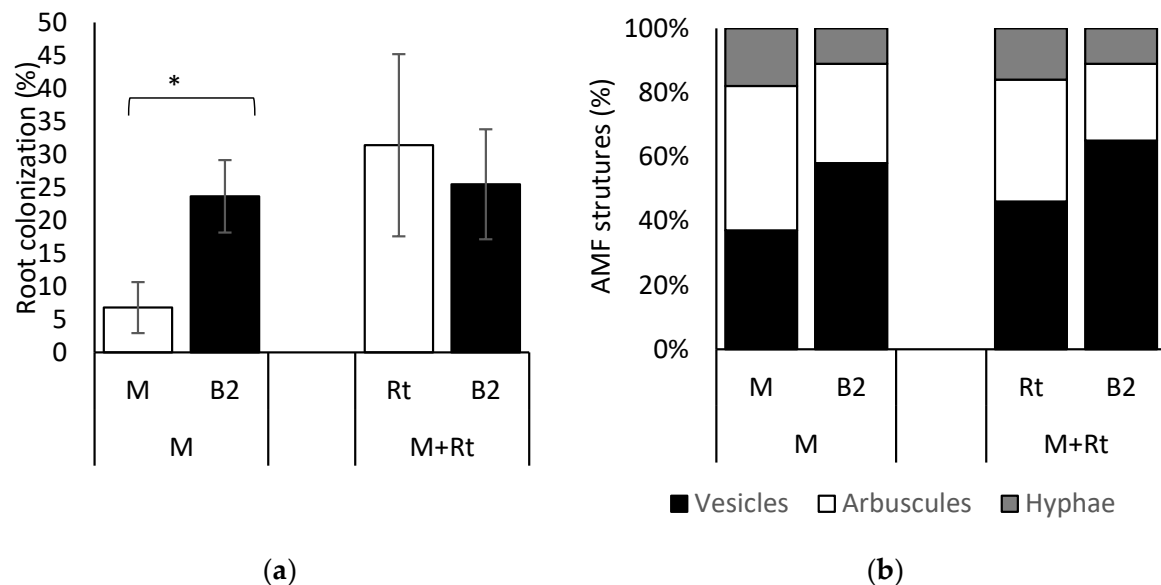


Figure 3. Effect of coinoculation of *Rhizobium tropici* and riboflavin (vitamin B2) in *Phaseolus vulgaris*. (a) Percentage of root colonization by Arbuscular mycorrhizal fungi (AMF) colonization percentage, and (b) percentage of colonization by AMF structures in roots with or without riboflavin and with or without *Rhizobium tropici* 35 days after inoculation. N = 4. Bars represent the mean ± standard deviation. M, AMF inoculum; B2, riboflavin (50 µM); Rt, *R. tropici* CIAT 899. *, significant differences (*t*-test, $p \leq 0.05$).

3.4. Inoculation with *R. tropici* Shifts the AMF Species Profile Colonizing *P. vulgaris*

The original inoculum was analyzed to determine the relative species composition which showed that *R. irregularis* was the predominant species (Figure 4). When the inoculum was used on *P. vulgaris*, *D. bernensis* became the dominant species, followed by *R. irregularis* and *C. pellucida* in smaller proportions. The main family that colonized the roots of *P. vulgaris* was the Glomeraceae.

Riboflavin had a small effect on the relative proportion of AMF species in the treatments inoculated with *R. tropici* (Figure 4), when inoculated with mycorrhiza only, the proportion of *G. macrocarpum* increased when riboflavin was added at low concentrations (Figure 4 right). Riboflavin altered the proportions of species recovered from *P. vulgaris* roots, by decreasing the dominance of *D. bernensis* in favor of *G. macrocarpum*. In the plant assays performed here, nodule dry weight in *P. vulgaris* was similar with or without riboflavin. Plants with riboflavin, mycorrhizal fungi and *R. tropici* had a significantly higher nodule dry weights than plants inoculated with both biological inoculants but without added riboflavin (Figure 1). The proportion of AMF species depended on coinoculation with *R. tropici* and host identity.

