Symbiotic Effectiveness of native Rhizobia Associated with non-native *Vicia* spp. and *Lathyrus* spp. From Magallanes

Eficacia simbiótica de Rizobios nativos asociados con especies no nativas de *Vicia* spp. y *Lathyrus* spp. de Magallanes

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ABSTRACT

The species *Vicia magellanica*, *V. nigricans* and *Lathyrus magellanicus* are part of the natural pastures of the Magallanes region. These species belong to the Fabaceae family and should be able to establish symbiotic associations with soil bacteria and thus fix atmospheric Nitrogen. The objective of this research was to evaluate root nodules formation and to determine the specificity and symbiotic effectiveness of rhizobia isolated from *Vicia sp* y *Lathyrus sp.* under controlled conditions. Thirteen rhizobial isolates were obtained from nodules collected from these species at three sites in the Magallanes region. Genetic fingerprinting identified five distinct strains. In nodulation trials with vetch (*V. atropurpurea*) and grass pea (*L. sativus*), all strains formed nodules on grass pea but were ineffective. The strains AG-298, AG-299 and AG-300 reached the highest nodulation index in grass pea. The strains AG-298, AG-299 and AG-300 were the only ones able to form root nodules in vetch, reaching dry matter levels like uninoculated control, and were therefore classified as ineffective. Rhizobia isolated from *Vicia sp.* and *Lathyrus sp.* in Magallanes, can induce nodulation but are ineffective in fixing nitrogen in *V. atropurpurea* and *L. sativus*.

RESUMEN

Las especies *Vicia magellanica*, *V. nigricans* y *Lathyrus magellanicus* son parte de las pasturas naturales de la región de Magallanes, aportando nitrógeno por medio de la fijación biológica de Nitrógeno atmosférico. El objetivo de esta investigación fue evaluar la formación de nódulos radiculares y determinar la especificidad y efectividad simbiótica de rizobios aislados desde plantas de los géneros *Vicia* y *Lathyrus*. Se recolectaron nódulos de tres sitios de la región de Magallanes, obteniéndose 13 aislados bacterianos. A través de la determinación de la huella genética, se identificaron cinco cepas genéticamente distintas. En un ensayo bajo condiciones controladas se evaluó la capacidad de las cepas de formar nódulos en vicia (*Vicia atropurpurea*) y chícharo (*Lathyrus sativus*). En chícharo, todas las cepas formaron nódulos y obtuvieron un resultado similar en materia seca siendo clasificadas como inefectivas. Las cepas AG-298, AG-299 y AG-300 fueron las que presentaron mejores resultados en índice de nodulación. En el ensayo en vicia, las cepas AG-298, AG-299 y AG-300 fueron las únicas que formaron nódulos radicales alcanzando materia seca similar al testigo sin nitrógeno, siendo clasificadas como inefectivas. Esta investigación confirma la capacidad de formar nódulos de los rizobios aislados desde *Vicia* y *Lathyrus* en Magallanes, siendo inefectivos en fijar nitrógeno en *V. atropurupurea* y *L. sativus*.

Palabras clave: Fijación de nitrógeno, Leguminosas, Patagonia Austral.

INTRODUCTION

The Magallanes region, located in the southernmost part of Chile, has adverse climatic conditions characterized by low temperatures and strong winds, which limit soil mineralization processes. These conditions favor the accumulation of organic matter but also lead to nitrogen deficiency (Radic and McAdam, 2012). The main economic activity of the region is livestock farming, with 96% of the territory dedicated to natural grasslands (ODEPA, 2018). However, these grasslands face challenges such as reduced carrying capacity due to overgrazing, which leads to the degradation of flora and fauna, and water scarcity during the summer (Covacevich, 2006). Since most of these grasslands are not fertilized, nitrogen becomes a critical nutrient.

In this context, native forage legumes such as *Vicia nigricans*, *Vicia magellanica*, and *Lathyrus mage-*

llanicus are essential due to their ability to establish symbiotic relationships with rhizobia. This interaction increases soil fertility by fixing atmospheric nitrogen and making it available to plants, which helps mitigate the nitrogen deficit in the soil (Ahmad Mahmud *et al*. 2021). This not only reduces the need for nitrogen fertilizers but also increases grassland productivity (Radic and McAdam, 2012; Elizalde, 2002), which is both economically beneficial and sustainable for agriculture (Ivanoc *et al*., 2012).

