Tesis de Doctorado en Biotecnología – Facultad de Ciencias - Udelar

Identificación de cultivos de cobertura basados en especies de leguminosas tropicales: Combinación de criterios de eficiencia de uso de agua y de fijación de N.

Doctorando: Mag. Verónica Berriel

Director: Dr. Carlos Perdomo

Co-director: Dr. Jorge Monza

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Prof. Agr. Dra. Sabina Vidal

Dra. Elena Fabiano

Dra. Astrid Agorio.

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RESUMEN

Los cultivos de cobertura (CC) basados en leguminosas estivales pueden aportar nitrógeno (N) vía fijación simbiótica (FBN) y carbono al retornar restos vegetales al suelo, así como disminuir el riesgo de erosión al mantener el suelo cubierto con vegetación en un período entre cultivos comerciales. Sin embargo, los CC también compiten por el agua del suelo con el cultivo renta posterior y además su productividad puede verse afectada por los periodos de deficiencia hídrica que ocurren comúnmente durante el verano en Uruguay. Por esto, resulta relevante elegir para esta función una especie o variedad que no solo posea una alta tasa de FBN sino también una alta eficiencia en el uso del agua (EUA). En Uruguay se han realizado evaluaciones a campo del potencial de crecimiento de algunas de estas especies con resultado promisorio, pero no existe información acerca del potencial de su capacidad de FBN ni de la EUA de las mismas. En este trabajo se evaluó en cámara de crecimiento y bajo diferentes regímenes hídricos la masa de FBN acumulada y la EUA de algunas especies de leguminosas con potencial uso como CC estival, empleando tanto técnicas convencionales como otras basadas en el uso de isótopos estables (^{13}C, ^{15}N y ^{18}O). Las especies evaluadas fueron *Crotalaria juncea*, *Crotalaria spectabilis*, *Crotalaria ochroleuca* y *Cajanus cajan*. Se identificó que *C. cajan* y *C. ochroleuca* fueron las especies que fijaron más N (1,8 veces más que *C. juncea* y *C. spectabilis*). También se propuso un método para la determinación de un componente clave que impacta en la cuantificación de la FBN, el denominado valor B. Por otro lado, se registró que las especies de rizobios nativos presentes en suelos agrícolas de Uruguay generan nódulos efectivos en estas especies, lo que abre la puerta a la producción de inoculantes comerciales. Además, se registró que durante períodos de deficiencia hídrica estas mismas dos especies fueron en ese orden las que incrementaron más su EUA (entre 2 y 1,4 veces, respectivamente). Asimismo, se confirmó que la metodología isotópica basada en la variación de la abundancia natural de ^{18}O y de ^{13}C permite indexar para un mismo ambiente en forma práctica y rápida la EUA de estas especies. Sin embargo, estos resultados deben ser confirmados en

posteriores ensayos de campo para poder recomendar el uso de *C. cajan* como CC de verano. Esta metodología también podría utilizarse para realizar un screening rápido de especies promisorias de leguminosas a ser usadas como CC en otros periodos del año.

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Introducción

Los cultivos de cobertura (CC) se utilizan para evitar que el suelo permanezca descubierto sin vegetación o parcialmente cubierto por malezas entre cultivos comerciales también denominados renta. Esto se debe a que los suelos sin vegetación son más susceptibles a la erosión hídrica, ya que la presencia de plantas protege al suelo del impacto erosivo de la lluvia (Feitosa et al., 2015) y al absorber agua y nitrato disminuyen la lixiviación de este anión. Además, los CC proveen otros beneficios eco-sistémicos, ya que promueven la biodiversidad de los sistemas e incorporan materia orgánica al suelo (Steenwerth and Belina, 2008; Boyhan et al., 2016), entre otros.

Luego de la supresión de los CC, la biomasa aérea queda dispersa sobre la superficie del suelo y forma un colchón o mulch, que contribuye aún más a reducir la erosión, atenúa las variaciones de temperatura del suelo y contribuye a controlar las malezas y mejorar el aporte de nutrientes. La biomasa de raíces, por otro lado, mejora la estructura del suelo al incrementar la macroporosidad y la síntesis de materia orgánica estable (humus) (Colazo and Garay, 2020).

Los beneficios de los CC se relacionan estrechamente con las especies utilizadas. Las gramíneas son las especies más usadas (Clark, 2007), ya que crecen rápidamente y producen gran cantidad de biomasa devolviendo el nitrato y amonio absorbido en forma de N orgánico. Por otro, lado las crucíferas son también muy utilizadas por su potencial para reducir la compactación de los suelos y mejorar la infiltración del agua (Bertolotto and Marzetti 2017), además de producir compuestos alelopáticos que ejercen una supresión de malezas (Menan et al., 2020). Las leguminosas como CC pueden elevar el nivel de N en el suelo, debido a su capacidad de realizar fijación biológica de nitrógeno (FBN) asociadas a rizobios específicos, enriqueciendo al suelo con este nutriente (Landriscini et al., 2019).

