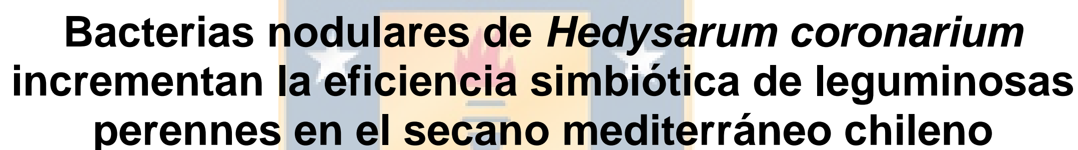
Universidad de Concepción
Dirección de Postgrado
Facultad de Agronomía -Programa de Magister en Ciencias Agronómicas



Bacterias nodulares de *Hedysarum coronarium* incrementan la eficiencia simbiótica de leguminosas perennes en el secano mediterráneo chileno

Tesis para optar al grado de Magister en Ciencias Agronómicas con Mención en Producción y Protección Vegetal

PAOLA CONSTANZA SOBARZO PALMA
CHILLÁN-CHILE

2019

Profesor Guía: Macarena Gerding González
Dpto. de Producción Vegetal, Facultad de Agronomía
Universidad de Concepción

BACTERIAS NODULARES DE *HEDYSARUM CORONARIUM* INCREMENTAN LA EFICIENCIA SIMBIÓTICA DE LEGUMINOSAS PERENNES EN EL SECANO MEDITERRÁNEO CHILENO

Aprobada por:

Macarena Gerding González ______________________
Ing. Agrónoma, PhD Profesora Guía

Marisol Vargas Concha ______________________
Ing. Agrónoma, Dr. Evaluadora Interna

Carlos Ovalle Molina ______________________
Ing. Agrónomo, Dr. Evaluador Externo

Rosemarie Wilckens Engelbreit ______________________
Lic. en Biología, Dr. rer. nat. Directora (s) Programa

AGRADECIMIENTOS

Esta investigación fue financiada por Fondo Nacional de Desarrollo Científico y Tecnológico, FONDECYT Nº1180821 y FONDECYT N°1180829

TABLA DE CONTENIDOS

	Página
Resumen	viii
Summary	ix
Capítulo 1. Introducción general	1
Hipótesis	4
Objetivo general	4
Objetivos específicos	4
Referencias	5
Capítulo 2. Can native nodule associated rhizobia, increase the symbiotic efficiency of perennial legumes in the Mediterranean dryland of Chile?	12
Abstract	13
Introduction	14
Materials and methods	17
Results	22
Discussion	26
Conclusions	30
Acknowledgements	31
References	31
Figures	41
Tables	47
Capítulo 3. Conclusiones generales	50

ÍNDICE DE FIGURAS Y TABLAS

Página

Figure 1 Phylogenetic tree of the 16S rRNA gene from 5 isolates (in bold) and type strains of closely related species. 41

Figure 2 Number of nodules and nodule effectivity in *Hedysarum coronarium* plants inoculated with different endophytic strains, 17 weeks after sowing. 42

Figure 3 Number of nodule initials induced in *Hedysarum coronarium* seedlings on time, inoculated with *Rhizobium sullae* WSM1592 alone and in co-inoculation with nodular endophytes. 42

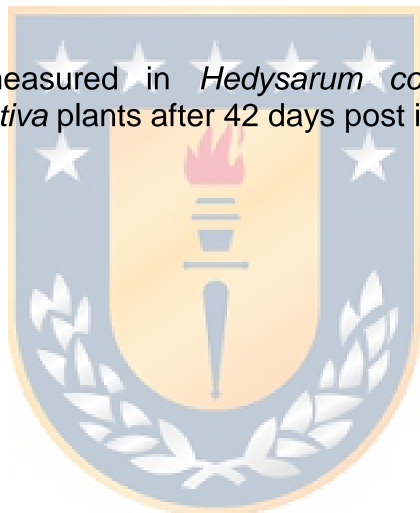
Figure 4 Number of nodule initials induced on *Medicago sativa* seedlings on time, inoculated with *Ensifer meliloti* AG118 and WSM2141 alone and in co-inoculation with nodular endophytes. 43

Figure 5 Root depth of *Hedysarum coronarium* seedlings collected at 7, 10, 14, 17 and 26 days after inoculation with *Rhizobium sullae* WSM1592 alone and in co-inoculation with nodular endophytes. 44

Figure 6 Root depth of *Medicago sativa* seedlings collected at 7, 10, 17, 21 and 26 days after inoculation with *Ensifer meliloti* AG118 and WSM2141 alone and in co-inoculation with *Hedysarum coronarium* nodular endophytes. 45

Figure 7 Mean shoot dry weights of *Hedysarum coronarium* (left) and *Medicago sativa* (right) inoculated with rhizobia alone and in co-inoculation with nodular endophytes expressed as a percentage of N^+ uninoculated treatment fertilized with nitrogen. N^- denotes the uninoculated treatment without nitrogen. Plants were harvested 42 days after inoculation. 46

Table 1	Nodule score system used to evaluate nodulation (adapted from Howieson and Ewing, 1989).	47
Table 2	Variables measured in *Hedysarum coronarium* plants inoculated with different endophytic strains, after 17 weeks of growing.	47
Table 3	Indole acetic acid produced by commercial *Rhizobium sullae* WSM1592, *Ensifer meliloti* WSM2141, Chilean native *E. meliloti* AG118 and *Hedysarum coronarium* nodular endophytes.	48
Table 4	Comparison of the area under the nodulation progression curve (AUNPC) for *Hedysarum coronarium* and *Medicago sativa* inoculated with *Rhizobium sullae* and *Ensifer meliloti* alone and in co-inoculation with nodular endophytes.	48
Table 5	Variables measured in *Hedysarum coronarium* and *Medicago sativa* plants after 42 days post inoculation.	49



BACTERIAS NODULARES DE *HEDYSARUM CORONARIUM* INCREMENTAN LA EFICIENCIA SIMBIÓTICA DE LEGUMINOSAS PERENNES EN EL SECANO MEDITERRÁNEO CHILENO

ROOT NODULE BACTERIA FROM *HEDYSARUM CORONARIUM* INCREASE SYMBIOTIC EFFICIENCY OF PERENNIAL LEGUMES IN CHILEAN MEDITERRANEAN DRYLAND

RESUMEN

Alfalfa (*Medicago sativa*) y sulla (*Hedysarum coronarium*) se introdujeron en el secano chileno para aumentar la disponibilidad de forraje y hacer frente a la sequía. Su desempeño simbiótico en asociación con rizobios ha sido inestable en campo y la ocupación de nódulos por parte de los inoculantes ha sido deficiente. Este estudio tuvo como objetivo evaluar la eficiencia simbiótica de rizobios, aislados de nódulos de sulla cultivada en secano, en co-inoculación con los simbiontes *Rhizobium sullae* y *Ensifer meliloti*. La diversidad de las cepas se evaluó mediante secuenciación de 16S rRNA. La capacidad de inducir nódulos radiculares fue evaluada en sulla. Las cepas, aparentemente incapaces de inducir la nodulación, fueron caracterizadas y co-inoculadas con el simbionte específico de alfalfa y sulla para evaluar su efecto en la nodulación y el crecimiento de las plantas. La mayoría de las cepas se identificaron como *Rhizobium sullae*, y algunas como *Mesorhizobium* spp y *Rhizobium* spp. Las cepas *Mesorhizobium* T3 y *Rhizobium* T5 produjeron ácido indolacético, aumentaron la eficiencia simbiótica y el crecimiento de las raíces de ambas leguminosas al co-inocularlas con sus rizobios específicos. Las bacterias nodulares de sulla en el secano mediterráneo chileno se identificaron como *Rhizobium sullae*, *Mesorhizobium* spp. y *Rhizobium* spp. Las cepas de los dos últimos géneros pudieron aumentar la tasa de nodulación y el desarrollo de la raíz en alfalfa y sulla.

SUMMARY

Lucerne (*Medicago sativa*) and sulla (*Hedysarum coronarium*) were introduced in Chilean Mediterranean dryland to increase forage availability and to cope with drought. Their symbiotic performance in association with rhizobia has been unstable in field and nodule occupancy by the rhizobial inoculants has been deficient. This study aimed to test the symbiotic efficiency of root nodule bacteria isolated from sulla in Chilean drylands, on their own and in co-inoculation with *Rhizobium sullae* and *Ensifer meliloti, Hedysarum coronarium* and *Medicago sativa* specific symbionts. The diversity of the strains obtained from the field was assessed through RAPD-PCR fingerprinting and 16S rRNA sequencing. Field strains ability to induce nodulation was assessed in sulla. Strains, apparently unable to nodulate sulla, were characterized *in vitro* and were assessed for their effect in nodulation and plant growth in Lucerne and sulla in co-inoculation with their specific symbiont. Most strains were identified as *Rhizobium sullae*, and some as *Mesorhizobium* spp and *Rhizobium* spp. The strains *Mesorhizobium* T3 and *Rhizobium* T5, produced indole acetic acid and increased sulla and lucerne symbiotic efficiency and root growth in co-inoculation with specific rhizobia. *Conclusions*: Root nodule bacteria from sulla in Chilean Mediterranean drylands were identified as *Rhizobium sullae*, *Mesorhizobium* spp. and *Rhizobium* spp. Strains of the last two genera were able to increase nodulation rate and root development in lucerne and sulla plants.

CAPÍTULO 1

INTRODUCCIÓN GENERAL

El fitomicrobioma se define como todos los microorganismos que se asocian a una planta colonizando tanto la rizósfera como la filósfera (Quiza et al. 2015). Estos microorganismos incluyen taxones procariontes y eucariontes, y pueden colonizar la superficie (especies epífitas) o partes internas del hospedero (Quiza et al. 2015; Ibáñez et al. 2017). Los microorganismos procariontes que pueden detectarse dentro de los tejidos de plantas aparentemente sanas se consideran endofitos (Schulz y Boyle 2006).

Los rizobios pertenecen al grupo alfa-proteobacteria y prosperan en diversos entornos, ya sea en el suelo como bacterias de vida libre o como simbiontes dentro de nódulos radiculares de leguminosas, donde fijan nitrógeno atmosférico (Masson-Boivin y Sachs 2018). La simbiosis leguminosa-rizobio evolucionó hace aproximadamente 58 millones de años y acontece en el 88% de las especies leguminosas (Sprent 2007; Sachs et al. 2011). Esta interacción comienza en el suelo, donde las plantas y los rizobios intercambian moléculas de señal difusibles, esencialmente flavonoides liberados por la leguminosa y factores Nod secretados por la bacteria (Ferguson et al. 2010). Una vez que se logra el reconocimiento mutuo, se produce una infección y la planta desarrolla nódulos, que son los nuevos órganos radiculares que albergan a las bacterias (Gourion et al. 2015). Estos nódulos proporcionan el ambiente ideal para que los rizobios fijen nitrógeno para la planta hospedera a cambio de carbohidratos (Ferguson et al. 2019). La relación simbiótica entre las plantas y los rizobios es delicada y puede romperse si uno de los simbiontes está siendo dañado por el otro (Aguilar et al. 2018). Es ampliamente aceptado que los nódulos constituyen un nicho especial para la fijación de nitrógeno, y que está colonizado principalmente por rizobios (Xiao et al. 2017). Sin embargo, éstos a menudo pueden estar ocupados por un microbioma bacteriano filogenéticamente diverso (Lu et al. 2017). Estas bacterias endofíticas residen intercelularmente o intracelularmente dentro de los tejidos del huésped y, en general, poseen ventajas en comparación con sus contrapartes de vida libre ya que están

protegidas del estrés ambiental y de la competencia microbiana (Sturz et al. 2000). Las bacterias dentro de los nódulos de la raíz, además de los rizobios, recientemente han sido descritas como endófitos no rizobiales (ENR) (De Meyer et al. 2015), endófitos de nódulos (Velásquez et al. 2013) o bacterias asociadas a nódulos (BAN) (Rajendran et al. al. 2012). En este escrito, se usará la abreviatura BAN, ya que posee un alcance más amplio que las otras dos designaciones (Martínez-Hidalgo e Hirsch 2017). En general, los miembros de las BAN son endófitos bacterianos no rizobianos y no nodulantes de géneros como *Azospirillum*, *Pantoea*, *Klebsiella*, *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Streptomyces* (Ibáñez et al. 2009; De Meyer et al. 2015; Lai et al. 2015; Subramanian et al. 2015; Cassán y Diaz-Zorita 2016; Sreevidya et al. 2016), entre otros. Sin embargo, las BAN también pueden ser rizobios que no son capaces de inducir nodulación en la leguminosa de la que fueron aislados, tal como informaron Tariq et al. (2014) para cepas de *Ochrobactrum* aisladas de *Pisum sativum* y Chen et al. (2014) para cepas de *Burkholderia* aisladas de *Arachys duranensis*. Además, Hakim et al. (2018) estudiaron la distribución relativa de rizobios endofíticos en los nódulos de raíz de *Vigna radiata*, una leguminosa promiscua, e informaron que los nódulos contenían rizobios del género *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* y *Rhizobium*, donde sólo los géneros *Bradyrhizobium* y *Ensifer* poseían genes fijadores de nitrógeno, lo que sugiere que las bacterias *Mesorhizobium* y *Rhizobium* eran BAN.

Condiciones ambientales, como el estrés por sequía (Staudinger et al. 2016) o temperaturas extremas (Ryalls et al. 2013), cada una sola o en combinación, podrían afectar el desarrollo de nódulos, el crecimiento de leguminosas y finalmente la biomasa total de la planta (Jian et al. 2019). Una alternativa diferente al uso de la inoculación tradicional de rizobios utilizando una sola cepa bacteriana, para mejorar el crecimiento de las leguminosas en entornos adversos, ha sido el uso de la co-inoculación entre rizobios y otras bacterias con características que promueven el crecimiento de las plantas (Chiboub et al. 2018; Armendariz et al. 2019; Ju et al. 2019) o el uso de la inoculación triple, incluyendo dos bacterias y micorriza (Abd-Alla et al. 2019). Algunas BAN pueden ayudar a los rizobios a extender su rango de hospedadores (Liu et al. 2010), mejorar la nodulación y la fijación de N_2 en simbiosis con leguminosas (Peix et al. 2015). Se ha demostrado que la co-inoculación de

rizobios junto con los miembros de BAN puede mejorar el crecimiento, la longitud de la raíz, el número de nódulos y el peso seco de las leguminosas (Bai et al. 2002; Ibáñez et al. 2009; Mishra et al. 2009; Rajendran et al. 2012) aunque la co-inoculación también puede aumentar la tolerancia de las plantas al estrés por sequía (Silva et al. 2019). En contraste con estos efectos positivos, algunas BAN pueden reducir la aptitud nodulante del rizobio a través de la exclusión competitiva en la rizósfera (Gano-cohen et al. 2016), mientras que otros pueden estar presentes como organismos oportunistas en el hábitat rico en nitrógeno provisto por el nódulo (Hoque et al. 2011).

Sulla, *Hedysarum coronarium,* y alfalfa, *Medicago sativa*, son importantes leguminosas forrajeras en la cuenca del Mediterráneo (Annicchiarico et al. 2014; Benabderrahim et al. 2015). En Chile, estas especies han surgido, en los últimos años, como alternativas forrajeras para los productores de ganado en el secano Mediterráneo chileno (Ovalle et al. 2015; del Pozo et al. 2017). Los suelos de la mayoría de los sistemas agrícolas de secano están degradados debido a la erosión, el agotamiento de nutrientes y la baja capacidad de infiltración y retención de agua, lo que en consecuencia conduce a una baja productividad y sostenibilidad (Martínez et al. 2011). La producción ganadera en esta área se basa en pasturas anuales y está fuertemente limitada por la falta de forraje disponible desde el verano hasta el otoño, debido a la falta de lluvia, la única fuente de agua durante estos meses (del Pozo et al. 2017). Sulla y alfalfa, a diferencia de las pasturas anuales, tienen raíces pivotantes y más profundas que les permiten tolerar las condiciones de sequía de esta área agrícola (Douglas 1984; Zhang et al. 2018), lo que podría resultar en la producción de forraje verde en los meses de escasez.

Sulla y alfalfa pueden establecer relaciones simbióticas con rizobios del género *Rhizobium* y *Ensifer*, respectivamente. Sulla se asocia a *Rhizobium sullae* (Squartini et al. 2002) mientras que el simbionte específico de alfalfa es *Ensifer meliloti* (De Lajudie et al. 1994). La relación entre el sulla y su rizobio es altamente específica (Casella et al. 1984; Glatzle et al. 1986), la leguminosa tiende a ser un nodulador 'tímido' y las plántulas jóvenes desarrollan rápidamente síntomas de deficiencia de nitrógeno cuando la nodulación es inadecuada (Drew et al. 2012) por lo que es esencial inocular a sulla cuando la especie se cultiva fuera de su hábitat

natural (Sulas et al.2017) ya que la leguminosa rara vez nodula con rizobios del suelo (Drew et al. 2012).