The Fabaceae family, to which *Vicia* and *Lathyrus* belong, is distributed from the tropics to the Arctic regions, encompassing a great diversity of species (Lewis *et al*., 2005). In particular, *V. nigricans* and *V. magellanica* stand out for their significant potential for protecting and restoring degraded soils, due to their root development and soil exploration capacity (Blackhall *et al*., 2021). Vicia species can be herbaceous with extensive root development, while in the genus Lathyrus, the species known as "sweet peas" are notable. In Magallanes, *L. magellanicus* is found from Rancagua to Tierra del Fuego, divided into varieties: var. *ovalifolius* and var. *heterocirrhus* (Lara y Cruz, 1987).

The ability of legumes to establish an effective symbiosis with rhizobia depends on an exchange of chemical and molecular signals, starting with the release of flavonoids from the plant roots. These compounds activate the *Nod* genes of the rhizobia, allowing them to secrete Nod factors. Once recognition between the rhizobium and the legume occurs, root invasion and nodule formation begin (Gourion *et al*., 2015; León, 2019), where nutrient exchange takes place. Rhizobia belong to the family Rhizobiaceae within the alpha-proteobacteria subdivision (Hernández *et al*., 2012).

This interaction is highly specific, and its effectiveness may vary between legume species and rhizobia (Garabato, 2018; Dary, 2015). To date, no detailed studies have been conducted on the symbiotic interactions between rhizobia and species of the genera *Vicia* and *Lathyrus* in the Magallanes Region, although it is known that there are differences in the specificity and effectiveness of the symbiosis between both genera (Covacevich and Ruz, 1996).

The aim of this research is to determine the specificity and symbiotic effectiveness of rhizobia isolated from *L. sativus* (pea) and *V. atropurpurea* (forage vetch) in Magallanes grasslands, specifically in *V. atropurpurea* and *L. sativus*.

MATERIAL AND METHODS

Collection and Isolation of Rhizobia

Whole plants of *Vicia* and *Lathyrus* were collected from three sites in the Magallanes Region: Los Cisnes (C10), La Leona (C19), and Portada (C21) (Figure 1).