Actualmente en Uruguay coexisten varios esquemas de rotación, pero una de las más extendidas es la rotación trigo soja. El cultivo de soja representa el 90 % del área agrícola de verano (917.000 ha) seguida por el trigo, con un área sembrada en invierno estimada en 237.000 ha (DIEA, 2021). La expansión de la soja es reciente; antes del año 2000 no era relevante y con la adopción de la siembra directa pasó a ser el cultivo más importante (Netzeband y Arbeleche, 2016). Además, este proceso estuvo acompañado por el abandono de los sistemas de rotación agrícolas-ganaderos y el comienzo de la agricultura continua (García Prechac et al., 2010).

La rotación soja-trigo en nuestro país no es sustentable desde el punto de vista ambiental ni productivo. Se ha constatado que estas rotaciones superan el límite de tolerancia de la erosión si se deja el suelo desnudo entre estos cultivos renta (Morón et al., 2012). La causa de esta erosión es la alta relación lluvia-evapotranspiración que ocurre en el período entre la cosecha de la soja (abril-mayo) y la siembra de trigo (junio y agosto). Para evitar este problema, actualmente el Ministerio de Ganadería Agricultura y Pesca de Uruguay obliga a planificar las rotaciones de cultivos o de cultivos y pasturas (Decreto 405/2008), para mantener la tasa de erosión anual por debajo del nivel de tolerancia. Esto ha llevado a que el uso de CC se haya extendido a casi la totalidad de área agrícola, porque sin su implantación el riesgo de erosión es elevado. Las gramíneas usadas como CC invernales en Uruguay se han destacado por presentar una buena implantación, capacidad de cubrir rápidamente el suelo, alta producción de biomasa y capacidad de retener nutrientes en el sistema (Sawchik et al., 2015).

A pesar de este consolidado desarrollo de los CC de invierno, podría también haber un nicho para el uso de CC de verano, para sustituir las rotaciones trigo – pradera en el caso de productores agrícola-ganaderos que prefieran hacer la transición a agricultura continua de invierno. En este caso, la pradera mezcla de gramínea y leguminosa podría ser sustituidas por leguminosas de verano. La ventaja de esta sustitución es el tiempo en que se pueden lograr altas fijaciones de N. Por ejemplo, existen datos para Uruguay de fijación de N de cultivos puros de las leguminosas invernales más usadas (trébol blanco, lotus, trébol rojo y alfalfa) en su año más productivo (segundo año) de 229, 226, 308 y 366 kg N/ha respectivamente (García et al., 1994). En el caso de praderas mixtas, estas pueden aportar hasta 190 kg de N/ha (García Lamothe, 1998). En cambio, información de Brasil indica que las leguminosas tropicales *Crotalaria juncea*, *C. spectabilis*, *C. ochroleuca* y *Cajanus cajan* son capaces de fijar en solo 90 días 450, 120, 200 y 180 kg N/ha respectivamente (Brseeds, 2021). La entrada de N vía fijación biológica es una característica deseable en las rotaciones, ya que permite el ahorro de fertilizantes nitrogenados para el cultivo siguiente (Sawchick, 2001) y evita la dependencia excesiva de estas fuentes químicas. De todos modos, para las condiciones de Uruguay no se conoce cuál es el potencial de fijación de N de estas especies tropicales ni el efecto residual en términos de aporte de N para los cultivos de invierno.

Estas leguminosas estivales también pueden ser usadas como CC cuando no se puede sembrar el cultivo renta de verano, como la soja, debido a sequías, excesos hídricos o a situaciones de retraso en la cosecha del trigo (Ceretta e Ibañez, 2010). Esto es debido a que el retraso en la siembra de soja conduce a la pérdida del potencial de rendimiento (Hu y Wiatrak, 2012), y en esta situación el productor debe evaluar si las ganancias obtenidas superarán los costos. En caso de decidir no sembrar soja, los CC de verano serían una buena alternativa. Además de las ventajas de la alta capacidad de fijar N de las leguminosas estivales en un corto período, estas especies también proveen otros servicios como la supresión de nematodos y malezas, el menor uso de herbicidas, etc.

Algunas especies de *Crotalaria* son utilizadas para el manejo de nematodos incluyendo *Meloidogyne* sp. y *Rotylenchulus reniformis*. Aunque se ha constatado que los efectos residuales son solo de algunos meses (McSorley et al., 1994). Con respecto al control de malezas, el uso de CC puede reducir la germinación y el desarrollo de las semillas de malezas (Weston 1996; Ohno et al. 2000), por lo cual son una forma ecológicamente adecuada y económicamente más rentable de combate. El control de las malezas resulta fundamental, ya que estas generan una reducción en la eficiencia de los recursos como los fertilizantes y el agua de riego, además de fortalecer la población de otros organismos como plagas. Si esto no se logra, se puede reducir drásticamente el rendimiento y calidad del cultivo. Debido a este efecto supresor de malezas y nematodos, existe también un nicho para la introducción de estas especies de leguminosas tropicales en rotaciones hortícolas.