Ovalle y col. (2015) introdujeron y estudiaron el establecimiento y la productividad de *Hedysarum coronarium*, *Medicago sativa* y otras leguminosas perennes para proporcionar nuevas opciones para la producción ganadera en las regiones de secano del centro-sur de Chile. Los autores establecieron sulla en Cauquenes y la inocularon con la cepa *Rhizobium sullae* WSM1592 (Yates et al. 2015). Sin embargo, la persistencia de sulla y la producción de biomasa fueron bajas, aparentemente porque el inoculante específico no persistió ya que en gran parte de las plantas no se encontraron nódulos en sus raíces. Las escasas plantas supervivientes de sulla y que mostraron mayor vigor (verdor y altura de la planta) se encontraban noduladas pero con bacterias distintas al inoculante original (Ovalle et al. 2015).

HIPOTESIS

Las plantas de *Hedysarum coronarium* poseen bacterias nodulares que ayudan a los rizobios a inducir nodulación y a las leguminosas a aumentar el rendimiento simbiótico y el crecimiento aéreo y radicular.

OBJETIVO GENERAL

Evaluar el efecto de bacterias endófitas de nódulos de *Hedysarum coronarium*, al usarlos en co-inoculación con *Rhizobium sullae* en *Hedysarum coronarium* y con *Ensifer meliloti* en *Medicago sativa*.

OBJETIVOS ESPECÍFICOS

- Evaluar la efectividad simbiótica de las cepas bacterianas colectadas a partir de nódulos *Hedysarum coronarium* cultivada en el secano interior de Chile, en su hospedero original.
- Evaluar el efecto promotor de crecimiento vegetal de bacterias nodulares aisladas desde *H. coronarium*, en *Medicago sativa* y en su hospedero original.

- Evaluar el efecto en la nodulación de bacterias nodulares aisladas desde *H. coronarium*, en *Medicago sativa* y en su hospedero original al co-inocular con los rizobios específicos.

REFERENCIAS

Abd-Alla MH, Nafady NA, Bashandy SR, Hassan AA (2019) Mitigation of effect of salt stress on the nodulation, nitrogen fixation and growth of chickpea (*Cicer arietinum* L.) by triple microbial inoculation. Rhizosphere 10:100148. https://doi.org/10.1016/j.rhisph.2019.100148

Aguilar A, Mora Y, Dávalos A, et al (2018) Analysis of genome sequence and symbiotic ability of rhizobial strains isolated from seeds of common bean (*Phaseolus vulgaris*). BMC Genomics 19:645. https://doi.org/10.1186/s12864-018-5023-0

Annicchiarico P, Ruisi P, Di Miceli G, Pecetti L (2014) Morpho-physiological and adaptive variation of Italian germplasm of sulla (*Hedysarum coronarium* L.). Crop Pasture Sci 65:206–213. https://doi.org/10.1071/CP13342

Armendariz AL, Talano MA, Olmos Nicotra MF, et al (2019) Impact of double inoculation with *Bradyrhizobium japonicum* E109 and *Azospirillum brasilense* Az39 on soybean plants grown under arsenic stress. Plant Physiol Biochem 138:26–35. https://doi.org/10.1016/j.plaphy.2019.02.018

Bai Y, D’Aoust F, Smith DL, Driscoll BT (2002) Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. Can J Microbiol 48:230–238. https://doi.org/10.1139/w02-014

Benabderrahim MA, Hamza H, Haddad M, Ferchichi A (2015) Assessing the drought tolerance variability in Mediterranean alfalfa (*Medicago sativa* L.) genotypes under arid conditions. Plant Biosyst 149:395–403. https://doi.org/10.1080/11263504.2013.850121

Casella S, Gault RR, Reynolds KC, et al (1984) Nodulation studies on legumes exotic to Australia: *Hedysarum coronarium*. FEMS Microbiol Lett 22:37–45.

https://doi.org/10.1111/j.1574-6968.1984.tb00350.x

Cassán F, Diaz-Zorita M (2016) *Azospirillum* sp. in current agriculture: From the laboratory to the field. Soil Biol Biochem 103:117–130. https://doi.org/10.1016/j.soilbio.2016.08.020

Chen JY, Gu J, Wang ET, et al (2014) Wild peanut *Arachis duranensis* are nodulated by diverse and novel *Bradyrhizobium* species in acid soils. Syst Appl Microbiol 37:525–532. https://doi.org/10.1016/j.syapm.2014.05.004

Chiboub M, Jebara SH, Saadani O, et al (2018) Physiological responses and antioxidant enzyme changes in *Sulla coronaria* inoculated by cadmium resistant bacteria. J Plant Res 131:99–110. https://doi.org/10.1007/s10265-017-0971-z

De Lajudie P, Willems A, Pot B, et al (1994) Polyphasic taxonomy of rhizobia: Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. Int J Syst Bacteriol 44:715–733. https://doi.org/10.1099/00207713-44-4-715

De Meyer SE, De Beuf K, Vekeman B, Willems A (2015) A large diversity of non-rhizobial endophytes found in legume root nodules in Flanders (Belgium). Soil Biol Biochem 83:1–11. https://doi.org/10.1016/j.soilbio.2015.01.002

del Pozo A, Ovalle C, Espinoza S, et al (2017) Water relations and use-efficiency, plant survival and productivity of nine alfalfa (*Medicago sativa* L.) cultivars in dryland Mediterranean conditions. Eur J Agron 84:16–22. https://doi.org/10.1016/j.eja.2016.12.002

Douglas GB (1984) Seed production of sulla - A plant for soil conservation. Proc New Zeal Grassl Assoc 45:239–242

Drew E, Herridge D, Ballard R, et al (2012) Inoculating legumes: A practical guide. 71

Ferguson BJ, Indrasumunar A, Hayashi S, et al (2010) Molecular analysis of legume nodule development and autoregulation. J Integr Plant Biol 52:61–76. https://doi.org/10.1111/j.1744-7909.2010.00899.x

Ferguson BJ, Mens C, Hastwell AH, et al (2019) Legume nodulation: The host

controls the party. Plant Cell Environ 42:41–51. https://doi.org/10.1111/pce.13348

Gano-cohen KA, Stokes PJ, Blanton MA, et al (2016) Nonnodulating *Bradyrhizobium* spp. modulate the benefits of Legume-Rhizobium mutualism. Appl Environ Microbiol 82:5259–5268. https://doi.org/10.1128/AEM.01116-16.Editor

Glatzle A, Schulte-Batenbrock T, Brockwell J (1986) Symbiotic incompatibility between two forage species of *Hedysarum*, grown in Morocco, and their homologous rhizobia. FEMS Microbiol Lett 37:39–43

Gourion B, Berrabah F, Ratet P, Stacey G (2015) Rhizobium-legume symbioses: The crucial role of plant immunity. Trends Plant Sci 20:186–194. https://doi.org/10.1016/j.tplants.2014.11.008

Hakim S, Mirza BS, Zaheer A, et al (2018) Retrieved 16S rRNA and nifH sequences reveal co-dominance of *Bradyrhizobium* and *Ensifer* (*Sinorhizobium*) strains in field-collected root nodules of the promiscuous host *Vigna radiata* (L.) R. Wilczek. Appl Microbiol Biotechnol 102:485–497. https://doi.org/10.1007/s00253-017-8609-6

Hoque MS, Broadhurst LM, Thrall PH (2011) Genetic characterization of root-nodule bacteria associated with *Acacia salicina* and *A. stenophylla* (Mimosaceae) across south-eastern Australia. Int J Syst Evol Microbiol 61:299–309. https://doi.org/10.1099/ijs.0.021014-0

Ibáñez F, Angelini J, Taurian T, et al (2009) Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. Syst Appl Microbiol 32:49–55. https://doi.org/10.1016/j.syapm.2008.10.001

Ibáñez F, Tonelli ML, Muñoz V, et al (2017) Bacterial endophytes of plants: Diversity, invasion mechanisms and effects on the host. In: Maheshwari DK (ed) Endophytes: Biology and Biotechnology. Sustainable Development and Biodiversity. Springer Cham, pp 25–40

Jian L, Bai X, Zhang H, et al (2019) Promotion of growth and metal accumulation of alfalfa by coinoculation with *Sinorhizobium* and *Agrobacterium* under copper and

zinc stress. PeerJ 7:. https://doi.org/10.7717/peerj.6875

Ju W, Liu L, Fang L, et al (2019) Impact of co-inoculation with plant-growth-promoting rhizobacteria and rhizobium on the biochemical responses of alfalfa-soil system in copper contaminated soil. Ecotoxicol Environ Saf 167:218–226. https://doi.org/10.1016/j.ecoenv.2018.10.016

Lai WA, Asif A, Lin SY, et al (2015) *Paenibacillus medicaginis* sp. nov. a chitinolytic endophyte isolated from a root nodule of alfalfa (*Medicago sativa* L.). Int J Syst Evol Microbiol 65:3853–3860. https://doi.org/10.1099/ijsem.0.000505

Leach JE, Triplett LR, Argueso CT, Trivedi P (2017) Communication in the phytobiome. Cell 169:587–596. https://doi.org/10.1016/j.cell.2017.04.025

Liu J, Wang ET, Ren DW, Chen WX (2010) Mixture of endophytic agrobacterium and *Sinorhizobium meliloti* strains could induce nonspecific nodulation on some woody legumes. Arch Microbiol 192:229–234. https://doi.org/10.1007/s00203-010-0543-2

Lu J, Yang F, Wang S, et al (2017) Co-existence of rhizobia and diverse non-rhizobial bacteria in the rhizosphere and nodules of *Dalbergia odorifera* seedlings inoculated with *Bradyrhizobium elkanii*, *Rhizobium multihospitium*-Like and *Burkholderia pyrrocinia*-like strains. Front Microbiol 8:. https://doi.org/10.3389/fmicb.2017.02255

Martínez-Hidalgo P, Hirsch AM (2017) The nodule microbiome: N_2-fixing rhizobia do not live alone. Phytobiomes J 1:70–82. https://doi.org/10.1094/pbiomes-12-16-0019-rvw

Martínez GI, Ovalle C, Pozo A., et al (2011) Influence of conservation tillage and soil water content on crop yield in dryland compacted Alfisol of central Chile. Chil J Agric Res 71:615–622. https://doi.org/10.4067/S0718-58392011000400018

Masson-Boivin C, Sachs JL (2018) Symbiotic nitrogen fixation by rhizobia — the roots of a success story. Curr Opin Plant Biol 44:7–15. https://doi.org/10.1016/j.pbi.2017.12.001

Mishra PK, Mishra S, Selvakumar G, et al (2009) Coinoculation of *Bacillus thuringeinsis*-KR1 with *Rhizobium leguminosarum* enhances plant growth and nodulation of pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.). World J Microbiol Biotechnol 25:753–761. https://doi.org/10.1007/s11274-009-9963-z

Ovalle C, Espinoza S, Barahona V, et al (2015) Lucerne and other perennial legumes provide new options for rain fed livestock production in the Mediterranean-climate region of Chile. Cienc e Investig Agrar 42:461–474. https://doi.org/10.4067/S0718-16202015000300014

Peix A, Ramírez-bahena MH, Velázquez E, et al (2015) Bacterial Associations with legumes. CRC Crit Rev Plant Sci 34:17–42. https://doi.org/10.1080/07352689.2014.897899

Quiza L, St-Arnaud M, Yergeau E (2015) Harnessing phytomicrobiome signaling for rhizosphere microbiome engineering. Front Plant Sci 6:. https://doi.org/10.3389/fpls.2015.00507

Rajendran G, Patel MH, Joshi SJ (2012) Isolation and characterization of nodule-associated *Exiguobacterium* sp. from the root nodules of fenugreek (*Trigonella foenum-graecum*) and their possible role in plant growth promotion. Int J Microbiol 2012:. https://doi.org/10.1155/2012/693982

Ryalls JMW, Riegler M, Moore BD, et al (2013) Effects of elevated temperature and CO_2 on aboveground-belowground systems: A case study with plants, their mutualistic bacteria and root/shoot herbivores. Front Plant Sci 4:445. https://doi.org/10.3389/fpls.2013.00445

Sachs JL, Skophammer RG, Regus JU (2011) Evolutionary transitions in bacterial symbiosis. Proc Natl Acad Sci U S A 108:10800–10807. https://doi.org/10.1073/pnas.1100304108

Schulz B, Boyle C (2006) What are Endophytes? In: Schulz B, C. Boyle, T. N. Sieber (eds) Microbial Root Endophytes. Soil Biology, 9th edn. Springer-Verlag Berlin Heidelberg, Berlin, pp 1–13

Silva ER, Zoz J, Oliveira CES, et al (2019) Can co-inoculation of *Bradyrhizobium* and

Azospirillum alleviate adverse effects of drought stress on soybean (*Glycine max* L. Merrill.)? Arch Microbiol 201:325–335. https://doi.org/10.1007/s00203-018-01617-5

Sprent JI (2007) Evolving ideas of legume evolution and diversity: A taxonomic perspective on the occurrence of nodulation. New Phytol 174:11–25. https://doi.org/10.1111/j.1469-8137.2007.02015.x

Squartini A, Struffi P, Döring H, et al (2002) *Rhizobium sullae* sp. nov. (formerly '*Rhizobium hedysari*'), the root-nodule microsymbiont of *Hedysarum coronarium* L . Int J Syst Evol Microbiol 52:1267–1276

Sreevidya M, Gopalakrishnan S, Kudapa H, Varshney RK (2016) Exploring plant growth-promotion actinomycetes from vermicompost and rhizosphere soil for yield enhancement in chickpea. Brazilian J Microbiol 47:85–95. https://doi.org/10.1016/j.bjm.2015.11.030

Staudinger C, Mehmeti-Tershani V, Gil-Quintana E, et al (2016) Evidence for a rhizobia-induced drought stress response strategy in *Medicago truncatula*. J Proteomics 136:202–213. https://doi.org/10.1016/j.jprot.2016.01.006

Sturz A V., Christie BR, Nowak J (2000) Bacterial endophytes: Potential role in developing sustainable systems of crop production. CRC Crit Rev Plant Sci 19:1–30. https://doi.org/10.1080/07352680091139169

Subramanian P, Kim K, Krishnamoorthy R, et al (2015) Endophytic bacteria improve nodule function and plant nitrogen in soybean on co-inoculation with *Bradyrhizobium japonicum* MN110. Plant Growth Regul 76:327–332. https://doi.org/10.1007/s10725-014-9993-x

Sulas L, Piluzza G, Salis M, et al (2017) Cropping systems sustainability: Inoculation and fertilisation effect on sulla performances in a new cultivation area. Ital J Agron 12:938. https://doi.org/10.4081/ija.2017.938

Tariq M, Hameed S, Yasmeen T, et al (2014) Molecular characterization and identification of plant growth promoting endophytic bacteria isolated from the root nodules of pea (*Pisum sativum* L.). World J Microbiol Biotechnol 30:719–725.

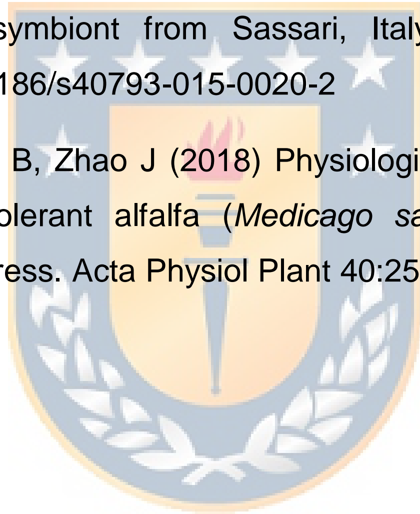
https://doi.org/10.1007/s11274-013-1488-9

Velásquez E, Martínez-Hidalgo P, Carro L, et al (2013) Nodular endophytes: An untapped diversity. In: Rodelas González MB, González-López J (eds) Beneficial Plant Microbial Interactions: Ecology and Applications. CRC Press, Boca Raton, pp 214–236

Xiao X, Chen W, Zong L, et al (2017) Two cultivated legume plants reveal the enrichment process of the microbiome in the rhizocompartments. Mol Ecol 26:1641–1651. https://doi.org/10.1111/mec.14027

Yates R, Howieson J, De Meyer SE, et al (2015) High-quality permanent draft genome sequence of *Rhizobium sullae* strain WSM1592; a *Hedysarum coronarium* microsymbiont from Sassari, Italy. Stand Genomic Sci 10:44. https://doi.org/10.1186/s40793-015-0020-2

Zhang C, Shi S, Wang B, Zhao J (2018) Physiological and biochemical changes in different drought-tolerant alfalfa (*Medicago sativa* L.) varieties under PEG-induced drought stress. Acta Physiol Plant 40:25. https://doi.org/10.1007/s11738-017-2597-0



CAPÍTULO 2

Can native nodule associated rhizobia, increase the symbiotic efficiency of perennial legumes in the Mediterranean dryland of Chile?