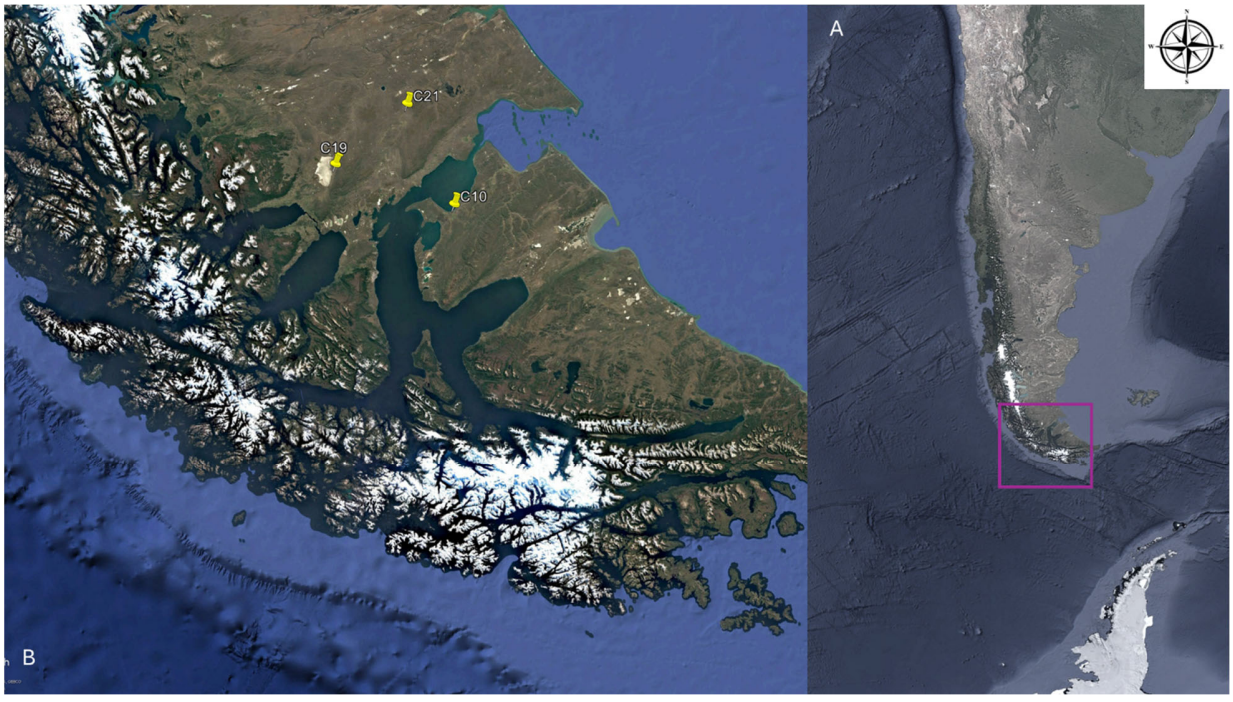

Figure 1. A) Panoramic view of the location of Patagonia. B) Location of sampling sites.
Figura 1. A) Vista panorámica de la Patagonia. B) Ubicación de los lugares de muestreo.

The root nodules were extracted and stored in tubes containing silica gel and hydrophilic cotton. The dehydrated samples were transported to the Bacteriology Laboratory of the Faculty of Agronomy, Chillán campus of the University of Concepción, where the experiments and rhizobia isolation were carried out under sterile conditions. The nodules were rehydrated by immersion in sterile distilled water for one hour and superficially disinfected with 70% ethanol for 30 s, followed by 4% sodium hypochlorite for 90 s and 6 washes with sterile distilled water to remove the disinfectants. The nodules were squeezed with sterile forceps, and the contents were inoculated into a mannitol yeast extract agar (YMA) medium with Congo red as an indicator.

The colonies obtained were incubated at 25°C for two to six days, depending on the growth rate of each rhizobia. Colonies with typical rhizobial characteristics on YMA were subcultured on YMA + Congo red until pure colonies were obtained (Matos *et al*., 2002; Hernández *et al*., 2012). The isolates obtained were cryopreserved in 20% glycerol at -80°C.

Genetic Diversity of Rhizobia

The genetic diversity of the isolated strains was evaluated through amplification of the genetic fingerprint using polymerase chain reaction (PCR). First, cell preparation was performed by suspending three loopfuls of pure colonies in 1 mL of sterile saline solution (0.89% NaCl). Each suspension was centrifuged at 9000 rpm for 3 min, the supernatant was removed, and the cells were resuspended in sterile saline solution, repeating this process 3 times. Suspensions were standardized to an optical density (OD) of 6.0 at a wavelength of 600 nm (Modified from Gerding *et al*., 2012).

Random Amplified Polymorphic DNA (RAPD) amplification (Alberti *et al*., 2011) was performed using the primer RPO1 (5' AATTTTCAAGCGTCGTGCCA 3'), which amplifies conserved and variable regions of the *nif* gene (Richardson *et al*., 1995; Gerding *et al*., 2012). The PCR cycling conditions were a cell lysis step of 5 min at 95ºC, followed by 5 cycles at 94ºC for 30 s; 5ºC for 10 s, and 72ºC for 90 s; and then 35 cycles at 94ºC for 30 s, 55ºC for 25 s and 72ºC for 90 s and a final extension at 72ºC for 5 min.

Amplification was verified by electrophoresis in 2% (w/v) agarose gels stained with 10,000X Gel Red™ Nucleic Acid Stain (Biotium), using the Kappa universal ladder as a marker. Electrophoresis was performed in buffered tanks with 1xTAE (Tris-acetate 40mM, EDTA 1mM, pH 8.0) at 100V for three hours (Gerding, 2011). The bands obtained were visualized with a UV transilluminator, and band patterns were analyzed to identify genetically distinct strains. Cladograms were constructed based on genetic distances between isolates using the NEIGHBOR application of Phylips software and visualized with MEGA 5.2 (Tamura *et al*., 2007). Only isolates with unique amplified band patterns were further evaluated.

Symbiotic Effectiveness Experiment

The nitrogen fixation effectiveness of rhizobial strains isolated from *Vicia* spp. and *Lathyrus* spp. was evaluated. The strains were cultured in mannitol yeast broth and incubated at 25°C for five days with agitation.