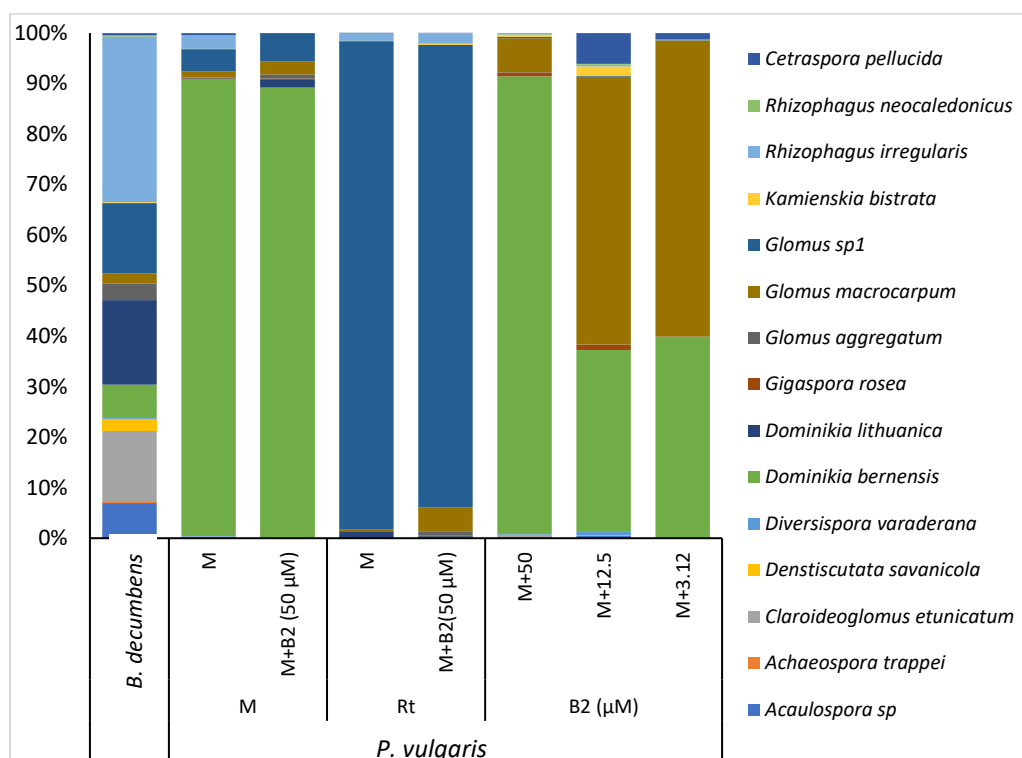


Figure 4. Arbuscular mycorrhizal fungi (AMF) composition and relative abundances in *Phaseolus vulgaris* root determined by a sequencing analysis of mycorrhizal SSU rRNA amplicons. Left, original inoculum obtained from *Brachiaria decumbens* Stapf (Poaceae). M, arbuscular mycorrhizal fungi inoculation; B2, irrigated with 50 µM riboflavin n; Rt, *Rhizobium tropici* CIAT 899 inoculation; C, control without inoculation.

4. Discussion

Numerous studies have reported the beneficial effects of mycorrhiza in *P. vulgaris* plants using fungal monocultures, especially *R. irregularis* [14,60]. *R. irregularis* is widely used because it can be cultivated in vitro [61] and is considered a model species [62]. In contrast, here we used here an inoculum composed of multiple species. AMF species diversity is usually associated with higher plant biomass production and nutrient uptake [21]. The use of a multispecies inoculum may increase the possibilities of achieving beneficial effects [33,44].