Paola Sobarzo-Palma[a], Macarena Gerding[a], Carlos Ovalle[b], Tamara Quezada[a], Marisol Vargas[a] and Jorge Campos[a]

[a] Universidad de Concepción, Facultad de Agronomía, Chillán, Chile.

[b] Instituto de Investigaciones Agropecuarias, CRI-La Cruz, La Cruz, Chile.

To whom correspondence should be addressed:

Macarena Gerding, Laboratorio de Bacteriología, Universidad de Concepción/ Av. Vicente Méndez 595, Chillán, Chile. Tel: (42) 2208755; E-mail: mgerding@udec.cl

Paola Sobarzo; E-mail: psobarzo@udec.cl

Keywords: *Hedysarum coronarium*, *Medicago sativa*, IAA, *Mesorhizobium*, root development

REVISTA: PLANT AND SOIL

FECHA DE ENVÍO: 18 diciembre 2019

Abstract

Aims: Lucerne (*Medicago sativa*) and sulla (*Hedysarum coronarium*) were introduced in Chilean Mediterranean dryland to increase forage availability and to cope with drought. Their symbiotic performance in association with rhizobia has been unstable in field and nodule occupancy by the rhizobial inoculants has been deficient. This study aimed to test the symbiotic efficiency of rhizobia isolated from sulla nodules in Chilean drylands, in co-inoculation with *Rhizobium sullae* and *Ensifer meliloti* symbionts.

Methods: The diversity of the strains was assessed through 16S rRNA sequencing. Strains symbiotic effectiveness was assessed in sulla. Strains, apparently unable to induce nodulation, were characterized and were co-inoculated with the specific symbiont in lucerne and sulla to assess their effect in nodulation and plant growth.

Results: Most strains were identified as *Rhizobium sullae*, and some as *Mesorhizobium* spp. and *Rhizobium* spp. The strains *Mesorhizobium* T3 and *Rhizobium* T5, produced indole acetic acid and increased sulla and lucerne symbiotic efficiency and root growth in co-inoculation with specific rhizobia.

Conclusions: Root nodule bacteria from sulla in Chilean Mediterranean drylands were identified as *Rhizobium sullae*, *Mesorhizobium* spp. and *Rhizobium* spp. Strains of the last two genera were able to increase nodulation rate and root development in lucerne and sulla plants.

Abbreviations:

WSM Western Soil Microbiology
CFU Colony-forming unit
DAI Days After Inoculation
IAA Indole-3-actic acid
OD Optical Density

Introduction

The phytobiome has been described as plants, their environment, and the organisms that interact with them, which together influence plant health and productivity (Leach et al. 2017). The phytomicrobiome is a subset of the phytobiome, which is defined as all the microorganisms that colonize everything connected to the plant body, i.e., the rhizosphere and the phyllosphere, and includes all the directly associated endophytes and epiphytes (Quiza et al. 2015). Microorganisms interacting with plants include prokaryotic and eukaryotic taxa and can colonize the surface or internal parts of the host (Ibáñez et al. 2017). Those prokaryotic microorganisms that can be detected within the tissues of apparently healthy plant hosts are considered as endophytic bacteria (Schulz and Boyle 2006).

Rhizobia belong to the alpha-proteobacteria and thrive in diverse environments, either as free-living bacteria in soil or as symbionts within the root nodules of legumes, where they fix atmospheric nitrogen (Masson-Boivin and Sachs 2018). The legume–rhizobia symbiosis evolved approximately 58 million years ago and occurs in 88% of all legume species (Sprent 2007; Sachs et al. 2011). This interaction begins in the soil, where plants and rhizobia exchange diffusible signal molecules, essentially flavonoids released by the legume and Nod factors secreted by the bacteria (Ferguson et al. 2010). Once reciprocal recognition is achieved, an infection occurs and the plant develops nodules, which are new root organs that house the bacteria (Gourion et al. 2015). These nodules provide the ideal environment for biological nitrogen fixation, which the rhizobia perform for their host plant in exchange for carbohydrates (Ferguson et al. 2019). The symbiotic relationship between plants and rhizobia is delicate and can be broken if one of the symbionts is impaired by the other (Aguilar et al. 2018). It is widely accepted that legume root nodules constitute a special niche for nitrogen fixation, mainly colonized by rhizobia (Xiao et al. 2017). Nevertheless, nodules are often occupied by a phylogenetically diverse bacterial microbiome (Lu et al. 2017). These endophytic bacteria reside intercellular or intracellularly within host tissues and are, in general, more advantageous as compared to free-living counterparts by being protected from environmental stresses and microbial competitions (Sturz et al. 2000). Bacteria inside root nodules, other than rhizobia, are recently known as non-rhizobial endophytes (NRE) (De Meyer et al. 2015), nodule endophytes (Velásquez et al. 2013), or nodule-associated bacteria (NAB) (Rajendran et al. 2012). In this paper, the abbreviation NAB will be used, since it is

broader in scope than the other two designations (Martínez-Hidalgo and Hirsch 2017). Generally, NAB members are non-rhizobia and non-nodulating bacterial endophytes from genera including *Azospirillum*, *Pantoea*, *Klebsiella*, *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Streptomyces* (Ibáñez et al. 2009; De Meyer et al. 2015; Lai et al. 2015; Subramanian et al. 2015; Cassán and Diaz-Zorita 2016; Sreevidya et al. 2016), between others. However, NAB can also be rhizobia that cannot induce nodulation in the legume host from which they were isolated, as reported Tariq et al. (2014) for *Ochrobactrum* strains isolated from *Pisum sativum* and Chen et al. (2014) for *Burkholderia* strains isolated from *Arachys duranensis*. Also, Hakim et al. (2018) studied the relative distribution of endophytic rhizobia in root nodules of *Vigna radiata*, a promiscuous legume host, and reported that nodules contained rhizobia from *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium* genus, where only *Bradyrhizobium* and *Ensifer* rhizobia possessed nitrogen fixing genes, suggesting that *Mesorhizobium* and *Rhizobium* bacteria were NAB.

Many environmental conditions, such as drought stress (Staudinger et al. 2016) or extreme temperature (Ryalls et al. 2013), each one alone or in combination, could affect nodule development, legume growth and finally total plant biomass (Jian et al. 2019). A different alternative to the use of the traditional rhizobia single inoculation, for enhancing legume growth in adverse environments, has been the use of co-inoculation between rhizobia and other bacteria that exerts plant growth promoting traits (Chiboub et al. 2018; Armendariz et al. 2019; Ju et al. 2019) or the use of triple inoculation, including two bacteria and mycorrhiza (Abd-Alla et al. 2019). Some NAB can help rhizobia to extend their host range (Liu et al. 2010), and improve nodulation and N_2 fixation in symbiosis with legumes (Peix et al. 2015). It has been shown that rhizobia co-inoculation along with NAB members can enhance growth, root length, nodule number and shoot dry weight in legumes (Bai et al. 2002; Ibáñez et al. 2009; Mishra et al. 2009; Rajendran et al. 2012) but co-inoculation can also increase plant tolerance to drought stress (Silva et al. 2019). Nevertheless, in contrast to these positive effects, some NAB may be able to reduce the fitness of nodulating rhizobia via competitive exclusion in the rhizosphere (Gano-cohen et al. 2016), while others can serve as opportunistic organisms in the nitrogen-rich habitat (Hoque et al. 2011).

Hedysarum coronarium and *Medicago sativa*, known as sulla and lucerne respectively, are important forage legumes in the Mediterranean basin (Annicchiarico et al. 2014; Benabderrahim et al. 2015) which make the perfect species to provide forage alternatives to livestock producers in the Chilean Mediterranean dryland (Ovalle et al. 2015; del Pozo et al. 2017). The soils of most dryland farming systems are highly degraded because of erosion, nutrient depletion and low water infiltration and holding capacity, which consequently lead to low productivity and sustainability (Martínez et al. 2011). Livestock production in this area is based on annual pastures and is heavily constrained by the lack of available forage from summer to autumn (del Pozo et al. 2017) due to the lack of rain, the only source of water during these months. Sulla and lucerne, unlike annual pastures, have pivoting and deepening roots that allow them to tolerate the drought conditions of this agricultural area (Douglas 1984; Zhang et al. 2018), which could result in forage production during lack of green fodder.

Sulla and lucerne can both establish symbiotic relationships with rhizobia from the genus *Rhizobium* and *Ensifer* respectively. Sulla associates to *Rhizobium sullae* (Squartini et al. 2002) while lucerne specific symbiont is *Ensifer meliloti* (De Lajudie et al. 1994). The relationship between sulla and its rhizobia is highly specific (Casella et al. 1984; Glatzle et al. 1986), the legume tends to be a 'shy' nodulator and young seedlings quickly develop nitrogen deficiency symptoms when nodulation is inadequate (Drew et al., 2012) so it is essential to inoculate sulla when the species is cultivated outside its natural habitat (Sulas et al. 2017) since the legume rarely nodulates with background soil rhizobia (Drew et al. 2012).

Ovalle et al. (2015) introduced and studied the establishment and productivity of *Hedysarum coronarium*, *Medicago sativa*, and other perennial legumes to provide new options for livestock production to the dryland regions of central-southern Chile. The authors established sulla in Cauquenes and the legume was inoculated with *Rhizobium sullae* strain WSM1592 (Yates et al. 2015). However, sulla persistence and biomass production was low, apparently because the specific inoculant did not persist since the surviving plants were nodulated with different bacteria to the inoculant. From the same experiment established by Ovalle et al. (2015), some surviving sulla plants showed greater vigor (greenness and plant height) and when examining their roots, they were profusely nodulated. We hypothesize that *Hedysarum coronarium* plants possess NAB that helped the rhizobia and the legume to increase symbiotic

performance and tolerate the drought stress induced by Chilean Mediterranean dryland. The aim of this study was to evaluate the beneficial or detrimental effect of these endophytes in co-inoculation with *Rhizobium sullae* WSM1592 on *Hedysarum coronarium* and with *Ensifer meliloti* on *Medicago sativa*.

Materials and methods

Nodule collection and bacteria isolation

Surviving plants of *H. coronarium* from field experiments in Chilean dryland (Ovalle et al. 2015) were collected from two field sites at INIA Cauquenes Experimental Station. Complete plants (n=31) were excavated and the roots were kept in plastic bag in a cooler (10°C) until used in laboratory.

In the laboratory, nodules were removed from the root systems and were surface sterilized by immersion in 70% (v/v) ethanol for 30 s, followed by immersion in 30 % (v/v) sodium hypochlorite for 90 s and six washes in sterile deionized water (modified from Hungria et al. 2016). Surface sterilized nodules were crushed and nodule contents streaked on yeast mannitol agar (YMA) plates. After incubation at 28 °C for 3 to 15 days, depending on the rate of growth of the strain, the bacteria were re-streaked from individual colonies onto fresh YMA plates. Each isolate was cryopreserved in 20% glycerol in ultra-freezer at -80°C (Oskouei et al. 2010).

Molecular fingerprinting

To prepare the template for PCR reactions, loopfuls of bacteria from pure culture in YMA plates were transferred to Eppendorf tubes with 1 ml of sterile saline solution (0.89% w/v NaCl). The Eppendorf tubes were centrifuged at 8000 rpm for 8 min, followed by the removal of the supernatant. The bacterial pellet was resuspended in 500 µl of saline solution and centrifuged to remove the supernatant (modified from Gerding et al., 2017). This was performed twice to wash the cells. Cell template was finally standardized to an optical density (OD) of 6.0 and 1.5 at 600 nm wavelength (Gerding et al. 2017).
The genetic diversity of the isolates was assessed at the strain level by molecular fingerprinting using the primers RPO1 (5′ AATTTTCAAGCGTCGTGCCA 3′) (Richardson et al. 1995). The PCR reaction mix for RPO1 contained 1.0 µl of cell template (OD_{600nm} 6.0), 0.5

µl of Taq DNA polymerase (Invitrogen), 0.5 µl of RPO1 primer (100 nM; Integrated DNA Technologies), 1.2 µl of 50 mM $MgCl_2$ (Invitrogen), 2.0 µl 10X PCR Rxn Buffer (Invitrogen), 0.4 µl of 10 mM dNTP and 14.4 µl of UltraPure grade water Hyclone® (Thermo Scientific) to total 20 µl (modified from Richardson et al. (1995)). PCR was conducted on an iCycler (BIORAD) and the cycling conditions were a cell lysis step of 5 min at 95 °C, followed by 5 cycles at 94 °C for 30 s; 50 °C for 10 s and 72 °C for 90 s; and then 35 cycles at 94 °C for 30 s, 55 °C for 25 s and 72 °C for 90 s and a final extension at 72 °C for 5 min. The PCR products were electrophoresed in 2 % (w/v) agarose gels (Thermo Fisher Scientific) pre-stained with 10,000X in water Gel Red™ Nucleic Acid Stain (Biotium). Generuler 100 bp DNA Ladder (Thermo Fisher Scientific) was used as a marker. Electrophoresis was carried out in tanks buffered with 1xTAE (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0) at 100 V for 3.5 h. Bands were visualized in a UV transilluminator. To determine genetically distinct strains, a matrix indicating presence or absence of bands was prepared, which was used to construct a cladogram based on the genetic distance between isolates using the NEIGHBOR application of the Phylips program and then the MEGA 7.0 software (Kumar et al. 2016) to visualize the cladogram.

Sequencing of the 16S rRNA gene

To investigate phylogenetic relationships among the strains, the gene encoding 16S rRNA was amplified by PCR using universal primers for bacteria 27F (5′-AGAGTTTGATCCTG GCTCAG-3′) and 1492R (5′-GGCTACCTTGTTACGACTT- 3′) (Ritchie et al. 1997). The PCR reaction mixture comprised 2 µl of cell suspension, 0.2 µl of Taq DNA polymerase (Invitrogen Life Technologies), 2.5 µl of forward and reverse primers, 1.5 µl of 50 mM $MgCl_2$ (Invitrogen), 5 µl 10X PCR Rxn Buffer (Invitrogen), 1 µl of 10 mM dNTPs and 35.3 µl of Ultra Pure grade water Hyclone® (Thermo Scientific) to total 50 µl for a single reaction. The cycling parameters were: 5 min at 95 °C followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s and a final hold at 72 °C for 7 min. The PCR products were analyzed in 1.2 % (p/v) agarose gel electrophoresis stained with GelRed Nucleic Acid Gel StainTM (Biotium, Hayward, CA). Purified amplicons were sequenced by Macrogen Inc. (Seoul, Korea), and used for phylogenetic analysis.

Phylogenetic analysis

The chromatograms for partial 16S rRNA sequences, were analyzed and edited with Geneious software (Biomatters Ltd, NZ). Sequence alignments and phylogenetic analyses were conducted in MEGA7 (Kumar et al. 2016). The alignments were made by ClustalX and were edited manually. The phylogenetic tree was inferred by the Neighbor-Joining method (Saitou and Nei 1987) with a bootstrap analysis with 1000 replicates was performed to assess the support of the clusters (Mwenda et al. 2018). The genetic distances were computed using the Maximum Composite Likelihood method. The BLAST search tool from the National Centre for Biotechnological Information (NCBI) was used to find species closely related to strains isolated and to download type strain's genetic sequences to be included in the phylogenetic trees.

Authentication experiment

An experiment using a closed vial system was set up to confirm the ability of the different root nodule strains to nodulate *Hedysarum coronarium* (Gerding et al. 2017). Seeds were surface sterilized by immersion in 70 % (v/v) ethanol for 1 min, followed by one soak in 3 % (v/v) sodium hypochlorite for 3 min and six washes in sterile deionized water, and the sterilized seeds were placed on 1.5 % (w/v) water agar plates and incubated for 72 h at 25°C in the dark. Once the radicle had emerged the seeds were transferred to hermetic plastic vials. Four seedlings were grown in 700 ml plastic vials, containing 350 ml of sterilized substrate consisting of equal parts of sand and pearlite. After planting the seedlings, 20 ml of sterile nutrient solution devoid of nitrogen was added to each vial. The nutrient solution contained 12.3 g L^{-1} $MgSO_4.7H_2O$; 6.8 g L^{-1} KH_2PO_4; 17.5 g L^{-1} K_2SO_4; 2.5 g L^{-1} Fe EDTA; 1.2 g L^{-1} $CaSO_4$; 0.464 mg L^{-1} H_3BO_3; 0.018 mg L^{-1} $Na_2MoO_4.2H_2O$; 0.539 mg L^{-1} $ZnSO_4.7H_2O$; 0.042 mg L^{-1} $MnSO_4,4H_2O$; 0.141 mg L_{-1} $CoSO_4.7H_2O$ and 0.125 mg L^{-1} $CuSO_4$ (CRS Plant Growth Nutrient Solution, Yates et al. 2016).