Por otro lado, los CC basados en leguminosas anuales invernales tienen como desventaja la variabilidad de implantación, producción de biomasa y capacidad de fijar N (Sawchik et al., 2015). Si bien cuanto mayor es el período de crecimiento primaveral de estos CC mayor será su producción de biomasa, la ventana de siembra entre los cultivos renta de verano e invierno limita su uso. Además, si se prolonga el ciclo de los CC de invierno, se puede retrasar la fecha de siembra del cultivo renta de verano y disminuir la reserva de agua del suelo.

Ventajas del uso de leguminosas estivales del género *Crotalaria* y de *Cajanus* como CC en las rotaciones de Uruguay

Entre los posibles CC estivales que podrían emplearse en Uruguay se encuentran especies de *Crotolaria* como *C. juncea*, *C. spectabilis* o *C. ochroleuca*. De estas especies la más utilizada ha sido *C. juncea* que ha demostrado tener potencial para producir altos niveles de biomasa y fijar N proporcionando al suelo (Balkcom y Reeves, 2005). Otra especie utilizada es el gandul (*Cajanus cajan*), que también proporciona una importante cantidad de N al suelo vía FBN (Balkcom and Reeves, 2005).

Tanto las especies del género *Crotalaria* como *Cajanus cajan* habitualmente no son inoculadas con rizobios, debido a que son noduladas por cepas autóctonas, al menos en Brasil (Neves y Rumjanek, 1997). Si bien su promiscuidad podría considerarse una ventaja ya que simplifica el manejo de las semillas, se ha reportado que la FBN puede incrementarse cuando estas especies se asocian simbióticamente con cepas del género *Bradyrhizobium* (MAPA, 2011). Por lo tanto, cuando se desea incrementar la producción de materia seca y la FBN de estos CC puede ser necesaria la inoculación con cepas específicas de *Bradyrhizobium* (Sikora et al., 1997).

Debido a que el rendimiento de los CC depende de factores ambientales como la disponibilidad de agua en el suelo y esta a su vez condiciona la FBN, sería necesario evaluar estas especies de CC en las condiciones climáticas de nuestro país, caracterizado por tener balances hídricos deficitarios en el período estival y bajos niveles de agua acumulada a nivel del suelo (Chiara, 2009).

Estimación del N fijado por las leguminosas

Una de las técnicas utilizadas para cuantificar el N fijado se basa en la medición de la variación de la abundancia natural de ^{15}N entre las especies fijadoras y no fijadoras. Para ello, es necesario determinar la composición isotópica de N de las especies fijadoras y no fijadoras, expresada en notación de delta ($\delta^{15}N$) y en unidades de partes por mil (‰). Esta diferencia se produce debido a que las plantas fijadoras toman la mayor parte de su N del aire, mientras que las no fijadoras lo absorben del suelo, el cual está más enriquecido en ^{15}N.

A partir de esta diferencia en los valores isotópicos, es posible estimar con la Ec. [1] la proporción de N que deriva de la FBN (Shearer y Khol, 1986).

$$\%Ndfa = \left(\frac{\delta^{15}N_{ref} - \delta^{15}N_{fix}}{\delta^{15}N_{ref} - B}\right) \times 100 \qquad \text{Ecuación 1}$$

Dónde:

% Ndfa es la proporción de N de la planta derivado de la FBN.

δ^{15}Nref: es el valor de $\delta^{15}N$ de la planta referencia (no fijadora).

δ^{15}Nfij: es el valor de $\delta^{15}N$ de la planta fijadora.

B es el valor de $\delta^{15}N$ de una planta fijadora creciendo en un medio sin N.

Para la estimación de la proporción de N fijada por una leguminosa, es necesario conocer la composición isotópica de N de la planta de referencia, así como el valor B. El valor B corresponde a la composición isotópica de ^{15}N de una leguminosa que durante todo su ciclo de vida creció en un medio libre de N mineral (Unkovich et al., 1994). En esta situación, todo el N acumulado por la planta proviene de la FBN. Este valor B resulta ser distinto al valor de $\delta^{15}N$ del aire porque los procesos de discriminación isotópica del N que ocurren durante el transporte y metabolismo provocan enriquecimientos o empobrecimientos de ^{15}N en distintas partes de la planta (Evans, 2001). El valor B varía con la especie vegetal y la cepa de rizobio con las que se asocian las leguminosas (Boddey et al., 2000), por esto su determinación para cada situación debe ser considerada o tenida en cuenta.

Eficiencia en el uso del agua

La eficiencia en el uso del agua (EUA) se puede definir a nivel de la planta como la relación entre la biomasa producida y el agua transpirada. Los fenómenos que le dan origen, en primera instancia, ocurren a nivel de los estomas en la hoja (Medrano et al., 2015). En condiciones de limitación hídrica o de baja o media demanda atmosférica el intercambio de estos gases CO_2 y H_2O por el estoma se ve disminuido (Pou et al., 2011). En esta situación se logra una mayor EUA cuando las especies logran mantener la capacidad de fijación de carbono con una menor tasa transpiratoria (Hubick and Gibson, 1993).