The trials were conducted in a phytotron under controlled conditions (24 ± 2°C) and LED light, using *L. sativus* (pea) and *V. atropurpurea* (forage vetch) as hosts. The seeds were disinfected by immersion in 70% ethanol (v/v) for 1 min, 3% sodium hypochlorite (v/v) for three minutes, and washed 6 times with sterile distilled water. Then, they were planted in pots disinfected with 3% sodium hypochlorite, filled with a substrate of sand and perlite (1:1), and watered with 20 mL of Nitrogen-free nutrient solution (Appendix 1). Four days later, they were inoculated with 1 mL of bacterial culture at an OD_{600}nm of 0.1 (equivalent to 10^7 CFU/mL).

The experimental design was completely random, with four replicates per treatment, consisting of the inoculation of five bacterial strains. Two control treatments were included, one without inoculation and nitrogen (N-), and another with nitrogen (N+) to which 10 mL of 0.1M KNO_3 was added. After 6-8 weeks, nodulation parameters, dry weight of aerial parts (DWA) and roots, dried at 60°C, were evaluated.

Nodulation parameters included the total number of nodules, percentage of active nodules, size, and location of nodules, to obtain the nodule scoring according to the scale from the Center for Rhizobium Studies (CRS, 2012).

$$\begin{aligned} Nodule\ scoring \\ &= (large\ crown\ nodules * 6) \\ &+ (medium\ crown\ nodules * 4) \\ &+ (small\ crown\ nodules * 2) \\ &+ (large\ lateral\ nodules * 3) \\ &+ (medium\ lateral\ nodules * 2) \\ &+ small\ lateral\ nodules \end{aligned}$$

Statistical analysis

Data were analyzed using ANOVA ($p \leq 0.05$) with mean comparisons by Tukey's test at the 95% confidence level for the dry shoot weight (PSA) data. Nodulation variables were analyzed using non-parametric Kruskal-Wallis tests, with mean comparisons by the Conover test. All analyses were performed with Infostat software (Di Rienzo *et al*., 2016).

RESULTS AND DISCUSSION

Rhizobia isolation

Thirteen root nodules of *Vicia* spp. and *Lathyrus* spp. were collected in the Magallanes region, all of the indeterminate type, characterized by the presence of a persistent apical meristem (Lima, 2012; Wang *et al*., 2002). From these nodules, 13 bacterial strains were isolated with typical characteristics of rhizobia: pale pink coloration, mucilaginous appearance, rapid growth, and absence of Congo red absorption, which allowed them to be distinguished from potential contaminants (Hungria *et al*., 2016).

Genetic fingerprinting of the isolates

Using the RAPDs-PCR technique and the construction of a cladogram (Figure 2), five genetically distinct strains were identified (AG-298, AG-299, AG-300, AG-301, and AG-302). The observed genetic diversity was low compared to previous studies in the region (Galaz, 2019).

Symbiotic effectiveness test

No nodulation was observed in the non-inoculated treatments (N- and N). In *L. sativus*, all strains induced nodule formation, but AG-301 and AG-302 showed a significantly lower nodulation index compared to AG-298, AG-299, and AG-300 (Figure 3). A higher nodulation index indicates early nodule formation, which may be relevant when performing field inoculations, as these strains would be more competitive and quickly occupy infection sites, preventing ineffective strains from using them (Gerding *et al*., 2014).

There were significant differences between the inoculation treatments and the nitrogen control (N+) in terms of dry shoot weight (DSW), with the latter being