After the emergence, sulla seedlings were inoculated with 1 ml of each genetically different strain (**T1**, **T2**, **T3**, **T4** and **T5**) in yeast mannitol broth liquid medium adjusted to an OD_{600nm} of 1, Two control treatments were included: nitrogen fertilized with 7.5 ml of 10 g L^{-1} KNO_3 uninoculated (N^+) and uninoculated devoid of nitrogen (N^-). Five plant replicates per treatment were grown in a temperature controlled phytotron at 25 ± 2 °C, under axenic conditions for 17 weeks. At harvest, plants were carefully removed from the vials, and washed in tap water.

Number of nodules, nodule score and nodule effectivity were measured. The nodule score was assessed using a modified version of Howieson and Ewing (1989) in Table 1, in which the total plant score is based upon the sum of each individual nodules scores which are themselves the product of their size and position. The variable nodule effectivity was determined dissecting each nodule to see its internal coloration (white, pink or red); if the coloration was red or pink the nodule was effective and if it was white, the nodule was considered ineffective. Two nodules were sampled from each plant and the re-isolated bacteria were fingerprinted with the primer RPO1 to confirm their identity (Gerding et al. 2017).

Compatibility between strains

The strains *Mesorhizobium* **T3** and *Rhizobium* **T5** (strains that showed an increase in nodulation in sulla roots), were tested for compatibility with *Rhizobium sullae* WSM1592 **T1**, *Ensifer meliloti* WSM2141, rhizobia strain used in Australia, and *E. meliloti* AG118 strain obtained from the Chilean Patagonia that showed the best symbiotic efficiency (Galaz 2019). To assess the compatibility, all the strains were standardized at OD_{600nm} of 1. One hundred µl of **T3** and **T5** were distributed superficially in YMA plates using a glass rod and immediately after the absorption, 3 drops of 10 µl of **T1**, WSM2141 and AG118 were deposited above to see if as the bacteria grew, a halo of inhibition was formed by any of them. Four replicates of each bacteria were included in the analysis.

IAA production capacity

To quantify IAA, a calibration curve was made with IAA concentration between 0 and 50 µg ml^{-1}. An aliquot of 0.6 ml of each dilution was mixed with 0.4 ml of reagent Salkowski (98 ml perchloric acid 35%, 2 ml 0.5 *M* $FeCl_3$) (Sarwar and Kremer 1995). After 30 min of incubation at ambient temperature the absorbance was measured at a wavelength of 530 nm in a spectrophotometer. Data of absorbance and IAA concentration was fitted to a linear regression ($R = 0.99$; $P < 0.001$) to obtain a calibration curve. To evaluate IAA production by each isolate, two loopfuls of cells were inoculated in 5 ml of standard nutrient broth and incubated at 24 ± 2°C in an orbital shaker at 160 rpm for 85 hours. One ml of the bacterial suspension was placed in Eppendorf tubes, and centrifuged at 5000 rpm for 3 min. A sample of 0.6 ml of the supernatant was mixed with 0.4 ml of reagent Salkowski and analyzed with a

spectrophotometer to determine IAA concentration as described above (Sepúlveda-Caamaño et al. 2018). After the determination, the OD of the strains was measured to standardize at the same optical density and to determine the amount of IAA of each rhizobia treatment.

Assessing the timing of nodule initiation and development on *H. coronarium* and *Medicago sativa*

The experiment was designed as the authentication essay with modifications. Surface sterilized germinated seeds of sulla and lucerne (Q31) were sowed in the hermetic vials and immediately inoculated with 1 ml of bacterial suspension in 1% w v^{-1} sucrose. All the rhizobia strains tested were compatible so the treatments used for sulla were: *R. sullae* WSM1592 alone, *R. sullae* WSM1592 + *Mesorhizobium* T3 and *R. sullae* WSM1592+ *Rhizobium* T5, and for lucerne: *E. meliloti* WSM2141 alone, *E. meliloti* WSM2141 + *Mesorhizobium* T3, *E. meliloti* WSM2141 + *Rhizobium* T5, *E. meliloti* AG118 alone, *E. meliloti* AG118 + *Mesorhizobium* T3 and *E. meliloti* AG118 + *Rhizobium* T5. The bacteria were standardized to an OD_{600nm} of 1 for each strain alone and 0.5 of each strain in co-inoculation (Fox et al. 2011). Forty-eight hermetic vials were included per experimental, since the evaluations on time were destructive. Sulla and lucerne plants were harvested 5, 7, 10, 14, 17, 21 and 26 DAI for nodule initials counting, and measurement of root depth and aerial height.

Plants were grown in a phytotron with 170.0 µmol s^{-1} for a maximum of 26 DAI and fertilized with a 20 ml of a nutrient solution devoid of nitrogen (refer to authentication experiment) at the moment of sowing. For nodule initials counting, roots were cleared in 10% w v^{-1} KOH at room temperature for 2 h, rinsed with water, then acidified in 0.25 M HCl for 5 min, stained in 0.1% (w/v) Brilliant green for 30 min and de-stained in water overnight (Fox et al. 2011). Nodule initials were distinguished from lateral root initials and were counted under a dissecting microscope at x 20 magnification (Cheng et al. 2002; Fox et al. 2011).

Symbiotic effectiveness of isolated strains in co-inoculation with *R. sullae* and *Ensifer meliloti.*

Germinated seeds of sulla and lucerne were sowed in 500 ml plastic pots. Immediately after sowing, plantlets were inoculated with 1 ml of bacteria suspended in 1% w v^{-1} sucrose which was standardized to an OD_{600nm} of 1 and applied to the root area. The bacterial treatments were

the same as used in the Nodule initiation and development experiment and two non-inoculated controls were also included (N- and N+) which were supplied weekly with a nutrient solution devoid of nitrogen and with 5 ml of 0.1 g L^{-1} KNO_3 solution, respectively (refer to authentication experiment). All plants were watered three times a week with 20 ml of DI water.

After 42 DAI shoots and roots of harvested plants were separated at the hypocotyl. Root and aerial height was measured, nodules were counted and individual nodules were scored using Table 1. The shoots and roots were then dried for 2 d at 60°C prior to weighing to assess dry matter production. The root area was determined using the ImageJ software. The roots were scanned, the images were converted to binary images, to associate the number of black pixels to a specific length, using ImageJ, to finally determine total root area.

Data analysis

Nodule initial counts were plotted on time, and the area under the nodulation progress curve (AUNPC) was calculated for each inoculation treatment (Sepúlveda-Caamaño et al. 2018). Number of nodules, nodule scoring, nodule effectivity, shoot height, dry matter, nitrogen effectivity, root depth, root area data and the AUNPC were subjected to an analysis of variance (ANOVA) which was carried out with the statistics software INFOSTAT version 2008 (Di Rienzo et al. 2008). The mean separation test used was the Fisher's least significant difference (LSD) test with α =0.05. Variables that did not meet some of the ANOVA assumptions were transformed to square root, and those that could not be adjusted despite having transformed them, were analyzed with non-parametric analysis, as in the case of nodule effectivity and root depth.

Results

Bacteria isolation and phylogeny based on 16S rRNA

Twenty nodules were collected from the first site and 51 from the second site resulting in 64 isolates. After analysis of the RPO1-PCR fingerprinting patterns, only 5 distinct strains were identified as **T1**, **T2**, **T3**, **T4** and **T5**.

A 690 to 1020 bp internal fragment of the 16S rRNA gene was successfully amplified for the 5 strains. The analysis of the 16S rRNA gene sequences (Fig. 1) allowed grouping 3 strains within the genus *Mesorhizobium* and 2 strains within the genus *Rhizobium*, supported with 95 and 99% bootstrap value respectively. Within the *Mesorhizobium* genus clade, 2 subclades were formed, both with 100% bootstrap value. The first subclade (A) included **T3**, **T4**, *M. ciceri*, *M. loti*, *M. ginshengii*, *M. shangrilense*, *M. australicum* and *M. cantuariense*. In the second subclade (B) strain **T2**, and the type strains *M. opportunistum*, *M. jarvisii*, *M. erdmanii*, *M. huakuii*, *M. amorphae* y *M. waimense* were grouped. Within the *Rhizobium* genus clade 2 subclades were formed with 100% and 99% bootstrap value for clade C and D respectively (Fig. 1). **T1** was grouped in clade C, which includes *Rhizobium sullae* strain WSM1592, and the type strain *Rhizobium sullae* IS123. Finally, **T5** was grouped in clade D together with *R. esperanzae*, *R. etli*, *R. acidisoli*, *R. aegyptiacum*, *R. lentis*, *R. binae*, *R. bangladeshense* y *R. anhuiense.*

Authentication of strains as root nodule bacteria

The pH of the starter fertilizer was 5.5 and the number of nodules per plant varied from 0 to 33. Nitrogenized (N+) and not nitrogenized (N-) treatments were included in all the statistical analyses because the plants of these controls formed nodules in their root systems. Due to nodulation in the uninoculated controls, the whole experiment was replicated twice, obtaining the same results.

In terms of nodule numbers, it was possible to observe significant differences between the treatments (P= 0.0101). *Rhizobium* (T5) and *Mesorhizobium* (T3) were the only treatments that differed from uninoculated controls (Fig. 2). Regarding nodule effectivity (P=0.01), only *Rhizobium* (T5) was able to reach higher values compared to uninoculated controls (Fig. 2). For the variable nodule scoring (Table 2), all the strains showed the greatest significant difference (P=0.0280) but in this case, *Mesorhizobium* (T3) and *Rhizobium* (T5) stood out compared with N+ and N- treatments.

Since controls had nodules, to confirm the ability of the strains to nodulate sulla, approximately 3 to 8 nodules were selected per treatment, bacteria were isolated and RPO1 RAPD-PCR was performed to confirm the strains identity. All the isolates obtained from

nodules from every inoculation treatment corresponded to the strain *Rhizobium sullae* WSM1592 (T1) through RPO1-PCR fingerprinting, even for isolates from treatments T2, T3, T4 and T5*,* suggesting that this strain is established as a seed endophyte in *H. coronarium*. Although apparently, strains T2, T3, T4 and T5 did not induce nodulation in *H. coronarium*, inoculation with *Rhizobium* sp. (T5) and *Mesorhizobium* (T3) had a positive effect in nodule effectivity, nodule score and number of nodules per plant (Table 2 and Fig. 2), stimulating sulla nodulation in 73% and 48% respectively in comparison with *Rhizobium sullae* WSM1592 (T1) by itself. Therefore, further experiments were planned to assess the plant growth promoting abilities and their nodule promoting abilities in co-inoculation rhizobia.

Production of Indole acetic acid by nodular endophytes and rhizobia

All the strains were able to produce IAA (Table 3). NAB strains isolated from *Hedysarum coronarium* nodules produced between 2.24 to 12.62 µg IAA ml^{-1}, where *Mesorhizobium* T3 outstood as the largest producer. Whereas, *Rhizobium sullae* WSM1592 was the lowest IAA producer with 1.40 µg ml^{-1}, followed by *Ensifer meliloti* WSM2141 with 3.60 µg ml^{-1}, and the largest producer was *Ensifer meliloti* AG118, a naturalised rhizobia isolated from Chilean Patagonia with 17.45 µg IAA ml^{-1} (Table 3).

The strain that produced the highest amount of IAA in medium without the addition of tryptophan was *Ensifer meliloti* AG118, followed by *Mesorhizobium* T3 and the strains that produced the lowest amount of IAA were *Rhizobium sullae* WSM1592, *Ensifer meliloti* WSM2141 and *Rhizobium* T5, without statistical differences between them (Table 3).

Assessing the timing of nodule initiation and nodule development on *H. coronarium* and *Medicago sativa*

In general terms there were no significant effects of co-inoculation in the timing of nodulation in *Hedysarum coronarium* (Fig. 3, Table 4) or in *Medicago sativa* (Fig. 4, Table 4). Only at day 10 after inoculation, the treatment *R. sullae* WSM1592 + *Mesorhizobium* T3 induced a higher number of nodule initials in *H. coronarium* in comparison to the other two treatments, however, this rise was not significantly different (P=0.0692) (Fig. 3).

In terms of root depth, sulla plants showed higher values 14 days after inoculation (P <0.01) with the *R. sullae* WSM1592 + *Mesorhizobium* sp. T3 treatment (Fig. 5) in comparison with *R. sullae* WSM1592 alone. For lucerne, it was also possible to observe differences using the Chilean native rhizobia and the Australian strain WSM2141 in co-inoculation with the nodular endophytes. The greatest depth in co-inoculation with respect to rhizobia alone was only achieved with *E. meliloti* AG118 + *Rhizobium* sp. T5 (P <0.01) 5 days after inoculation and for *E. meliloti* WSM2141 + *Mesorhizobium* sp. T3 (P <0.01) 10 days after inoculation (Fig. 6).

Symbiotic effectiveness of isolated strains in co-inoculation with *R. sullae* and *Ensifer meliloti*

For sulla, there were significant differences in shoot height (P=0.0494), shoot dry matter (P=0.0282), nitrogen efficiency (P=0.0225) and root area (P=0.0067), but there were no differences in number of nodules, nodule scoring, nodule effectivity and root depth (P>0.05). The N+ treatment, *Rhizobium sullae* WSM1592 alone and in co-inoculation with *Mesorhizobium* T3 and *Rhizobium* T5 all induced greater height in plants and greater dry weight compared with the N- control (Table 5). Root area in *H. coronarium* varied from 0.83 cm^2 to 7.40 cm^2, where the N+ control outstood with highest area, followed by *R. sullae* WSM1592 + *Mesorhizobium* T3, that was also significantly different from the N- control (Table 5).

Results in lucerne showed significant differences in nodulation, shoot height, shoot dry matter, nitrogen efficiency, root depth and root area, and non-significant differences in nodule scoring and nodule effectivity (Table 5). *Ensifer meliloti* WSM2141 and AG118 induced the same amount of nodules in lucerne roots (Table 5). The rhizobial treatment that induced the higher number of nodules in lucerne roots was *E. meliloti* WSM2141 + *Rhizobium* T5, treatment significantly different to *E. meliloti* AG118 + *Mesorhizobium* T3 and *E. meliloti* WSM2141+ *Mesorhizobium* T3 (Table 5). All *E. meliloti* strains, either alone or in co-inoculation with *Mesorhizobium* T3 and *Rhizobium* T5, reached the same height as the N^+ control, and all were statistically differentiated from the N^- control (Table 5). The highest shoot dry matter was reached by the N+ control. *E. meliloti* AG118 alone or in co-inoculation, and *E. meliloti* WSM2141 + *Rhizobium* T5 did not statistically differentiate from the control with N, however, *E. meliloti* WSM2141 alone or in co-inoculation with *Mesorhizobium* T3 could not reach the highest dry weight values, in spite of their statistical differences from the control

without N (Table 5). Lucerne N- and inoculated with *E. meliloti* AG118 reached the highest values in root depth and were different from *E. meliloti* WSM2141, N+ and *E. meliloti* AG118 + *Mesorhizobium* T3 (Table 5). The root area trait in lucerne plants varied from 3.56 cm^2 to 6.33 cm^2, where also N+ control outstood with highest area and was followed by all *E. meliloti* AG118 treatments alone or in co-inoculation. All *E. meliloti* WSM2141 treatments alone or in co-inoculation were not significantly different from the N- control (Table 5).

Regarding the nitrogen fixation effectiveness in sulla (Fig. 7), only co-inoculations were able to induce values significantly higher than the N- control. *R. sullae* WSM1592 alone only reached 60% of efficiency + dry weight, being classified as a partially effective strain; *R. sullae* WSM1592 with *Rhizobium* T5 reached around 70% and coinoculated with *Mesorhizobium* T3 it reached around 85%. Although both coinoculations differed significantly from the N- control, the first was classified as partially effective, and the second as an effective strain (Fig. 7). In the case of Lucerne, no significant differences were found between treatments in terms of percentage of mean shoot dry weight. In terms of nitrogen efficiency in lucerne, all rhizobia treatments differed from the control without N, however, *E. meliloti* WSM2141 alone and *E. meliloti* WSM2141 + *Mesorhizobium* T3 were classified as partially effective strains because they did not reach 75% efficiency (Fig. 7).

Discussion

Sulla (*Hedysarum coronarium*) and lucerne (*Medicago sativa*) have emerged as forage alternatives for Mediterranean drylands in central Chile. However, particularly in the case of sulla, their symbiotic relationship with rhizobia has not been as efficient as expected (Ovalle et al. 2015). From established sulla plants, root nodule bacteria other than the inoculant were isolated. Some of these strains were identified as *Mesorhizobium* spp, and were clustered close to strains associated with *Amorpha fruticosa* legumes, species of the *Astralagus* genus, with the *Sophora* genus, *Biserrula pelecinus*, *Lotus corniculatus* and *Cicer arietinum*. Strains of the *Rhizobium* genus in clade C (Fig 1) correspond to the symbionts reported for *Hedysarum coronarium*. *Rhizobium sullae* IS 123 is a type strain which was described by Squartini et al. (2002) and was isolated from nodules of *Hedysarum coronarium* in southern Spain. The strain WSM1592 was isolated in 1995 from *H. coronarium* sampled in Italy and is the current Australian commercial inoculant due its high effectivity in fixing atmospheric nitrogen (Yates

et al. 2015). The phylogenetic analysis of a study conducted by Yates et al. (2015) revealed that the strain WSM1592 is the most genetically related to the type strain IS 123. *Rhizobium* strains located in clade D are associated with pulses such as *Vicia faba*, *Lens culinaris*, *Phaseolus vulgaris* and the forage legume, *Trifolium alexandrinum*. Of all these legumes mentioned above, *B. pelecinus* and *L. corniculatus* were evaluated by Ovalle et al. (2004, 2015) in the same experimental station and inoculated with the rhizobial inoculants described above, which could explain the presence of these rhizobia strains in the soil. The rhizobial strains associated to pulses and *T. alexandrinum* are strains that are commonly found in Chileans soils due their extensive cultivation in the rainfed area.