La metodología de determinación de EUA, a nivel de planta entera en ensayos experimentales resulta engorrosa. Por esta razón, varios investigadores han preferido estimar la EUA a nivel de hoja a través del monitoreo de las concentraciones de CO_2 (gas) y vapor de H_2O, con equipos provistos de detectores infrarrojos (Polley et al., 2002). Esta metodología tiene la debilidad de suministrar datos puntuales o instantáneos, que pueden no reflejar la fisiología de la planta durante un período de tiempo.

Actualmente se ha desarrollado otra técnica de determinación de la EUA basada en la mayor discriminación isotópica del ^{13}C ($\Delta^{13}C$). Este proceso de discriminación o fraccionamiento del ^{13}C a nivel de la hoja es mucho mayor del que sufre el isótopo más abundante del carbono, el ^{12}C. Esta metodología permite con una única medida integrar la EUA a lo largo del tiempo (Santesteban et al., 2015). La utilización de $\Delta^{13}C$ como proxy de la EUA ha sido confirmada en diferentes especies C3 (Maxwell et al., 2018; Pronger et al., 2019; Castillo et al., 2017), aunque no lo ha sido en todas las leguminosas de grano (Turner et al., 2007).

La disponibilidad de agua es una de las variables ambientales que más afecta a $\Delta^{13}C$ (Hartman and Danin, 2010). En este sentido, existe una fuerte correlación negativa entre $\Delta^{13}C$ y la lluvia (Stewart et al., 1995), el agua contenida en el suelo (Farquhar et al., 1989) y los gradientes de humedad (Ehleringer y Cooper, 1988).

De igual forma que para $\Delta^{13}C$, la discriminación isotópica del ^{18}O ($\Delta^{18}O$) del agua está siendo adoptada como criterio de evaluación de la tasa transpiratoria y la conductancia estomática (Scheidegger et al., 2000). La base teórica se sustenta en que durante la transpiración de las plantas en la cavidad subestomática ocurre un proceso de fraccionamiento en el cual la molécula de agua que contiene al isótopo liviano del oxígeno (^{16}O) se evapora preferentemente respecto a la que tiene el isótopo pesado (^{18}O) (Farquhar et al., 2007). Por lo tanto, en hojas en condiciones de intensa transpiración hay mayor $\Delta^{18}O$ que en condiciones de escasa transpiración, por ejemplo, cuando hay baja disponibilidad hídrica (Werner et al., 2012).

El índice isotópico $\Delta^{18}O$ facilita la interpretación de los resultados de $\Delta^{13}C$ entre individuos que crecen en el mismo ambiente, ya que permite separar los efectos de la capacidad fotosintética de los de la conductancia estomática (Barbour et al., 2000). Esto es posible, debido a las diferencias entre $\Delta^{13}C$ y $\Delta^{18}O$, ya que el primero responde a cambios en la conductividad estomática y en la tasa fotosintética, mientras que el segundo es independiente de la tasa fotosintética y depende de la conductividad estomática. Por lo tanto, si estos dos índices isotópicos se utilizan conjuntamente permiten inferir la conductancia estomática y transpiración de especies vegetales (Barbour et al., 2007).

En los escenarios de cambio climático proyectados se prevé la disminución de la precipitación en varias regiones del planeta; por lo cual, es cada vez más necesario rediseñar las secuencias o rotaciones agrícolas de manera de mitigar el impacto del cambio climático sobre la productividad y sostenibilidad de las rotaciones (Condón, 2020; Hernández-Ochoa et al., 2019). En esta tesis se evaluaron los atributos de producción de biomasa, fijación de N y ET de *C.*

spectabilis, *C. juncea*, *C. ochroleuca* y *Cajanus cajan*, especies de leguminosas tropicales usadas como CC. La información generada podrá contribuir al diseño de rotaciones agrícolas que permitan incrementar los servicios ecosistémicos de estas rotaciones aún bajo un escenario de mayores eventos de déficit hídricos debido al impacto del cambio climático.

Objetivo general

Diseñar rotaciones bajo agricultura continúa con cultivo renta invernal y cultivos de coberturas estivales capaces de mantener balances positivos o neutros de N, con bajo riesgo de erosión y robustos frente al cambio climático.

Objetivos específicos

1. Identificar especies de leguminosas estivales con alta capacidad de fijación de N durante cortos períodos de tiempo y con alta eficiencia en el uso del agua.
2. Investigar acerca de la existencia en Uruguay de poblaciones de rizobios capaces de establecer simbiosis y formar nódulos con las leguminosas de origen tropical.
3. Optimizar y validar metodologías basadas en el uso de isótopos estables para estimaciones de valor B en estudios de fijación de N y de tasa transpiratoria y eficiencia en el uso de agua.