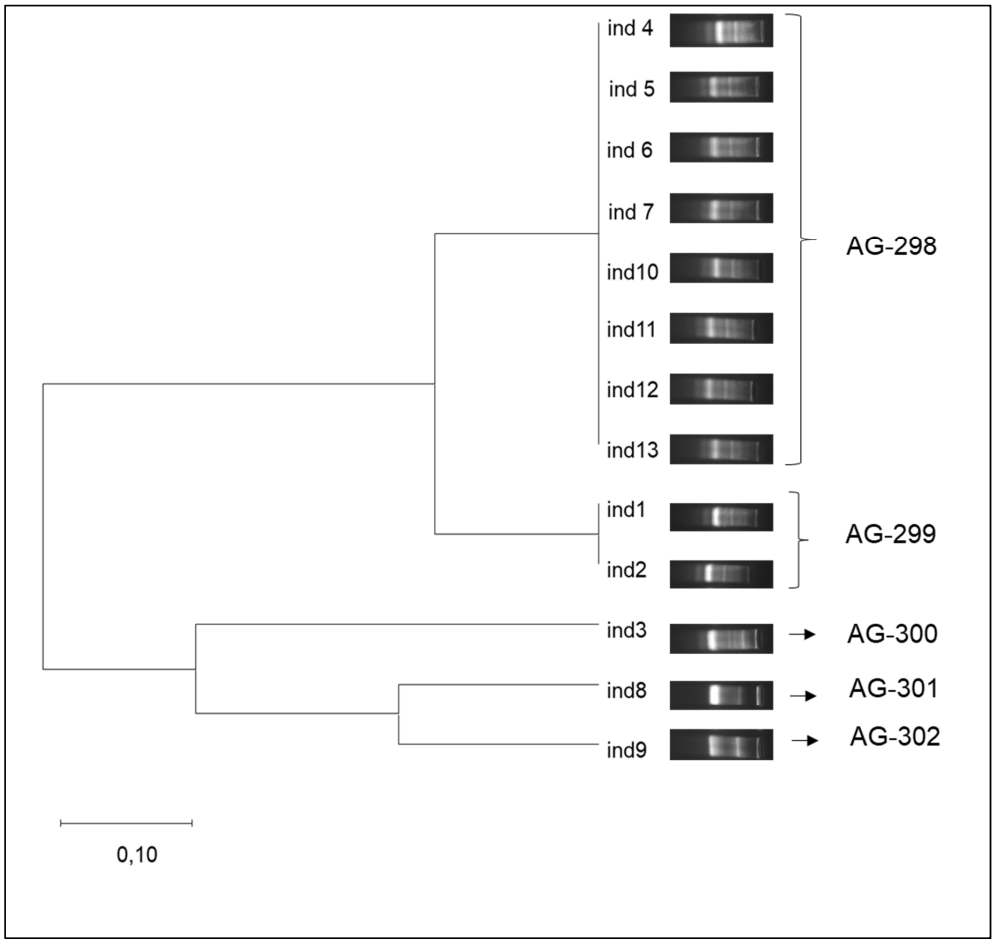

Figure 2. Cladogram of genetic relationships between rhizobia isolated from *Lathyrus magellanicus* and *Vicia magellanica*, according to the RAPDs–PCR product with the RPO1 primer.

Figura 2. Cladograma de las relaciones genéticas entre rizobios aislados desde *Lathyrus magellanicus* y *Vicia magellanica*, de acuerdo con el producto de RAPDs–PCR con el partidor RPO1.