Co-inoculation between *Rhizobium sullae* WSM1592 and the nodule associated rhizobia was completely accidental. The objective of the authentication test was to verify that the bacteria isolated from sulla nodules, effectively formed nodules and were able to fix atmospheric nitrogen with the original host. However, all the nodules that had reddish and pink coloration contained the *Rhizobium sullae* WSM1592 symbiont. One possible explanation for this is the possibility that the seeds contained endophytic *Rhizobium sullae*. This is supported by the fact that plants were inoculated and grew in hermetic and sterile vials, with sterilized substrate; and the experiment was replicated twice showing the same results. Diverse authors have reported endophytic bacteria in legume seeds (Oehrle et al. 2000; López-López et al. 2010; Mora et al. 2014; Aguilar et al. 2016, 2018; Alibrandi et al. 2018) but recently, it was demonstrated that nitrogen-fixing rhizobia is also found within legume seeds as endophytes (Mora et al. 2014). Aguilar et al. (2016) also observed nodule formation in uninoculated plants and they attributed it to seeds as a source of the bacteria. Additionally, plants collected from the second site had not been deliberately sown in that area, but emerged from the seeds that were in the soil from previous seasons experiments. In spite of this, it was possible to isolate *Rhizobium sullae* WSM1592 from nodules collected in this site, reinforcing the idea of endophytic rhizobia.

From these last results, however, we cannot assure that *R. sullae* WSM1592 is the only bacteria fixing nitrogen inside the nodule, due to the type of isolation that was performed. Identification of bacteria in nodules has traditionally relied on their cultivability when streaked on YMA plates (Lu et al. 2017). Nevertheless, such results do not provide direct information on the relative distribution of particular bacterial genera within the nodules (Xiao et al. 2017;

Hakim et al. 2019), because the method is significantly influenced by bacterial growth characteristics and/or incubation conditions (Hakim et al. 2019). For example, it is expected to see growth from nodule squashes begin with two to four days for classic fast growers, such as *Rhizobium* and *Ensifer* genus, while colony growth is seen from three to seven days for *Mesorhizobium* genus (Gao et al. 2004; Hungria et al. 2016).

What could be observed in the authentication experiment is that strains *Mesorhizobium* T3 and *Rhizobium* T5, isolated from sulla nodules, apparently promoted colonization by *Rhizobium sullae* WSM1592, an effect that was verified when performing the *in vivo* experiment in both sulla and lucerne.

The two experiments performed with sulla *in vivo* indicate that co-inoculation of *Rhizobium sullae* WSM1592, a highly specific symbiont of *Hedysarum coronarium*, together with the strains isolated from their nodules do not exert a negative effect on any of the measured variables, on the contrary, they promote symbiotic performance and root development. The advantages of co-inoculation versus using simple inoculation are reflected in: greater early root depth and nodule formation, greater nitrogen efficiency and root area 6 weeks post-inoculation and greater number of nodules and nodule scoring at the end of the experiment using the *R. sullae* WSM1592 + *Mesorhizobium* T3 combination for all cases. Inoculation with *R. sullae* WSM1592 + *Rhizobium* T5 is also beneficial versus single inoculation in terms of greater nitrogen efficiency 6 weeks post-inoculation, greater number of nodules and nodule scoring after 17 of growth. The results in lucerne confirm that co-inoculation of *Ensifer meliloti* with strains isolated from sulla nodules can also induce benefits on this species versus single inoculation, but this behavior depends on the *Ensifer meliloti* and the endophyte strain combination. During the first 10 days after inoculation, *E. meliloti* AG118 with *Rhizobium* T5 induced greater root length than with single inoculation, while for *E. meliloti* WSM2141 the same effect can be observed in co-inoculation with *Mesorhizobium* T3. Also lucerne plants inoculated with *E. meliloti* WSM2141 and *Rhizobium* T5 had significantly more nodules than plants co-inoculated with *Mesorhizobium* T3. An interesting observation is that *E. meliloti* AG118, alone and independent of the co-inoculant, can achieve the greatest values in all the variables measured (Table 5). Finally, the results reflect that, in lucerne, the *E. meliloti* WSM2141 + *Mesorhizobium* T3 combination is not harmful to the plant, however, it fails to

achieve maximum symbiotic benefits in association with the plant compared to other treatments such as *E. meliloti* WSM2141 + *Rhizobium* T5. On the other hand, single *E. meliloti* AG118 inoculation manages to match the symbiotic performance of *E. meliloti* WSM2141 + *Rhizobium* T5, with the advantage that this strain induces greater root depth and root area.

NAB members can provide beneficial services to their host plants in co-inoculation with rhizobia, improving the symbiotic performance through increase in nodulation, nitrogen fixation and leghemoglobin content (Mishra et al. 2009; Khalifa and Almalki 2015; Subramanian et al. 2015; Egamberdieva et al. 2017), also are capable of promoting the legume yield (Saïdi et al. 2013; Martínez-Hidalgo et al. 2015; Le et al. 2016; Egamberdieva et al. 2017) and root development (Khalifa and Almalki 2015). This is consistent with our results, which showed improvements in both symbiosis and root development in sulla and lucerne using NAB and rhizobia. The production of the auxin IAA has been reported for rhizobia of genera *Rhizobium* (Bhattacharjee et al. 2012; García-Fraile et al. 2012; Flores-Félix et al. 2013), *Ensifer* (Bianco and Defez 2010) and *Mesorhizobium* (Wdowiak-Wróbel and Małek 2016), reports that are coincident with our results since the WSM1592, WSM2141, AG118, T3 and T5 strains used in the *in vivo* essays, in both sulla and lucerne, belong to the *Rhizobium*, *Ensifer* and *Mesorhizobium* genus. All the benefits observed in terms of symbiosis and root depth in sulla when inoculated with *R. sullae* WSM1592 + *Mesorhizobium* T3 and in lucerne inoculated with *E. meliloti* AG118 alone can be explained by the high production of IAA by *Mesorhizobium* T3 and *E. meliloti* AG118. IAA is involved in the increase in the number of nodules (Boiero et al. 2007; Ali et al. 2008) and also enhances development of longer roots with an increased number of root hairs and lateral roots (Wdowiak-Wróbel and Małek 2016; Sindhu et al. 2019). An interesting case occurs with *E. meliloti* AG118 + *Mesorhizobium* T3 which, despite the negative induction of nodules, it was still classified as an effective treatment. This could be explained by the high amounts of IAA that both strains produce, amount that probably increased the activity of the nitrogenase enzyme in each nodule, enzyme which transforms atmospheric nitrogen into ammonia that plants can then use for growth (Smith 2002). Defez et al. (2019) reported that higher values of IAA produced by a genetically modified *E. meliloti* strain (56 μM IAA) increases the expression of nitrogen fixation genes and the activity of the nitrogenase enzyme compared to a poor IAA producing

E. meliloti strain (0.60 µM). Ali et al. (2008) also reported that application of IAA improved growth, leghemoglobin content and nitrogenase activity in mung bean plants. In addition, IAA has proven to be an important hormone to increase salinity resistance (Bianco and Defez 2009) and drought response in plants (Defez et al. 2017).

The greater induction of nodules and nitrogen fixation with the combination *E. meliloti* WSM2141 + *Rhizobium* T5 in lucerne is not explained by IAA production of WSM2141 or T5. *Rhizobium* T5 could have induced greater nodulation for other reasons, it could produce ACC deaminase that also improves nodulation (Shahzad et al. 2010; Zahir et al. 2011) or maybe another hormone such as cytokinins, which along with IAA, generate nodule primordia in response to rhizobia (Boivin et al. 2016).

The big difference between the two co-inoculations in sulla is that *R. sullae* WSM1592 + *Mesorhizobium* T3 is able to induce 13% more biomass than *R. sullae* WSM1592 + *Rhizobium* T5, expressed in N fixation effectiveness (Fig 7). Nitrogen fixation effectiveness delta is more pronounced when comparing *R. sullae* WSM1592 + *Mesorhizobium* T3 to *R. sullae* WSM1592 alone, with an increase of 24.6%. This delta is very important, especially in Mediterranean dryland where sulla is desired to be established, since drought stress causes severe effects on plant growth and impairs the process of nitrogen fixation (Furlan et al. 2017). As for the increase in root depth caused by the co-inoculation of both legumes during the first fourteen and 42 days after inoculation, the increase is beneficial for the establishment of forage species under rainfed conditions. In any of these perennial species, which are characterized by deep roots, the fact that it accelerates the deepening rate means that they will reach an underground water source in less time compared to the same legume inoculated with single rhizobia. In addition, if this acceleration in the deepening rate is accompanied by a greater production of secondary roots (root area), it is a perfect combination for the plant to generate higher nodulation points and also acquire nutrients that cannot be supplied by the rhizobial inoculant.

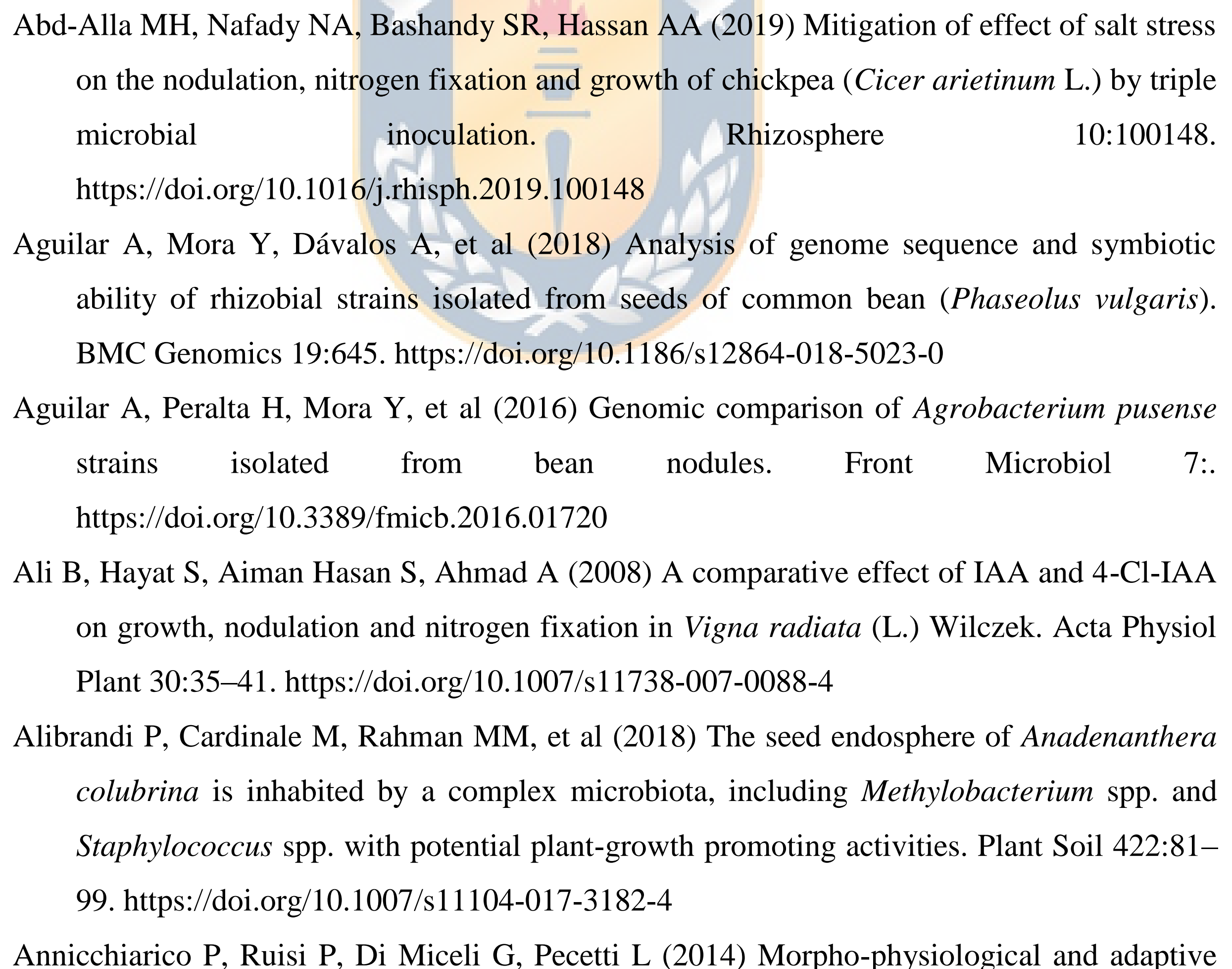
Conclusions

There are NAB associated *Hedysarum coronarium* that are rhizobia residing in Chilean soils. Some of these NAB are benefiting *H. coronarium* and *Medicago sativa* plants by increasing

number of nodules, nitrogen efficiency, depth and root area when used in co-inoculation with the specific nitrogen-fixing rhizobia of each legume. These findings could be promising for the establishment of legumes in drought stress conditions, as occurs in the Chilean dryland soils. However, future field essays are necessary to confirm our results, especially in this climate.

Acknowledgements

Paola Sobarzo-Palma was recipient of a Postgraduate Studies Scholarship from the Agronomy faculty at the University of Concepción. This study was carried out thanks to the financial support of the Fondo Nacional de Desarrollo Científico y Tecnológico, Project FONDECYT Nº 1180821 and FONDECYT N° 1180829.



References

Abd-Alla MH, Nafady NA, Bashandy SR, Hassan AA (2019) Mitigation of effect of salt stress on the nodulation, nitrogen fixation and growth of chickpea (*Cicer arietinum* L.) by triple microbial inoculation. Rhizosphere 10:100148. https://doi.org/10.1016/j.rhisph.2019.100148

Aguilar A, Mora Y, Dávalos A, et al (2018) Analysis of genome sequence and symbiotic ability of rhizobial strains isolated from seeds of common bean (*Phaseolus vulgaris*). BMC Genomics 19:645. https://doi.org/10.1186/s12864-018-5023-0

Aguilar A, Peralta H, Mora Y, et al (2016) Genomic comparison of *Agrobacterium pusense* strains isolated from bean nodules. Front Microbiol 7:. https://doi.org/10.3389/fmicb.2016.01720

Ali B, Hayat S, Aiman Hasan S, Ahmad A (2008) A comparative effect of IAA and 4-Cl-IAA on growth, nodulation and nitrogen fixation in *Vigna radiata* (L.) Wilczek. Acta Physiol Plant 30:35–41. https://doi.org/10.1007/s11738-007-0088-4

Alibrandi P, Cardinale M, Rahman MM, et al (2018) The seed endosphere of *Anadenanthera colubrina* is inhabited by a complex microbiota, including *Methylobacterium* spp. and *Staphylococcus* spp. with potential plant-growth promoting activities. Plant Soil 422:81–99. https://doi.org/10.1007/s11104-017-3182-4

Annicchiarico P, Ruisi P, Di Miceli G, Pecetti L (2014) Morpho-physiological and adaptive

variation of Italian germplasm of sulla (*Hedysarum coronarium* L.). Crop Pasture Sci 65:206–213. https://doi.org/10.1071/CP13342

Armendariz AL, Talano MA, Olmos Nicotra MF, et al (2019) Impact of double inoculation with *Bradyrhizobium japonicum* E109 and *Azospirillum brasilense* Az39 on soybean plants grown under arsenic stress. Plant Physiol Biochem 138:26–35. https://doi.org/10.1016/j.plaphy.2019.02.018

Bai Y, D'Aoust F, Smith DL, Driscoll BT (2002) Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. Can J Microbiol 48:230–238. https://doi.org/10.1139/w02-014

Benabderrahim MA, Hamza H, Haddad M, Ferchichi A (2015) Assessing the drought tolerance variability in Mediterranean alfalfa (*Medicago sativa* L.) genotypes under arid conditions. Plant Biosyst 149:395–403. https://doi.org/10.1080/11263504.2013.850121

Bhattacharjee RB, Jourand P, Chaintreuil C, et al (2012) Indole acetic acid and ACC deaminase-producing *Rhizobium leguminosarum* bv. *trifolii* SN10 promote rice growth, and in the process undergo colonization and chemotaxis. Biol Fertil Soils 48:173–182. https://doi.org/10.1007/s00374-011-0614-9