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Capítulo 1

Identificación rápida de cultivos promisorios de cobertura en base a su producción de biomasa, fijación biológica de nitrógeno y eficiencia de uso de agua: Aplicación de estos criterios a dos especies de *Crotalaria*

Un desafío del diseño de las rotaciones agrícolas incluye la elección de cultivos basados en los atributos de interés. Un atributo deseable es que los cultivos presenten una alta producción de biomasa en función del agua consumida, es decir una alta eficiencia en el uso del agua (EUA). Incluso en períodos de crecimiento sin limitación hídrica, los cultivos de cobertura (CC) basados en leguminosas estivales deben tener una alta EUA, ya que estos cultivos le restan agua almacenada en el suelo para el cultivo renta posterior. Pero, además, es deseable que estas especies tengan alta capacidad de fijación biológica de N (FBN), ya que esta característica puede mejorar el balance de N de la rotación. En este trabajo se compararon estos atributos en dos especies de *Crotalaria (C. spectabilis* y *C. juncea)* bajo condiciones controladas de alta temperatura y sin limitación hídrica, utilizando plantas no inoculadas y suelo como sustrato. La FBN se determinó por el método de abundancia natural de ^{15}N y la EUA se estimó por el método gravimétrico e isotópico (^{13}C). La especie *C. spectabilis* produjo un 72 % más de materia seca, acumuló 21% más de nitrógeno fijado y tuvo una EUA 28% superior a *C juncea.* Además, este ranking permaneció incambiado a través de dos metodologías de estimación de la EUA. Estos resultados se describen en detalle en el artículo que se presenta a continuación.

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Article

Cover Crop Selection by Jointly Optimizing Biomass Productivity, Biological Nitrogen Fixation, and Transpiration Efficiency: Application to Two *Crotalaria* Species

Verónica Berriel [1,*], Jorge Monza [2] and Carlos H. Perdomo [3]

[1] Centre for Applications of Nuclear Technology in Sustainable Agriculture (CATNAS), Soil and Water, Department, Agronomy College, University of the Republic, Av. Garzón 809, Montevideo CP 12.900, Uruguay
[2] Plant Biology Department, Agronomy College, University of the Republic, Av. Garzón 780, Montevideo CP 12.900, Uruguay; jmonza@fagro.edu.uy
[3] Soil and Water Department, Agronomy College, University of the Republic, Av. Garzón 780, Montevideo CP 12.900, Uruguay; chperdom@fagro.edu.uy
* Correspondence: vberriel@gmail.com or vberriel@fagro.edu.uy

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Abstract: *Crotalaria spectabilis* and *Crotalaria juncea* are cover crops (CC) that are used in many different regions. Among the main attributes of these species are their high potential for biomass production and biological fixation of nitrogen (BNF). Attempting to maximize these attributes, while minimizing water consumption through high transpiration efficiency (TE), is a challenge in the design of sustainable agricultural rotations. In this study, the relationship between biomass productivity, BNF, and TE in *C. spectabilis* and *C. juncea* was evaluated. For this purpose, an experiment was carried out under controlled conditions without water limitations and using non-inoculated soil. BNF was determined by the natural abundance of ^{15}N, while TE was estimated by several different methods, such as gravimetric or isotopic method (^{13}C). *C. juncea* produced 42% less dry matter, fixed 28% less nitrogen from the air, and had 20% less TE than *C. spectabilis*. TE results in both species were consistent across methodologies. Under simulated environmental conditions of high temperature and non-limiting soil water content, *C. spectabilis* was a relatively more promising species than *C. juncea* to be used as CC.

Keywords: *Crotalaria spectabilis*; *C. juncea*; ^{15}N natural abundance; ^{13}C isotopic composition; transpiration efficiency

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1. Introduction

The use of legumes as cover crops (CC) in agricultural rotations makes it possible to reduce the production costs associated with a lower use of nitrogenous fertilizers, which also results in environmental benefits [1,2]. CCs are also used to reduce soil erosion caused by high precipitation, minimize surface runoff, and provide channels to the subsurface layers of the soil, allowing an increased infiltration rate [3,4].

The use of the genus *Crotalaria*, in particular *C. juncea* and *C. spectabilis*, as CCs has been recommended for warm and temperate regions [5]. Some of the main attributes of these species are their rapid and high productivity of biomass (8 Mg ha^{-1}) [6–8] and their high content of foliar nitrogen, obtained by biological nitrogen fixation (BNF) at an average of 150 kg N ha^{-1} [9–11]. In addition, a characteristic of these species is that they have the ability to establish a promiscuous and functional symbiosis with the native rhizobia of the soil [12]. The biomass production of CCs, including *C. juncea* and *C. spectabilis*, is positively correlated with the recycling of nutrients, the entry of carbon (C) into the soil [13–15], and a decrease in the rate of erosion [3]. Furthermore, high concentrations of foliar N derived from BNF determine a low C/N ratio, which favors the rapid decomposition of plant remains [16,17]. The ease of degradation of this material also facilitates net N mineralization, which can be used by subsequent crops [18].