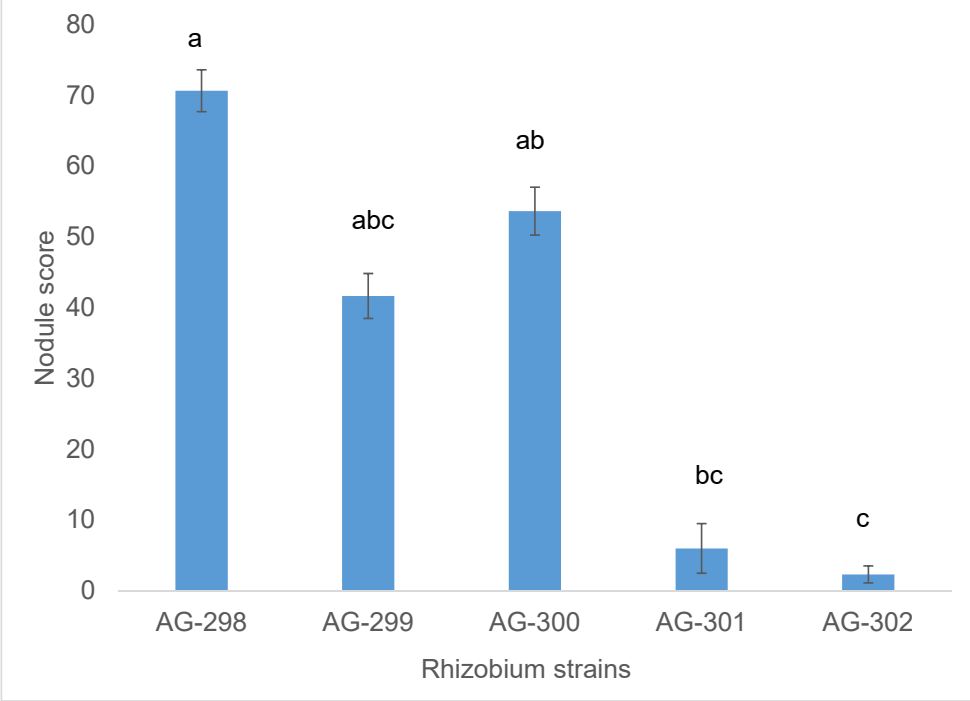

Figure 3. Nodulation index in *Lathyrus sativus* inoculated with different rhizobia strains isolated from root nodules of legumes in Magellanic grasslands.

Figura 3. Índice de nodulación en *Lathyrus sativus* inoculado con distintas de rizobios aislados desde nódulos radicales de leguminosas en praderas de Magallanes.

higher than the inoculated treatments ($p \leq 0.05$). There were no significant differences between the inoculated treatments and the nitrogen-free control (N-), suggesting that the strains were ineffective in *L. sativus*, as they did not exceed 50% of the DSW compared to the N+ treatment (Figure 4), classifying them as ineffective (Yates, 2016).

In *V. atropurpurea*, all strains induced nodulation, but again, AG-301 and AG-302 showed a lower nodule score compared to the others. The nodules formed showed a whitish internal color, indicating inactivity of leghemoglobin (Calvo, 2011; Fourmond and Léger, 2017).

AG-298 and AG-299 achieved a higher nodulation index compared to AG-301 and AG-302 in *V. atropurpurea* (Figure 5), and AG-301 did not form root nodules in this species. Significant differences ($p \leq 0.05$) were observed in the dry shoot weight (DSW) of *V. atropurpurea* between treatments and the nitrogen control (N+), with the former being significantly lower (Figure 6), confirming the ineffectiveness of the strains (Yates *et al*., 2016).

The low DSW in both inoculated species could be explained by the energy cost associated with the nodulation process and the lack of activation of genes related to biological nitrogen fixation (Wang *et al*., 2021; Bianco, 2020). Although nodulation occurred, it was ineffective, probably due to a lack of compatibility between rhizobial and host Nod factors, suggesting that the interaction did not occur properly (Parniske and Downie, 2003).

Nitrogen fixation also depends on the activation of the *NifH* genes, which is essential for the effectiveness of nodulation in a given host (Paredes, 2013). This could explain why, despite the induction of nodules, they were not effective in this study.

Ineffective nodulation in *L. sativus* and *V. atropurpurea* may be due to these species not being the original host of the isolated rhizobia, even though they belong to the same genus. However, there are cases where the same rhizobial species can form effective nodules in several species of the same genus, as is the case with *Trifolium* (Drew *et al*., 2012). Our results suggest that nodulation in the general *Lathyrus* and *Vicia* is highly specific, resulting in effective nodulation only in certain species, similar to what has been described for genera such as *Lotus* (Howieson *et al*., 2011) and some species of *Lathyrus* and *Vicia* (Grifti *et al*., 2020; Villadas *et al*., 2017).

Grifti *et al*. (2020) reported that *Rhizobium lagueerrae* is effective in *Lathyrus cicera* and *Vicia sativa*. Still, they obtained inconsistent results when inoculating both species with *Rhizobium leguminosarum*, which