Bianco C, Defez R (2010) Improvement of phosphate solubilization and *Medicago* plant yield by an indole-3-acetic acid-overproducing strain of *Sinorhizobium meliloti*. Appl Environ Microbiol 76:4626–4632. https://doi.org/10.1128/AEM.02756-09

Bianco C, Defez R (2009) *Medicago truncatula* improves salt tolerance when nodulated by an indole-3-acetic acid-overproducing *Sinorhizobium meliloti* strain. J Exp Bot 60:3097–3107. https://doi.org/10.1093/jxb/erp140

Boiero L, Perrig D, Masciarelli O, et al (2007) Phytohormone production by three strains of *Bradyrhizobium japonicum* and possible physiological and technological implications. Appl Microbiol Biotechnol 74:874–880. https://doi.org/10.1007/s00253-006-0731-9

Boivin S, Fonouni-farde C, Frugier F (2016) How auxin and cytokinin phytohormones modulate root microbe interactions. Front Plant Sci 7:. https://doi.org/10.3389/fpls.2016.01240

Casella S, Gault RR, Reynolds KC, et al (1984) Nodulation studies on legumes exotic to Australia: *Hedysarum coronarium*. FEMS Microbiol Lett 22:37–45. https://doi.org/10.1111/j.1574-6968.1984.tb00350.x

Cassán F, Diaz-Zorita M (2016) *Azospirillum* sp. in current agriculture: From the laboratory to

the field. Soil Biol Biochem 103:117–130. https://doi.org/10.1016/j.soilbio.2016.08.020

Chen JY, Gu J, Wang ET, et al (2014) Wild peanut *Arachis duranensis* are nodulated by diverse and novel *Bradyrhizobium* species in acid soils. Syst Appl Microbiol 37:525–532. https://doi.org/10.1016/j.syapm.2014.05.004

Cheng Y, Watkin ELJ, O'Hara GW, Howieson JG (2002) *Medicago sativa* and *Medicago murex* differ in the nodulation response to soil acidity. Plant Soil 238:31–39. https://doi.org/10.1023/A:1014273327527

Chiboub M, Jebara SH, Saadani O, et al (2018) Physiological responses and antioxidant enzyme changes in *Sulla coronaria* inoculated by cadmium resistant bacteria. J Plant Res 131:99–110. https://doi.org/10.1007/s10265-017-0971-z

De Lajudie P, Willems A, Pot B, et al (1994) Polyphasic taxonomy of rhizobia: Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. Int J Syst Bacteriol 44:715–733. https://doi.org/10.1099/00207713-44-4-715

De Meyer SE, De Beuf K, Vekeman B, Willems A (2015) A large diversity of non-rhizobial endophytes found in legume root nodules in Flanders (Belgium). Soil Biol Biochem 83:1–11. https://doi.org/10.1016/j.soilbio.2015.01.002

Defez R, Andreozzi A, Dickinson M, et al (2017) Improved drought stress response in alfalfa plants nodulated by an IAA over-producing *Rhizobium* strain. Front Microbiol 8:. https://doi.org/10.3389/fmicb.2017.02466

Defez R, Andreozzi A, Romano S, et al (2019) Bacterial IAA-delivery into Medicago root nodules triggers a balanced stimulation of C and N metabolism leading to a biomass increase. Microorganisms 7:403. https://doi.org/10.3390/microorganisms7100403

del Pozo A, Ovalle C, Espinoza S, et al (2017) Water relations and use-efficiency, plant survival and productivity of nine alfalfa (*Medicago sativa* L.) cultivars in dryland Mediterranean conditions. Eur J Agron 84:16–22. https://doi.org/10.1016/j.eja.2016.12.002

Di Rienzo JA, Casanoves F, Balzarini MG, et al (2008) Infostat - Software Estadístico. 336

Douglas GB (1984) Seed production of sulla - A plant for soil conservation. Proc New Zeal Grassl Assoc 45:239–242

Drew E, Herridge D, Ballard R, et al (2012) Inoculating legumes: A practical guide. 71

Egamberdieva D, Wirth SJ, Shurigin V V., et al (2017) Endophytic bacteria improve plant

growth, symbiotic performance of chickpea (*Cicer arietinum* L.) and induce suppression of root rot caused by *Fusarium solani* under salt stress. Front Microbiol 8:. https://doi.org/10.3389/fmicb.2017.01887

Ferguson BJ, Indrasumunar A, Hayashi S, et al (2010) Molecular analysis of legume nodule development and autoregulation. J Integr Plant Biol 52:61–76. https://doi.org/10.1111/j.1744-7909.2010.00899.x

Ferguson BJ, Mens C, Hastwell AH, et al (2019) Legume nodulation: The host controls the party. Plant Cell Environ 42:41–51. https://doi.org/10.1111/pce.13348

Flores-Félix JD, Menéndez E, Rivera LP, et al (2013) Use of *Rhizobium leguminosarum* as a potential biofertilizer for *Lactuca sativa* and *Daucus carota* crops. J Plant Nutr Soil Sci 176:876–882. https://doi.org/10.1002/jpln.201300116

Fox SL, O'Hara GW, Bräu L (2011) Enhanced nodulation and symbiotic effectiveness of *Medicago truncatula* when co-inoculated with *Pseudomonas fluorescens* WSM3457 and *Ensifer* (*Sinorhizobium*) *medicae* WSM419. Plant Soil 348:245. https://doi.org/10.1007/s11104-011-0959-8

Furlan AL, Bianucci E, Castro S, Dietz KJ (2017) Metabolic features involved in drought stress tolerance mechanisms in peanut nodules and their contribution to biological nitrogen fixation. Plant Sci 263:12–22. https://doi.org/10.1016/j.plantsci.2017.06.009

Galaz L (2019) Diversidad de rizobios asociados a praderas de alfalfa (*Medicago sativa*) en la Región de Magallanes. Universidad de Concepción

Gano-cohen KA, Stokes PJ, Blanton MA, et al (2016) Nonnodulating *Bradyrhizobium* spp. modulate the benefits of Legume-Rhizobium mutualism. Appl Environ Microbiol 82:5259–5268. https://doi.org/10.1128/AEM.01116-16.

Gao JL, Turner SL, Kan FL, et al (2004) *Mesorhizobium septentrionale* sp. nov. and *Mesorhizobium temperatum* sp. nov., isolated from *Astragalus adsurgens* growing in the northern regions of China. Int J Syst Evol Microbiol 54:2003–2012. https://doi.org/10.1099/ijs.0.02840-0

García-Fraile P, Carro L, Robledo M, et al (2012) *Rhizobium* promotes non-legumes growth and quality in several production steps: Towards a biofertilization of edible raw vegetables healthy for humans. PLoS One 7:e38122. https://doi.org/10.1371/journal.pone.0038122

Gerding M, Oyarzúa P, García L, et al (2017) Diversity and symbiotic effectiveness of

Adesmia spp. root nodule bacteria in central and southern Chile. Symbiosis 72:61–72. https://doi.org/10.1007/s13199-016-0440-9

Glatzle A, Schulte-Batenbrock T, Brockwell J (1986) Symbiotic incompatibility between two forage species of *Hedysarum*, grown in Morocco, and their homologous rhizobia. FEMS Microbiol Lett 37:39–43

Gourion B, Berrabah F, Ratet P, Stacey G (2015) Rhizobium-legume symbioses: The crucial role of plant immunity. Trends Plant Sci 20:186–194. https://doi.org/10.1016/j.tplants.2014.11.008

Hakim S, Mirza BS, Imran A, et al (2019) Illumina sequencing of 16S rRNA tag shows disparity in rhizobial and non-rhizobial diversity associated with root nodules of mung bean (*Vigna radiata* L.) growing in different habitats in Pakistan. Microbiol Res 231:126356. https://doi.org/10.1016/j.micres.2019.126356

Hakim S, Mirza BS, Zaheer A, et al (2018) Retrieved 16S rRNA and nifH sequences reveal co-dominance of *Bradyrhizobium* and *Ensifer* (*Sinorhizobium*) strains in field-collected root nodules of the promiscuous host *Vigna radiata* (L.) R. Wilczek. Appl Microbiol Biotechnol 102:485–497. https://doi.org/10.1007/s00253-017-8609-6

Hoque MS, Broadhurst LM, Thrall PH (2011) Genetic characterization of root-nodule bacteria associated with *Acacia salicina* and *A. stenophylla* (Mimosaceae) across south-eastern Australia. Int J Syst Evol Microbiol 61:299–309. https://doi.org/10.1099/ijs.0.021014-0

Howieson JG, Ewing MA (1989) Annual species of *Medicago* differ greatly in their ability to nodulate on acid soils. Aust J Agric Res 40:843–850

Hungria M, O'Hara GW, Zilli JE, et al (2016) Isolation and growth of rhizobia. In: Howieson J, Dilworth M (eds) Working with rhizobia. Australian Centre for International Agricultural Research, Cranberra. pp 39-60

Ibáñez F, Angelini J, Taurian T, et al (2009) Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. Syst Appl Microbiol 32:49–55. https://doi.org/10.1016/j.syapm.2008.10.001

Ibáñez F, Tonelli ML, Muñoz V, et al (2017) Bacterial endophytes of plants: Diversity, invasion mechanisms and effects on the host. In: Maheshwari DK (ed) Endophytes: Biology and Biotechnology. Sustainable Development and Biodiversity. Springer Cham, pp 25–40

Jian L, Bai X, Zhang H, et al (2019) Promotion of growth and metal accumulation of alfalfa

by coinoculation with *Sinorhizobium* and *Agrobacterium* under copper and zinc stress. PeerJ 7:. https://doi.org/10.7717/peerj.6875

Ju W, Liu L, Fang L, et al (2019) Impact of co-inoculation with plant-growth-promoting rhizobacteria and rhizobium on the biochemical responses of alfalfa-soil system in copper contaminated soil. Ecotoxicol Environ Saf 167:218–226. https://doi.org/10.1016/j.ecoenv.2018.10.016

Khalifa AYZ, Almalki MA (2015) Isolation and characterization of an endophytic bacterium, *Bacillus megaterium* BMN1, associated with root-nodules of *Medicago sativa* L. growing in Al-Ahsaa region, Saudi Arabia. Ann Microbiol 65:1017–1026. https://doi.org/10.1007/s13213-014-0946-4

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054

Lai WA, Asif A, Lin SY, et al (2015) *Paenibacillus medicaginis* sp. nov. a chitinolytic endophyte isolated from a root nodule of alfalfa (*Medicago sativa* L.). Int J Syst Evol Microbiol 65:3853–3860. https://doi.org/10.1099/ijsem.0.000505

Le XH, Franco CMM, Ballard RA, Drew EA (2016) Isolation and characterisation of endophytic actinobacteria and their effect on the early growth and nodulation of lucerne (*Medicago sativa* L.). Plant Soil 405:13–24. https://doi.org/10.1007/s11104-015-2652-9

Leach JE, Triplett LR, Argueso CT, Trivedi P (2017) Communication in the phytobiome. Cell 169:587–596. https://doi.org/10.1016/j.cell.2017.04.025

Liu J, Wang ET, Ren DW, Chen WX (2010) Mixture of endophytic agrobacterium and *Sinorhizobium meliloti* strains could induce nonspecific nodulation on some woody legumes. Arch Microbiol 192:229–234. https://doi.org/10.1007/s00203-010-0543-2

López-López A, Rogel MA, Ormeño-Orrillo E, et al (2010) Phaseolus vulgaris seed-borne endophytic community with novel bacterial species such as *Rhizobium endophyticum* sp. nov. Syst Appl Microbiol 33:322–327. https://doi.org/10.1016/j.syapm.2010.07.005

Lu J, Yang F, Wang S, et al (2017) Co-existence of rhizobia and diverse non-rhizobial bacteria in the rhizosphere and nodules of *Dalbergia odorifera* seedlings inoculated with *Bradyrhizobium elkanii*, *Rhizobium multihospitium*-Like and *Burkholderia pyrrocinia*-like strains. Front Microbiol 8:. https://doi.org/10.3389/fmicb.2017.02255

Martínez-Hidalgo P, Galindo-Villardón P, Igual JM, Martínez-Molina E (2015)

Micromonospora from nitrogen fixing nodules of alfalfa (*Medicago sativa* L.). A new promising plant probiotic bacteria. Sci Rep 4:6389. https://doi.org/10.1038/srep06389

Martínez-Hidalgo P, Hirsch AM (2017) The nodule microbiome: N_2-fixing rhizobia do not live alone. Phytobiomes J 1:70–82. https://doi.org/10.1094/pbiomes-12-16-0019-rvw

Martínez GI, Ovalle C, Pozo A., et al (2011) Influence of conservation tillage and soil water content on crop yield in dryland compacted Alfisol of central Chile. Chil J Agric Res 71:615–622. https://doi.org/10.4067/S0718-58392011000400018

Masson-Boivin C, Sachs JL (2018) Symbiotic nitrogen fixation by rhizobia — the roots of a success story. Curr Opin Plant Biol 44:7–15. https://doi.org/10.1016/j.pbi.2017.12.001

Mishra PK, Mishra S, Selvakumar G, et al (2009) Coinoculation of *Bacillus thuringeinsis*-KR1 with *Rhizobium leguminosarum* enhances plant growth and nodulation of pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.). World J Microbiol Biotechnol 25:753–761. https://doi.org/10.1007/s11274-009-9963-z

Mora Y, Díaz R, Vargas-Lagunas C, et al (2014) Nitrogen-fixing rhizobial strains isolated from common bean seeds: Phylogeny, physiology, and genome analysis. Appl Environ Microbiol 80:5644–5654. https://doi.org/10.1128/AEM.01491-14

Mwenda GM, O'Hara GW, De Meyer SE, et al (2018) Genetic diversity and symbiotic effectiveness of *Phaseolus vulgaris*-nodulating rhizobia in Kenya. Syst Appl Microbiol 41:291–299. https://doi.org/10.1016/j.syapm.2018.02.001

Oehrle NW, Karr DB, Kremer RJ, Emerich DW (2000) Enhanced attachment of *Bradyrhizobium japonicum* to soybean through reduced root colonization of internally seedborne microorganisms. Can J Microbiol 46:600–606. https://doi.org/10.1139/w00-030

Oskouei, Bekmen, Ellidokuz Y (2010) Evaluation of different cryoprotective agents in maintenance of viability of Helicobacter pylori in stock culture media. Brazilian J Microbiol 41:1038–1046

Ovalle C, Espinoza S, Barahona V, et al (2015) Lucerne and other perennial legumes provide new options for rain fed livestock production in the Mediterranean-climate region of Chile. Cienc e Investig Agrar 42:461–474. https://doi.org/10.4067/S0718-16202015000300014

Ovalle C, S SA, L AP, et al (2004) Atributos y antecedentes del comportamiento de *Biserrula pelecinus* L. nueva leguminosa forrajera anual para Chile mediterráneo. Agric Técnica

64:74–81

Peix A, Ramírez-bahena MH, Velázquez E, et al (2015) Bacterial Associations with legumes. CRC Crit Rev Plant Sci 34:17–42. https://doi.org/10.1080/07352689.2014.897899

Quiza L, St-Arnaud M, Yergeau E (2015) Harnessing phytomicrobiome signaling for rhizosphere microbiome engineering. Front Plant Sci 6:. https://doi.org/10.3389/fpls.2015.00507

Rajendran G, Patel MH, Joshi SJ (2012) Isolation and characterization of nodule-associated *Exiguobacterium* sp. from the root nodules of fenugreek (*Trigonella foenum-graecum*) and their possible role in plant growth promotion. Int J Microbiol 2012:. https://doi.org/10.1155/2012/693982

Richardson AE, Viccars LA, Watson JM, Gibson AH (1995) Differentiation of *Rhizobium* strains using the polymerase chain reaction with random and directed primers. Soil Biol Biochem 27:515–524. https://doi.org/10.1016/0038-0717(95)98626-Y

Ritchie DA, Edwards C, McDonald IR, Murrell JC (1997) Detection of methanogens and methanotrophs in natural environments. Glob Chang Biol 3:339–350. https://doi.org/10.1046/j.1365-2486.1997.00104.x

Ryalls JMW, Riegler M, Moore BD, et al (2013) Effects of elevated temperature and CO_2 on aboveground-belowground systems: A case study with plants, their mutualistic bacteria and root/shoot herbivores. Front Plant Sci 4:445. https://doi.org/10.3389/fpls.2013.00445

Sachs JL, Skophammer RG, Regus JU (2011) Evolutionary transitions in bacterial symbiosis. Proc Natl Acad Sci U S A 108:10800–10807. https://doi.org/10.1073/pnas.1100304108

Saïdi S, Chebil S, Gtari M, Mhamdi R (2013) Characterization of root-nodule bacteria isolated from *Vicia faba* and selection of plant growth promoting isolates. World J Microbiol Biotechnol 29:1099–1106. https://doi.org/10.1007/s11274-013-1278-4