For these reasons, in a sustainable production system, it is necessary that plant species used as CCs, if they are legumes, have a high BNF and also high biophysical gain rates (biomass productivity) in relation to the consumed or transpired water [19,20]; in other words, a high water use efficiency (WUE) or transpiration efficiency (TE). A low TE and excessive water consumption can not only waste soil water reserves, but can also induce a water deficit in the subsequent cash crop and reduce its yield [21]. For the genus of *Crotolaria*, there is little information about TE, so it was interesting to evaluate this attribute and its relationship with others that have been more studied, such as biomass production and BNF [6–8,10].

However, as there are different methodological approaches to assess TE, we needed to find a simple but robust indicator for these species. The reference technique consists of computing the ratio between total biomass productivity and transpired water during the whole crop cycle [20,22], providing an integrated value of TE for the entire plant growing period. Two other methods provide only a one-time "snapshot" of TE. The instantaneous foliar WUE is the ratio of the photosynthetic rate (A) to the transpiration rate (E), while the intrinsic foliar WUE is the proportion of A to stomatal conductance (g) [23,24]. In contrast, the ^{13}C isotopic composition (δ^{13}C) of plants with C3 photosynthetic metabolism has also been used to estimate the TE of plants in a time-integrated manner [25,26]. Through models, it is possible estimate from δ^{13}C the intrinsic WUE (iWUE) [25,27,28].

In a previous work, we compared the biomass productivity and the WUE of these two *Crotalaria* species, but under conditions of a moderate deficit of water in the soil. We found *C. spectabilis* showed superior behavior [29]. In this work, under controlled conditions and non-limited water, our objective was to relate the productivity of the biomass, BNF, and TE in these species. In addition, another secondary objective was to study the consistency between the methodologies that estimate TE, to understand its robustness and precision.

2. Materials and Methods

2.1. Plant Materials and Growing Conditions

Crotalaria juncea, *Crotalaria spectabilis* (obtained from Brseeds Sementes Co., Araçatuba, Brazil), and corns the seeds were planted in plastic pots containing 4 kg Argiudol soil from the south of Uruguay (latitude—34.6 S and longitude—55.6 W). Soil characteristics: soil organic carbon = 11.6 g kg^{-1} soil; organic matter = 20.0 g kg^{-1} soil; sand = 245 g kg^{-1} soil; silt = 487 g kg^{-1} soil; clay = 268 g kg^{-1} soil). The plants were not inoculated and noduled with the rhizobia in the soil. Ten days after the initial emergence of seedlings, the plants were thinned to one per pot, and perlite was placed on the soil surface to minimize water evaporation. The pots were kept in a growth chamber at 30 ± 3 °C, with variable relative humidity between 30% and 50%, and a light intensity of 1200 µmol m^{-2} s^{-1} with a 16/8 h cycle (light/dark). The growth chamber was continuously monitored by a computer system.

Soil moisture was kept constant at 100% (*w*/*w*) at container capacity for 75 days. The amount of water needed to achieve soil water capacity was estimated daily as the difference between the target gravimetric content and the actual water content in the soil. The sum of these daily differences was the evapotranspiration (ET) accumulated during the plant growing cycle. Transpiration (T) was determined as the accumulated loss of water from pots with plants, minus the average value determined in pots without plants and with perlite on the surface.

2.2. Biomass Productivity and Characteristics of Nodules

Seventy five days after starting the experiment (before flowering), the aerial parts of the plants (leaves, stems, and leaves + stems = shoots) were harvested and dried at 60 °C until they reached a constant weight, and then the dry mass of each plant was weighed. The roots were washed and the nodules were considered, according to their size, as larger or smaller nodules, the latter being about half the size of the large ones.

2.3. Determination of Transpiration Efficiency

Gravimetric method

The *TE* was calculated based on Equation (1) as the quotient between the biomass produced by the aerial part (shoot) and the accumulated plant transpiration throughout the experiment:

$$TE = \frac{shoot\ dry\ mass}{T}. \tag{1}$$

2.4. Gas Exchange Measurements

Intercellular CO_2 concentration, A, g, and E were determined using the youngest fully expanded leaf of all plants 70 days after sowing. These determinations were made using a portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, NE, USA); the photosynthetically active radiation was set to 1200 μmol m^{-2} s^{-2}, and the leaf temperature at 25 °C. The CO_2 concentration of the chamber was adjusted to 400 μL L^{-1}.