4.1. Changes on Arbuscular Mycorrhizal Fungi (AMF) Community Structure by the Plant

Host plants are important determinants of AMF community composition [63], but soil and environmental conditions may also have an influence. The effect of mycorrhizal and rhizobial coinoculation can be influenced by the host, rhizobia and AMF species. At the same time, plants can direct carbohydrate supply to the fungi that is best suited for the specific conditions [39,40]. Here, we obtained evidence of mycorrhizal selection by *P. vulgaris* that results in only a few species from an AMF mixture colonizing the roots. *P. vulgaris* selected *D. bernensis* from the other species present in the (Figure 4). In *B. decumbens* (the original host of the AMF mixture) a different species distribution was observed (Figure 4), where *R. irregularis* was most abundant in the plant roots. *R. irregularis* can be more competitive than other AMF species when co-inoculated [19,64], but in our results, the host and NFR influenced the abundance of each AMF species. Competing interactions between AMF species or other symbionts [65] are another important factor in the structure of the intraradical AMF community.

4.2. *R. Tropici* Effect and the Possible Mechanisms Influencing Arbuscular Mycorrhizal Fungi (AMF)

According to our results, *R. tropici* could be affecting the mycorrhizal composition in bean roots, favoring *Glomus* sp. as the dominant species and reducing the prevalence of *D. bernensis*. The direct mechanisms for this shift could be different, but some antifungals that are produced by *Rhizobium* might have an impact on the prevalence of the AMF species [66]. Breil et al. [67] reported that *Rhizobium* spp. produces trifolitoxin, which has antimicrobial activity that affects soilborne fungal pathogens [68]. Other mechanisms could be nutrient competition [69] since *R. tropici* can reach a large cell number on roots that would be a carbon sink. Otherwise, *R. tropici* could trigger plant defense responses [70] or have a signaling effect that conditions the colonization by different mycorrhizal species, favoring some species and inhibiting others. Inoculation with rhizobia may reduce colonization of AMF hyphae in roots, but this depends on the identity of the symbiont and nutrient availability [71]. AMF communities are influenced by available nutrients (nitrogen and phosphorus) [72], host interaction by other symbionts at the rhizosphere [73] or changes in plant innate immune responses [74]. We observed an increase in AMF root length colonization when *R. tropici* was coinoculated with the AMF mixture. Depending on the AMF species, root colonization may be positively, negatively or not significantly affected by the presence of NFB [75]. Tsikou et al. [75] found that *Mesorhizobium loti* favored root colonization of *C. etunicatum*, while it did not affect *F. mosseae*. In our study, *R. tropici* favored *Glomus* sp1 over *D. bernensis*, so in this system, the effect on colonization might depend on the mycorrhizal species.

In addition, *R. tropici* produces vitamins such as riboflavin [38] and plant hormones such as auxins [76] which may alter plant defense responses or plant metabolite production but could also be assimilated by fungi to alter the competitive ability of different mycorrhizal species. Therefore, we investigated the effects of exogenous riboflavin on mycorrhizal colonization in *P. vulgaris*.