Saitou N, Nei M (1987) The Neighbor-Joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Sarwar M, Kremer RJ (1995) Determination of bacterially derived auxins using a microplate method. Lett Appl Microbiol 20:282–285

Schulz B, Boyle C (2006) What are Endophytes? In: Schulz B, C. Boyle, T. N. Sieber (eds) Microbial Root Endophytes. Soil Biology, 9th edn. Springer-Verlag Berlin Heidelberg, Berlin, pp 1–13

Sepúlveda-Caamaño M, Gerding M, Vargas M, et al (2018) Lentil (*Lens culinaris* L.) growth promoting rhizobacteria and their effect on nodulation in coinoculation with rhizobia. Arch Agron Soil Sci 64:244–256. https://doi.org/10.1080/03650340.2017.1342034

Shahzad SM, Khalid A, Arshad M, et al (2010) Improving nodulation, growth and yield of *Cicer arietinum* L. through bacterial ACC-deaminase induced changes in root architecture. Eur J Soil Biol 46:342–347. https://doi.org/10.1016/j.ejsobi.2010.05.007

Silva ER, Zoz J, Oliveira CES, et al (2019) Can co-inoculation of *Bradyrhizobium* and *Azospirillum* alleviate adverse effects of drought stress on soybean (Glycine max L. Merrill.)? Arch Microbiol 201:325–335. https://doi.org/10.1007/s00203-018-01617-5

Sindhu SS, Sharma R, Sindhu S, Sehrawat A (2019) Soil fertility improvement by symbiotic rhizobia for sustainable agriculture. In: Panpatte DG, Jhala YK (eds) Soil Fertility Management for Sustainable Development. Springer Singapore, Singapore, pp 101–166

Smith BE (2002) Nitrogenase Reveals Its Inner Secrets. Science (80-) 297:1654–1655. https://doi.org/10.1126/science.1076659

Sprent JI (2007) Evolving ideas of legume evolution and diversity: A taxonomic perspective on the occurrence of nodulation. New Phytol 174:11–25. https://doi.org/10.1111/j.1469-8137.2007.02015.x

Squartini A, Struffi P, Döring H, et al (2002) *Rhizobium sullae* sp. nov. (formerly '*Rhizobium hedysari*'), the root-nodule microsymbiont of *Hedysarum coronarium* L . Int J Syst Evol Microbiol 52:1267–1276

Sreevidya M, Gopalakrishnan S, Kudapa H, Varshney RK (2016) Exploring plant growth-promotion actinomycetes from vermicompost and rhizosphere soil for yield enhancement in chickpea. Brazilian J Microbiol 47:85–95. https://doi.org/10.1016/j.bjm.2015.11.030

Staudinger C, Mehmeti-Tershani V, Gil-Quintana E, et al (2016) Evidence for a rhizobia-induced drought stress response strategy in *Medicago truncatula*. J Proteomics 136:202–213. https://doi.org/10.1016/j.jprot.2016.01.006

Sturz A V., Christie BR, Nowak J (2000) Bacterial endophytes: Potential role in developing sustainable systems of crop production. CRC Crit Rev Plant Sci 19:1–30. https://doi.org/10.1080/07352680091139169

Subramanian P, Kim K, Krishnamoorthy R, et al (2015) Endophytic bacteria improve nodule function and plant nitrogen in soybean on co-inoculation with *Bradyrhizobium japonicum* MN110. Plant Growth Regul 76:327–332. https://doi.org/10.1007/s10725-014-9993-x

Sulas L, Piluzza G, Salis M, et al (2017) Cropping systems sustainability: Inoculation and fertilisation effect on sulla performances in a new cultivation area. Ital J Agron 12:938. https://doi.org/10.4081/ija.2017.938

Tariq M, Hameed S, Yasmeen T, et al (2014) Molecular characterization and identification of plant growth promoting endophytic bacteria isolated from the root nodules of pea (*Pisum sativum* L.). World J Microbiol Biotechnol 30:719–725. https://doi.org/10.1007/s11274-013-1488-9

Velásquez E, Martínez-Hidalgo P, Carro L, et al (2013) Nodular endophytes: An untapped diversity. In: Rodelas González MB, González-López J (eds) Beneficial Plant Microbial Interactions: Ecology and Applications. CRC Press, Boca Raton, pp 214–236

Wdowiak-Wróbel S, Małek W (2016) Properties of *Astragalus* sp. microsymbionts and their putative role in plant growth promotion. Arch Microbiol 198:793–801. https://doi.org/10.1007/s00203-016-1243-3

Xiao X, Chen W, Zong L, et al (2017) Two cultivated legume plants reveal the enrichment process of the microbiome in the rhizocompartments. Mol Ecol 26:1641–1651. https://doi.org/10.1111/mec.14027

Yates R, Howieson J, De Meyer SE, et al (2015) High-quality permanent draft genome sequence of *Rhizobium sullae* strain WSM1592; a *Hedysarum coronarium* microsymbiont from Sassari, Italy. Stand Genomic Sci 10:44. https://doi.org/10.1186/s40793-015-0020-2

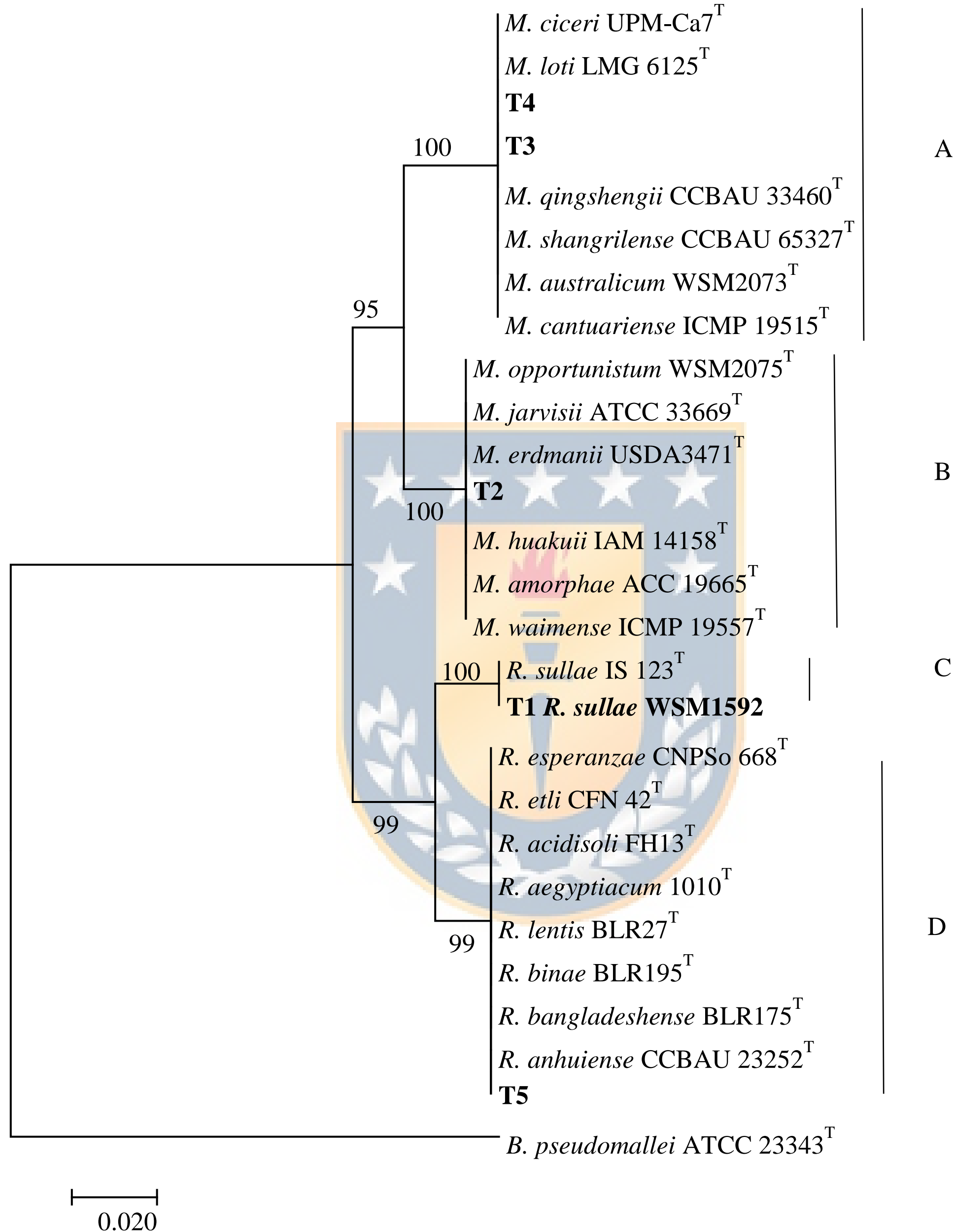
Yates RJ, Howieson JG, Hungria M, et al (2016) Authentication of rhiozbia and assessment of the legume symbiosis in controlled plant growth systems. In: Howieson J, Dilworth M (eds) Working with rhizobia. Australian Centre for International Agricultural Research, Cranberra. pp 73-108

Zahir ZA, Zafar-ul-Hye M, Sajjad S, Naveed M (2011) Comparative effectiveness of *Pseudomonas* and *Serratia* sp. containing ACC-deaminase for coinoculation with *Rhizobium leguminosarum* to improve growth, nodulation, and yield of lentil. Biol Fertil Soils 47:457–465. https://doi.org/10.1007/s00374-011-0551-7

Zhang C, Shi S, Wang B, Zhao J (2018) Physiological and biochemical changes in different drought-tolerant alfalfa (*Medicago sativa* L.) varieties under PEG-induced drought stress. Acta Physiol Plant 40:25. https://doi.org/10.1007/s11738-017-2597-0

FIGURES

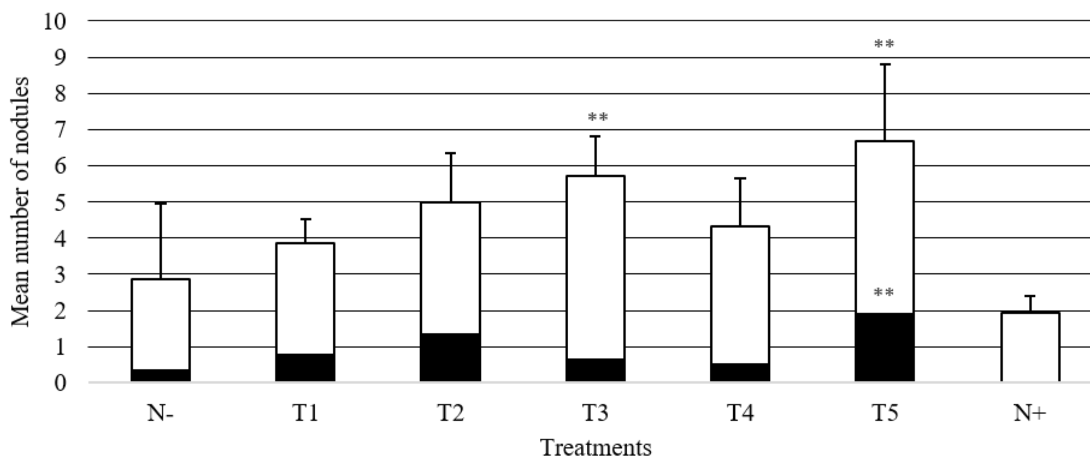
Fig. 1 Phylogenetic tree of the 16S rRNA gene from 5 isolates (in bold) and type strains of closely related species.



Phylogenetic tree constructed using Neighbor-joining statistical method based on the Maximum Composite Likelihood model in MEGA7 (Kumar et al. 2016). There was a total of 1021 positions in the final dataset, and node supports higher than 50% are labelled with a bootstrap value (1000 replicates). The sequence of *Burkholderia pseudomallei* ATCC 23343^{T} was included as an outgroup. Bar indicates five nucleotide substitutions per 100 nucleotides.

Source: Own elaboration

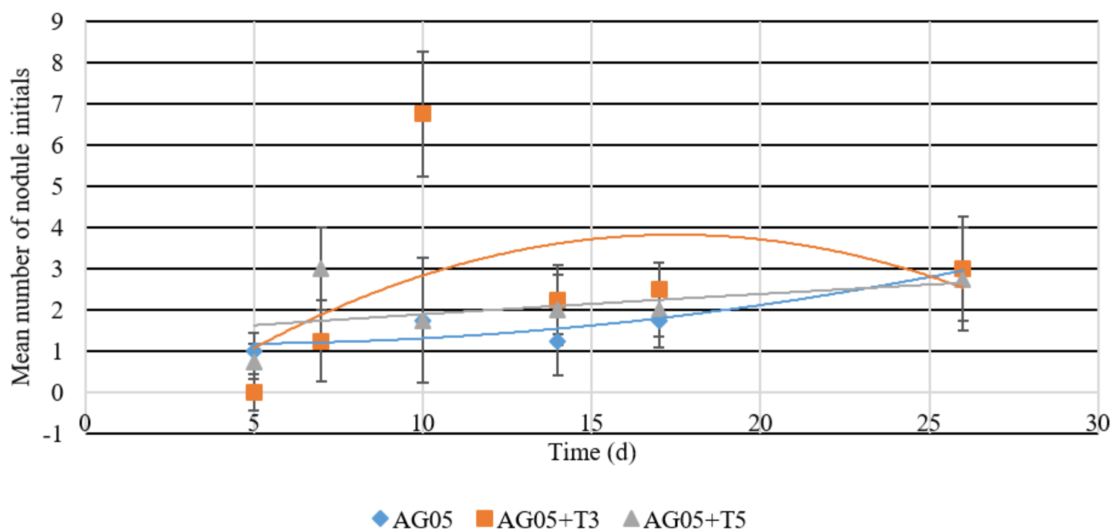
Fig. 2 Number of nodules and nodule effectivity in *Hedysarum coronarium* plants inoculated with different endophytic strains, 17 weeks after sowing.



[*] Treatments significantly different to both uninoculated controls.

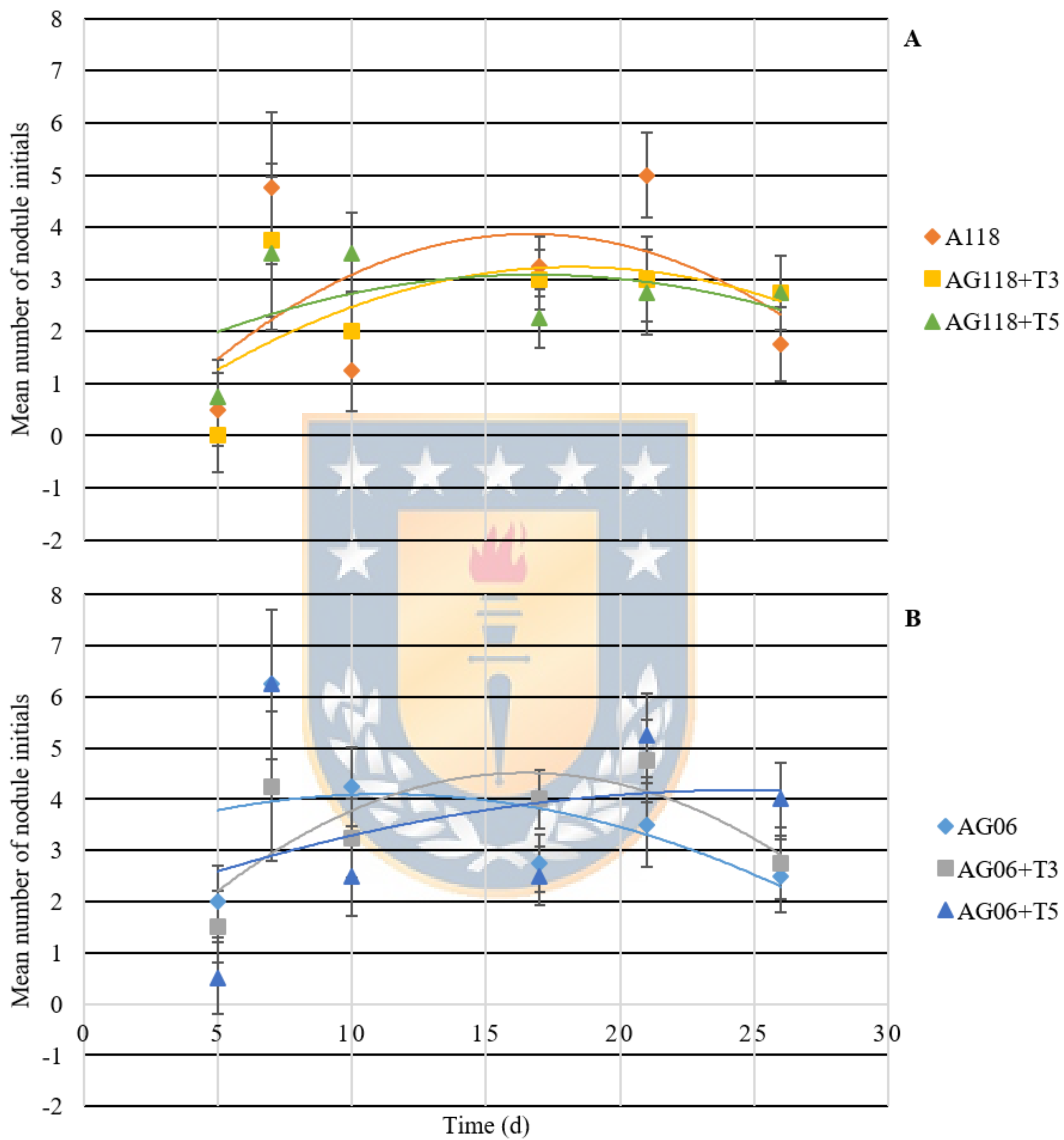
Source: Own elaboration

Fig. 3 Number of nodule initials induced in *Hedysarum coronarium* seedlings on time, inoculated with *Rhizobium sullae* WSM1592 alone and in co-inoculation with nodular endophytes.