2.5. Determination of Nitrogen Concentration and Stable Isotopic Composition of Plant Parts

Samples from different plant parts (leaves, stems, and leaves + stems = shoots) were first ground with a fixed and mobile knife mill (Marconi MA-580) until a particle size of less than 2 mm was achieved, and then with a rotary mill (SampleTek 200 vial Rotator). Determination of N-total concentration and natural abundance of ^{13}C and ^{15}N was determined on a Flash EA 1112 elemental analyzer coupled to a Thermo Finnigan DELTAplus mass spectrometer (Bremen, Germany). Isotopic relationships were expressed in delta notation (δ) in parts per thousand (‰), using the following equation [30]:

$$\delta^{13}C\ or\ \delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000. \tag{2}$$

Carbon ^{13}C isotope discrimination ($\Delta^{13}C$) was calculated according to Farquhar et al. [25], where $\delta^{13}C_{atmosphere}$ is the $\delta^{13}C$ value of air (−8‰) and $\delta^{13}C_{plant}$ is the $\delta^{13}C$ value of the plant sample:

$$\Delta^{13}C = \left(\frac{\delta^{13}C_{atmosphere} - \delta^{13}C_{plant}}{1 + \frac{\delta^{13}C_{atmosphere}}{1000}} - 1\right) \times 1000. \tag{3}$$

The ratios between the intercellular (in the plant) and air CO_2 concentration and the intrinsic WUE (iWUE) were determined from the following equations [25]:

$$iWUE = \frac{Ci}{Ca} = \frac{\Delta^{13}C_{plant} - 4.4}{22.6}[4]. \tag{4}$$

Biological nitrogen fixation was estimated with Equation [6], according to Unkovich et al. [25]:

$$BNF = \left(\frac{\delta^{15}N_{ref} - \delta^{15}N_{fix}}{\delta^{15}N_{ref} - B}\right) \times 100, \tag{5}$$

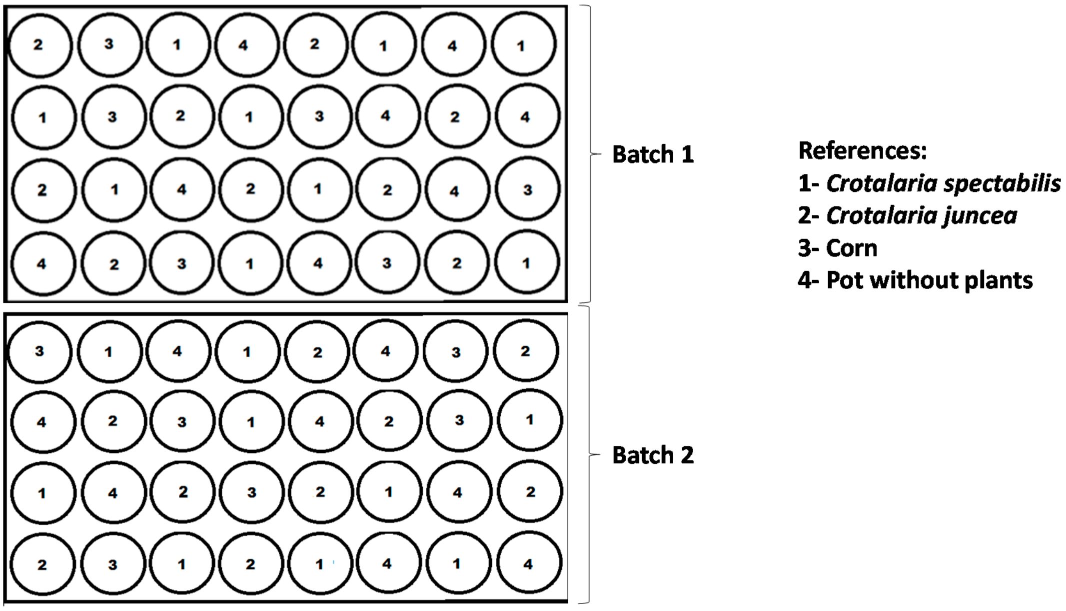
where:

BNF is the percentage of N in the plant, derived from BNF.
δ^{15}Nref is the δ^{15}N value of the non-fixing reference plant.
δ^{15}Nfix is the δ^{15}N value of the fixing plant.
B is the δ^{15}N value of a fixing plant growing in N-free growth medium.

Corn was the non-fixing reference plant used, with an δ^{15}N isotopic composition of −8‰ (average value of 12 plants), while in *C. juncea* and *C. spectabilis*, the reported B values of −2.25‰ [31] and −1.0‰ [32] were respectively assumed.

2.6. Experimental Setup

A completely randomized design was used; the pot was the experimental unit and the species was considered the treatment. The experiment was repeated in the same plant growth chamber in two time periods (with the same set of environmental parameters and the same duration in time), that were named batch 1 and batch 2. Nine pots of each *Crotalaria* species were used in each batch. Close to the *C. spectabilis* and *C. juncea* pots, six pots with corn plants and eight with soil but without plants were randomly placed. Between the two batches, 17 plants of *C. spectabilis* and 14 of *C. juncea* plants culminated the experiment. The scheme of the experiment is shown in Figure 1.



2	3	1	4	2	1	4	1
1	3	2	1	3	4	2	4
2	1	4	2	1	2	4	3
4	2	3	1	4	3	2	1

3	1	4	1	2	4	3	2
4	2	3	1	4	2	3	1
1	4	2	3	2	1	4	2
2	3	1	2	1	4	1	4

Figure 1. Design of the experiment. Circles represent the pots in the plant growth chamber.