4.3. Effect of Riboflavin in the Mycorrhizal Fungi in *Phaseolus vulgaris*

Riboflavin increased mycorrhizal colonization and the presence of vesicles in AMF-colonized roots. The vesicles are the primary lipid and P storage structures of AMF and larger amounts can serve as an important resource sink to provide for the colonized plants [77]. Riboflavin produced by *R. tropici* may be responsible for the switch in species dominance in the roots. Previous studies showed that riboflavin increased CO₂ content in *Medicago sativa* roots [33] up to 100% [78]. Becard et al. [37] reported that an increase in CO₂ content could favor AMF root colonization. It is likely that AMF benefit from an increase in CO₂ promoted by riboflavin produced by rhizobia in the roots. It has been reported that elevated CO₂ significantly stimulates root biomass and AMF gene abundance [79]. In addition, arbuscular mycorrhizal fungi enhance nutrient accumulation in wheat when exposed to elevated CO₂ [80]. It has been suggested that elevated atmospheric CO₂ may shift carbon flux from roots to associated microbial communities, particularly arbuscular mycorrhizal fungi, as they are key players in carbon transfer actors between plants and the soil [81]. Although the promotion of a symbiotic association between AMF and host has been documented, elevated CO₂ in *Pisum sativum* L. [82] roots has no effect on mycorrhizal development of some AMF strains [82]; implying that there may be some interspecific fungal differences considered [83]. Lumichrome, a riboflavin derived flavin, applied to *Lotus japonicus* and *Solanum lycopersicum* promoted nodulation of *Mesorhizobium loti* (in *L. japonicus*) and hyphal formation of *Glomus intraradices* (*R. intraradices*) and *Glomus mosseae* (*F. mosseae*), delaying the maturation of the arbuscule [84]. Our results support the suggestion that increased respiration in roots, caused by exogenous riboflavin administration, also alters and promotes AMF symbiosis [37].

The Glomeraceae family produces a large amount of intraradical biomass [85], while Gigasporaceae produce more extraradical hyphae [85,86]. In our molecular analysis, we found higher abundance of Glomeraceae in most treatments (*Dominikia* and *Glomus*). The initial inoculum had a large relative abundance of *R. irregularis*, which is usually the first

species to colonize the plants (within two weeks) compared to species such as *Glomus aggregatum* (four weeks) [64]. *R. irregularis* has the advantage of colonizing a larger portion of the root and reproducing when conditions are appropriate [64].

The pattern of root colonization is of special interest. When co-inoculated with *Bradyrhizobium* CB1015, the proportion of *F. mosseae* vesicles increased in mung bean (*Vigna radiata* (L.) R. Wilczek) [87]. Mycorrhizal colonization increased not only with *R. tropici*, but also with riboflavin, which also increased vesicle percentage as compared to the control (Figure 2b). In general, an increase in arbuscular fungi corresponds to an increase in phosphorus content in the root. [88]. When plants grow in stressful environment, the colonization of AMF roots is stimulated, affecting the amount of vesicles and arbuscules [89].

The development of an AMF inoculum for legumes should consider both AMF species identity and effects on plant performance and yield. This study provides the basis for future studies to further explore the interaction of AMF inoculum with efficient rhizobia, and how riboflavin, a vitamin produced by rhizobia can increase AMF colonization on a concentration basis.

5. Conclusions

Riboflavin can increase AMF root colonization in *P. vulgaris* plants, depending on the concentration. To our knowledge, this is the first work reporting riboflavin-induced changes in colonization by arbuscular mycorrhizal fungi. Coinoculation of AMF with *R. tropici* CIAT899 also increases AMF root colonization but is not further increased by the addition of 50 μ M riboflavin.

We found that the AMF composition changed from the AMF mixture originally used as inoculum. *P. vulgaris* may have a preference for *D. bernensis* from a diverse AMF mixture, while riboflavin may exert a selection pressure on AMF species at a certain concentration.

Coinoculation of AMF with *R. tropici* CIAT899 at a concentration of 50 μ M had little to no effect on AMF species composition. Our data suggest that secondary metabolites and the presence of other symbionts such as *R. tropici* may influence AMF species composition in mycorrhizal roots.

Author Contributions: Conceptualization, J.B., S.L.C.-R., N.M.M. and E.M.-R.; methodology, S.L.C.-R. and E.M.-R.; formal analysis, J.B.; investigation, J.B.; resources, E.M.-R., S.L.C.-R. and N.M.M.; data curation, J.B.; writing—original draft preparation, J.B.; writing—review and editing, E.M.-R., S.L.C.-R.; supervision, E.M.-R. and S.L.C.-R.; funding acquisition, S.L.C.-R., N.M.M. and E.M.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: Authors would like to thank M. Dunn and M. Rosenblueth for reading and commenting on the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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