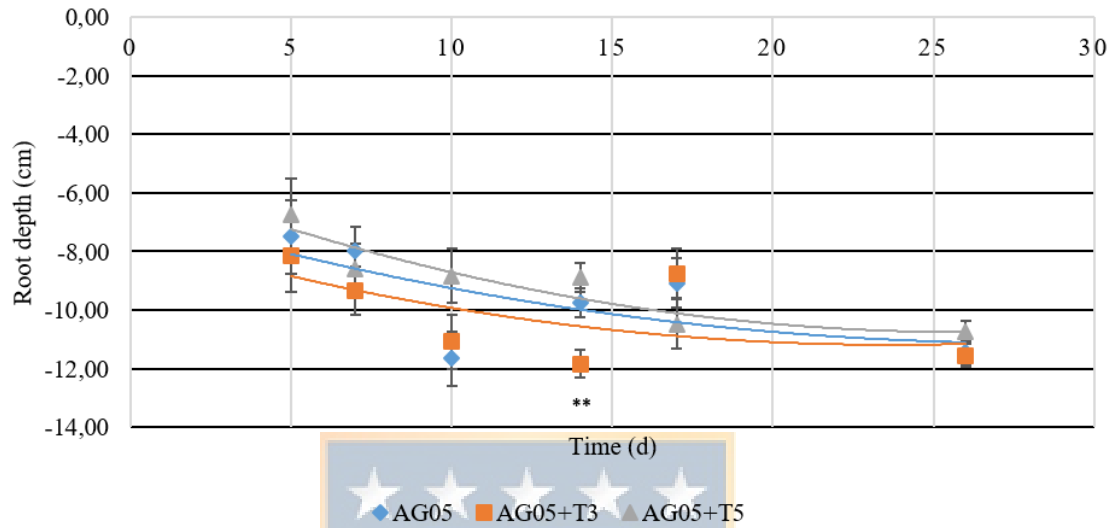
Source: Own elaboration

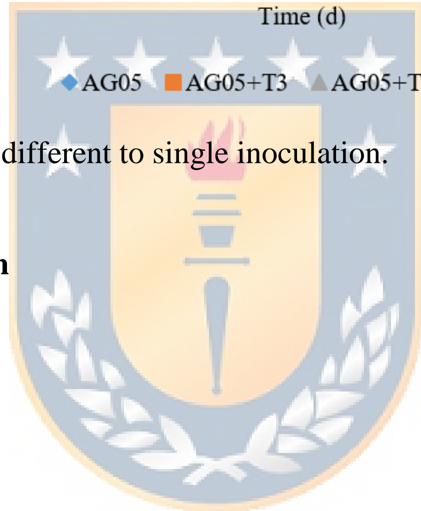
Fig. 4 Number of nodule initials induced on *Medicago sativa* seedlings on time, inoculated with *Ensifer meliloti* AG118 and WSM2141 alone and in co-inoculation with nodular endophytes.



Source: Own elaboration

Fig. 5 Root depth of *Hedysarum coronarium* seedlings collected at 7, 10, 14, 17 and 26 days after inoculation with *Rhizobium sullae* WSM1592 alone and in co-inoculation with nodular endophytes.

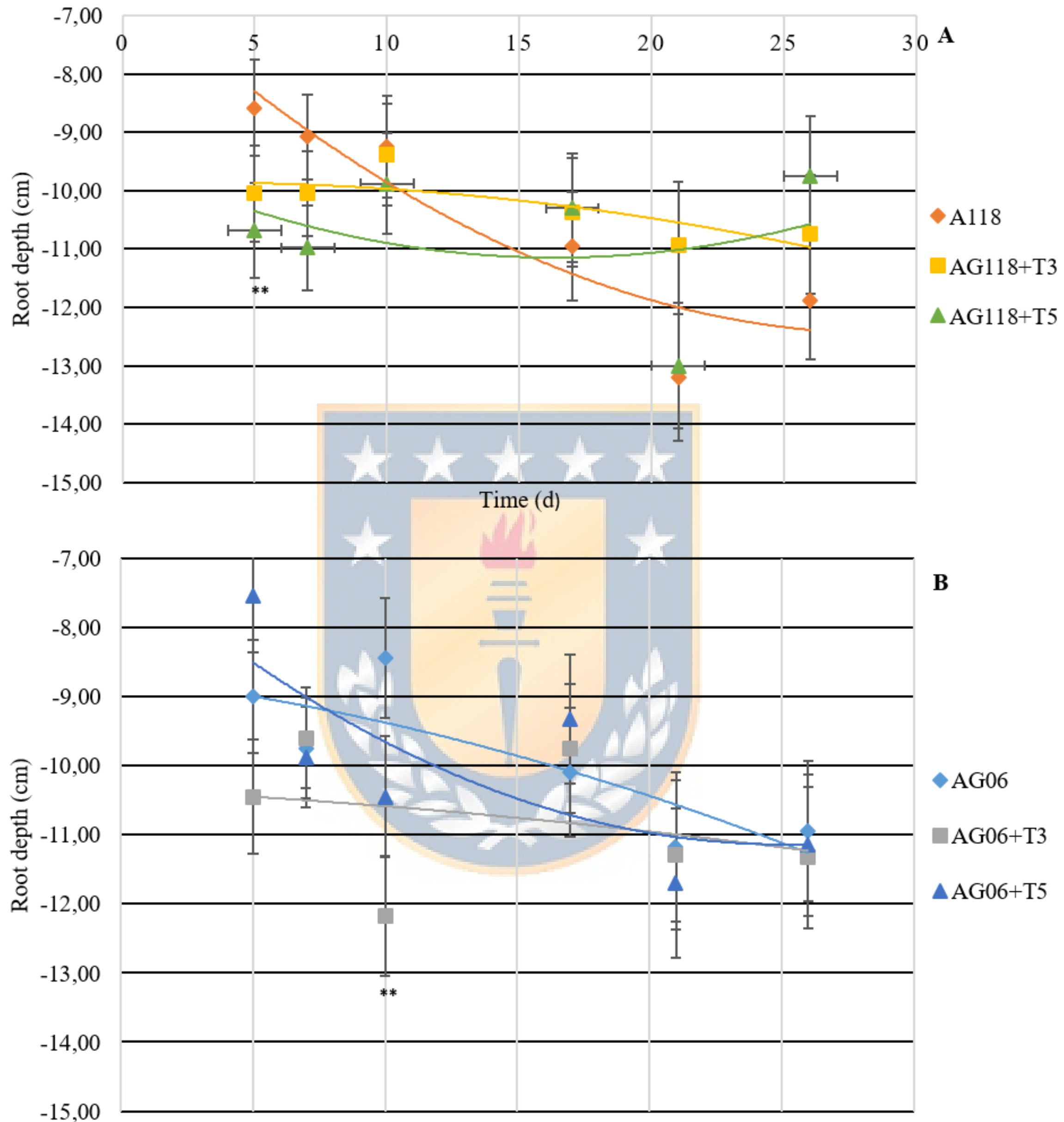




[*] Treatments significantly different to single inoculation.

Source: Own elaboration

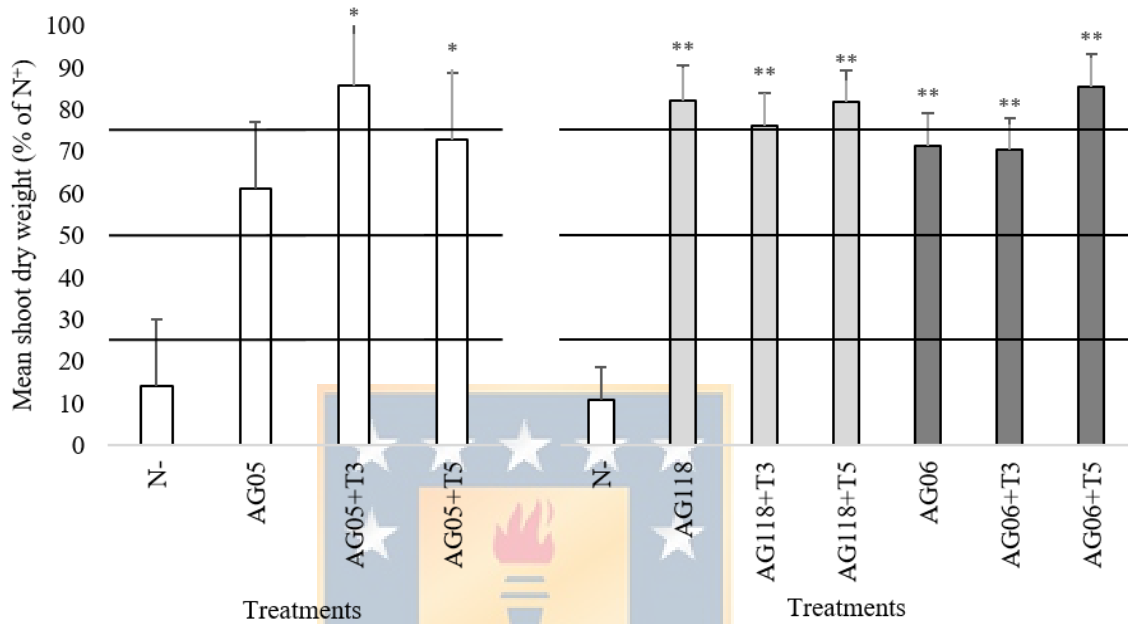
Fig. 6 Root depth of *Medicago sativa* seedlings collected at 7, 10, 17, 21 and 26 days after inoculation with *Ensifer meliloti* AG118 and WSM2141 alone and in co-inoculation with *Hedysarum coronarium* nodular endophytes.



* Treatments significantly different to single inoculation.

Source: Own elaboration

Fig. 7 Mean shoot dry weights of *Hedysarum coronarium* (left) and *Medicago sativa* (right) inoculated with rhizobia alone and in co-inoculation with nodular endophytes expressed as a percentage of N^+ uninoculated treatment fertilized with nitrogen. N^- denotes the uninoculated treatment without nitrogen. Plants were harvested 42 days after inoculation.



* Treatments significantly different to N^- control.

Source: Own elaboration

TABLES

Table 1. Nodule score system used to evaluate nodulation (adapted from Howieson and Ewing, 1989)

Size	Weighting	Position of nodule	Weighting
Large 5-8 mm	3	On upper tap (0-5 cm) or on lateral roots within 1 cm of upper tap	2
Medium 3-4 mm	2		
Small <1-2 mm	1	Nodules elsewhere on root system	1

Source: Own elaboration

Table 2 Variables measured in *Hedysarum coronarium* plants inoculated with different endophytic strains, after 17 weeks of growing.

Treatment	**Nodule scoring**	**Nodule effectivity (%)**
N-	3.53	12.50
T1	7.89	20.42
T2	9.26	26.95
T3	11.90*	11.19
T4	8.56	12.20
T5	12.40*	28.30*
N+	3.66	0c

[*] Treatments significantly different to both uninoculated controls.

Source: Own elaboration

Table 3 Indole acetic acid produced by commercial *Rhizobium sullae* WSM1592, *Ensifer meliloti* WSM2141, Chilean native *E. meliloti* AG118 and *Hedysarum coronarium* nodular endophytes.

Treatment	**IAA production DO_{600nm} 1 (μg ml^{-1})**
Rhizobium sullae WSM1592	1.40d
Mesorhizobium sp. (T2)	8.37c
Mesorhizobium sp. (T3)	12.62b
Mesorhizobium sp. (T4)	10.61bc
Rhizobium sp. (T5)	2.24d
Ensifer meliloti (AG118)	17.45a
Ensifer meliloti (WSM2141)	3.60d

Source: Own elaboration

Table 4 Comparison of the area under the nodulation progression curve (AUNPC) for *Hedysarum coronarium* and *Medicago sativa* inoculated with *Rhizobium sullae* and *Ensifer meliloti* alone and in co-inoculation with nodular endophytes.

Treatment	**AUNPC *H. coronarium***	**AUNPC *M. sativa***
Rhizobium sullae WSM1592 alone	37.47a	-
R. sullae WSM1592+ *Mesorhizobium* T3	62.31a	-
R. sullae WSM1592 + *Rhizobium* T5	41.87a	-
Ensifer meliloti AG118 alone	-	20.22a
E. meliloti AG118 + *Mesorhizobium* T3	-	13.28a
E. meliloti AG118 + *Rhizobium* T5	-	14.88a
Ensifer meliloti WSM2141 alone	-	17.67a
E. meliloti WSM2141 + *Mesorhizobium* T3	-	26.25a
E. meliloti WSM2141 + *Rhizobium* T5	-	20.51a

Source: Own elaboration

Table 5 Variables measured in *Hedysarum coronarium* and *Medicago sativa* plants after 42 days post inoculation.

Treatment	n	Number of nodules	Nodule scoring	Nodule effectivity (%)	Shoot height (cm)	Shoot dry matter (g)	Root depth (cm)	Root area (cm^2)
Hedysarum coronarium								
N-	6	-	-	-	3.08b	0.01b	12.48a	0.83c
WSM1592	6	5.33a	11.33a	80.79a	8.10a	0.06a	15.15a	3.38bc
WSM1592+T3	5	5.42a	12.22a	75.84a	7.43a	0.08a	19.66a	3.80ab
WSM1592+T5	6	4.17a	13.83a	89.29a	7.73a	0.07a	16.38a	2.37bc
N+	4	-	-	-	9.04a	0.10a	18.28a	7.40a
Medicago sativa								
N^-	11	-	-	-	6.77b	0.01c	18.57a	3.56b
AG118	10	9.39ab	22.14a	95.84a	22.73a	0.11ab	17.41a	6.05a
AG118+T3	12	8.92b	21.17a	94.21a	20.70a	0.11ab	13.63b	5.19a
AG118+T5	12	12.67ab	23.67a	91.50a	23.83a	0.11ab	16.90ab	5.87a
WSM2141	11	11.42ab	18.60a	88.67a	21.66a	0.10b	14.66b	4.92ab
WSM2141+T3	12	8.75b	17.92a	97.22a	21.43a	0.10b	15.34ab	5.06ab
WSM2141+T5	11	13.99a	24.64a	99.48a	22.14a	0.12ab	14.85ab	4.98ab
N^+	9	-	-	-	23.41a	0.14a	13.87b	6.33a

Source: Own elaboration

CAPÍTULO 3

CONCLUSIONES GENERALES

En suelo chilenos, existen cepas bacterianas asociadas a nódulos de *Hedysarum coronarium* distintas del inoculante original para esta especie.

Algunas de estas bacterias nodulares benefician a plantas de *H. coronarium* y *Medicago sativa* al ser co-inoculadas con los rizobios específicos para ambas leguminosas, aumentando el número de nódulos, la eficiencia del nitrógeno, la profundidad y el área de la raíz.

En *H. coronarium*, la co-inoculación con el rizobio específico *Rhizobium sullae* WSM1592 y la bacteria nativa *Mesorhizobium* T3 aumenta la eficiencia de fijación de nitrógeno de *R. sullae* WSM1592 en aproximadamente 25%.

En *M. sativa*, *Ensifer meliloti* AG118 por sí sola alcanza una excelente eficiencia de fijación de nitrógeno, mayor profundidad de raíz y también mayor área de la raíz. La cepa *Ensifer meliloti* WSM2141 aumenta su efectividad simbiótica en alfalfa al ser co-inoculada con la bacteria nodular *Rhizobium* T5.

Por lo tanto, sería aconsejable co-inocular ambas especies de leguminosas cuando se utiliza *Rhizobium sullae* WSM1592 para *Hedysarum coronarium* y *Ensifer meliloti* WSM2141 para *Medicago sativa*.

Estos hallazgos podrían ser promisorios para el establecimiento de leguminosas en condiciones de estrés, como ocurre en el secano mediterráneo chileno, sin embargo, es necesario realizar ensayos en terreno, especialmente en este clima, para confirmar estos resultados.