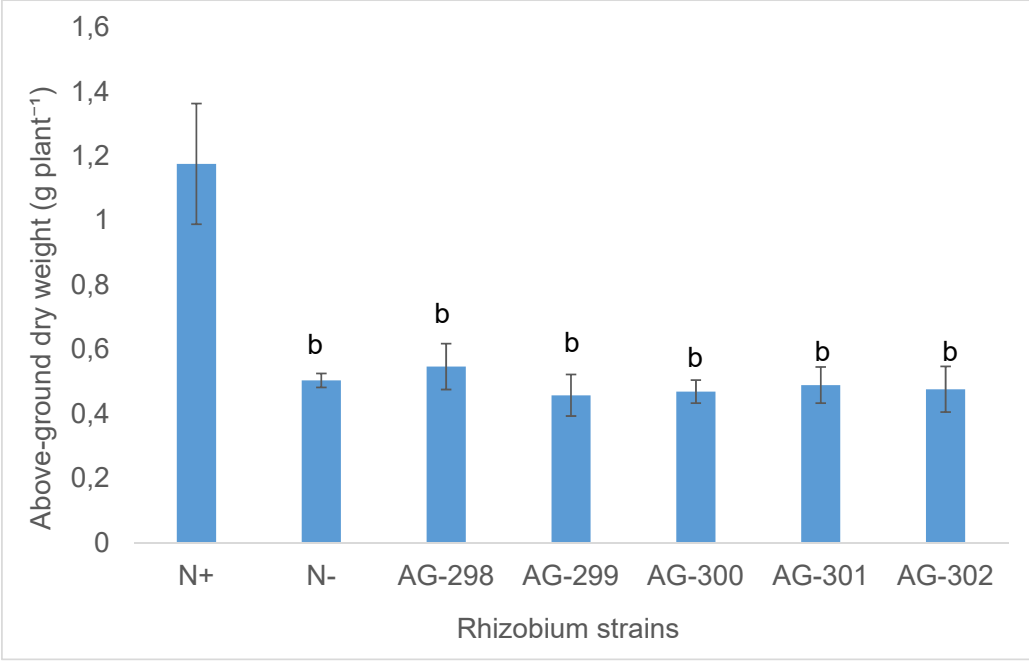

Figure 4. Shoot biomass of *Lathyrus sativus* inoculated with different rhizobia strains isolated from root nodules of legumes in Magellanic grasslands.

Figura 4. Biomasa aérea de *Lathyrus sativus* inoculado con distintas cepas de rizobios aislados desde nódulos radicales de leguminosas en praderas de Magallanes.

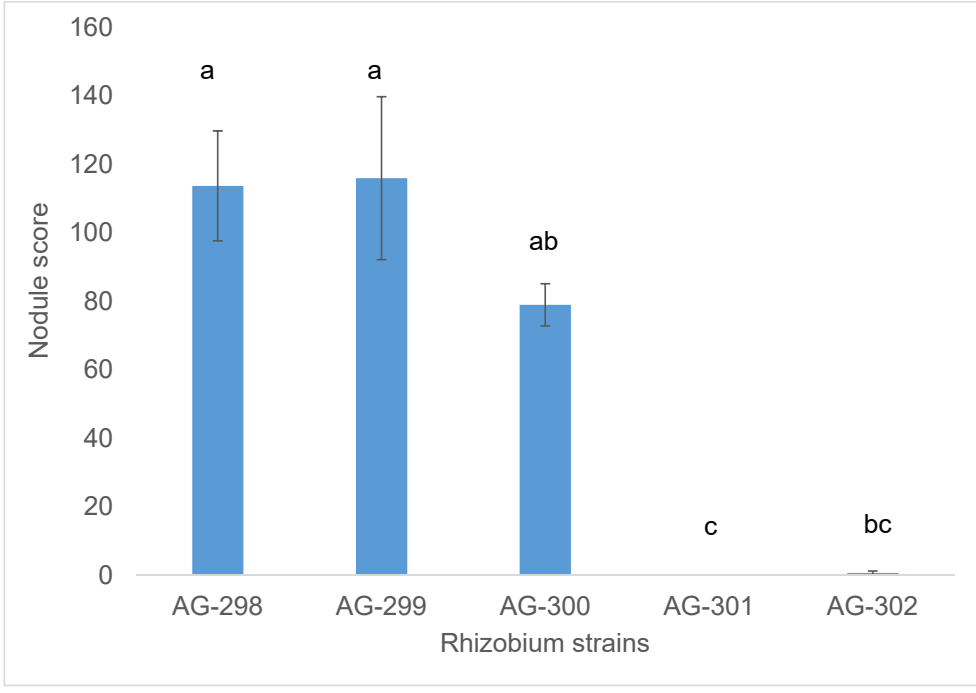

Figure 5. Nodulation index in *Vicia atropurpurea* inoculated with different rhizobia strains isolated from root nodules of legumes in Magellanic grasslands.

Figura 5. Índice de nodulación en *Vicia atropurpurea*, inoculado con distintas cepas de rizobios aislados desde nódulos radicales de leguminosas en praderas de Magallanes.

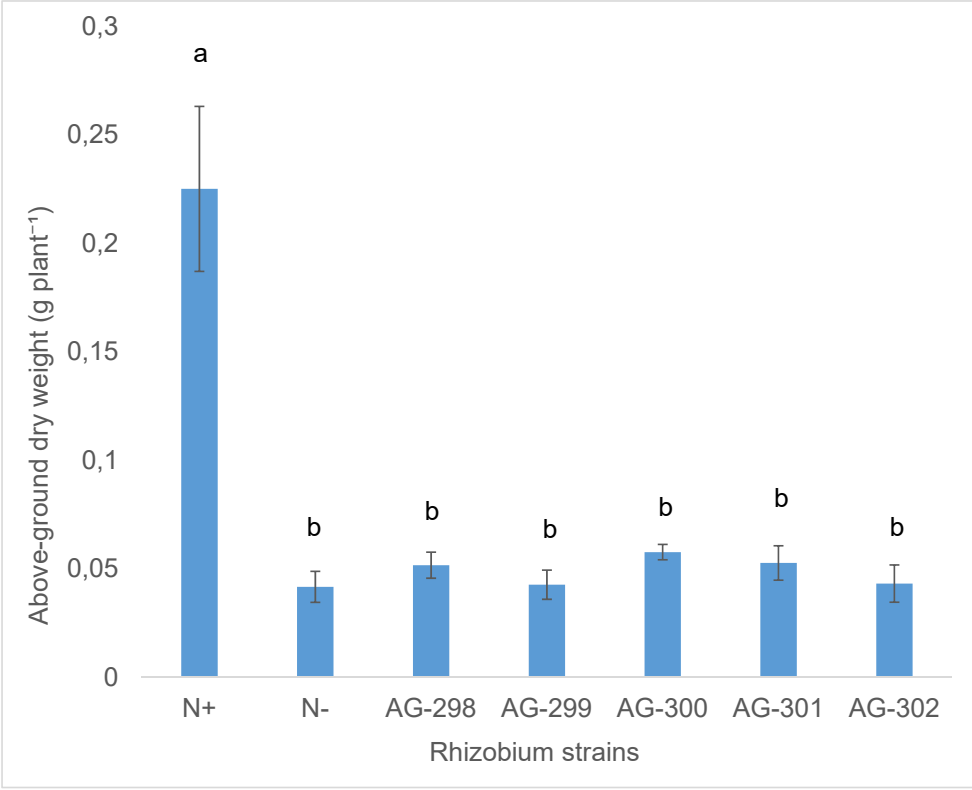

Figure 6. Shoot biomass of *Vicia atropurpurea* inoculated with different rhizobia strains isolated from root nodules of legumes in Magellanic grasslands.

Figura 6. Biomasa aérea de *Vicia atropurpurea* inoculada con distintas cepas de rizobios aislados desde nódulos radicales de leguminosas en praderas de Magallanes.

has been described as the most frequent symbiont in *Lathyrus* spp. in Canada (Drouin *et al*., 1996) and also in *Lathyrus* spp. and *Vicia* spp. in grasslands in Spain (Villadas *et al*., 2017).

Although the bacterial species was not identified, the characteristics of the incubated colonies and its growth rate suggest that they belong to the genus *Rhizobium*, which is consistent with previous studies on interactions in *Vicia* and *Lathyrus* (Delgado *et al*., 2007; Grifti *et al*., 2020). This study confirms the ability of the isolates to induce nodules, but their effectiveness in these species requires further evaluation. If effective, these strains could be key to the restoration of grasslands in the Magallanes region, as they are likely adapted to the edaphoclimatic conditions of the area, which is crucial given the challenge that low soil temperatures pose for nodulation (Fernández, 2003; Jiménez, 2007).

CONCLUSIONS

Bacteria isolated from root nodules of *Lathyrus* spp. and *Vicia* spp. in natural grasslands of the Magallanes region demonstrated the ability to induce nodulation in the legumes *Lathyrus sativus* and *Vicia atropurpurea*. However, the obtained strains were symbiotically ineffective, as they failed to stimulate a significant increase in biomass production in either species.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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