2.7. Statistical Analysis

In order to test if there was a difference in five variables (foliar concentration of N, T, TE, A, and E) in each *Crotalaria* species between the two batches, we carried out a Shapiro–Wilks test to evaluate normality, an F-test and a Student's *t*-test. According to the results obtained, the F-test showed that the variances could be considered as equal because the p-value was superior to 0.05. In the Student's *t*-test, the null hypothesis (the differences between means is equal to 0) could not be rejected in any of the species at a significance level of 0.05. Within a specie, no statistically significant difference at $\alpha = 0.05$ was found between batches for any of the evaluated parameters. For this reason, the data for the two batches were pooled for each species.

In the pooled data, also the normality was evaluated with the Shapiro–Wilks test, while the assumption of equality of variances was evaluated with Levene's test. After, the species effect was analyzed by ANOVA in those variables with a normal distribution (N, T, TE, A, E, and A/E), and by the Kruskal Wallis test for variables without a normal distribution (shoot dry mass, g, A/g, $\delta^{15}N$, BNF, $\delta^{13}C$, and iWUE). Pearson correlation analyses were also performed. The statistical packages InfoStat [33] and XLSTAT [34] were used in the statistical analyses.

3. Results and Discussion

3.1. Biomass and Nitrogen Productivity from Fixation

In simulated environmental conditions, with a high temperature and non-limited soil water availability, the two species differed both in terms of biomass productivity (Tables 1 and 2) and foliar N concentration (Tables 1 and 3). *C. spectabilis* was the species that produced the highest biomass and had the higher leaf N concentration (Table 1). All *C. spectabilis* plants and 57% of *C. juncea* presented large pink nodules. The remaining 43% of the *C. juncea* plants also had pink nodules, but these were small. The same trend with respect to nodulation was observed between the two analyzed batches of *C. juncea* plants, most of them presented larger and a minority smaller nodules.

Due the species of the genus *Crotalaria* sp. showing promiscuous behavior and establishing more or less efficient symbiosis with rhizobia from the soil, the plants were not inoculated. Therefore, in this experiment, the symbiotic efficiency of the rhizobia strains present in the soil was evaluated. The difference in the size of *C. juncea* nodules may be a consequence of its nodulation by less efficient and competitive strains, as has been observed in white clover [35].

When were compared the biomass productivity and leaf N concentration in the two *C. juncea* groups (with larger and smaller nodules), a statistically significant difference in favor of the group with larger nodules was found (Tables 1 and 3). Furthermore, shoot dry matter and foliar N concentration were correlated positively with each other (shoot dry mass = 2.4415 × [N] + 0.0286, $R^2 = 0.3783$, $p = 0.0004$). This finding is in agreement with the findings of Adams et al. [36], which stated that an increase in foliar N concentration favors photosynthetic capacity [37].

The ^{15}N isotopic composition of the leaves ($\delta^{15}N$) significantly varied between the two species; while the $\delta^{15}N$ mean in *C. spectabilis* was negative, in *C. juncea* it was positive (Table 1). Contrarily, when only the *C. juncea* group with large nodules was included in this comparison, no significant difference was found (Table 2). In turn, the mean values of $\delta^{15}N$ in the *C. juncea* groups with larger and smaller nodules were different, being negative in the first group and positive in the second (Table 1), although they were always less than the $\delta^{15}N$ values of the reference plant. Negative values of $\delta^{15}N$ would indicate that the main N source was atmospheric N_2 acquired by BNF, while positive values seem to point to the soil as the main N source.

The BNF proportion, estimated form the average $\delta^{15}N$ values of whole plants, was higher in *C. spectabilis* than in *C. juncea* (Table 1). On the contrary, there was no difference in BNF between these two species when only the *C. juncea* plants with large nodules were compared with *C. spectabilis* plants (Table 2). Within the *C. juncea* plants, the BNF values were close to 85% in the group with larger nodules, but decreased to 45% in the group with smaller nodules (Table 1). In *C. spectabilis*, on the other hand, all individuals had BNF values equal to or greater than 90% (Table 1). In any case, the BNF proportion was high for both species, which is in agreement with reports from Brazilian authors [11,38]. Overall, this result suggests that *C. spectabilis* maintained high BNF values in the simulated environment, while *C. juncea* showed high variability among plants. This result contrasts, however, with that of another Uruguayan field study, in which these species, despite having been inoculated, failed to nodulate [17].

Table 1. Mean values of total dry matter (Total DM), transpired water mass (T), foliar N concentration (N_{leaf}), net photosynthesis (A), leaf stomatal conductance (g), instantaneous transpiration rate (E), ^{13}C isotopic composition ($\delta^{13}C$), ^{15}N isotopic composition ($\delta^{15}N$), transpiration efficiency (TE), foliar intrinsic water efficiency (A/g), foliar instantaneous water efficiency (A/E), intrinsic water efficiency of the whole plant (iWUE), and proportion of biological N fixation (BNF) in *Crotalaria spectabilis* and *C. juncea*, evaluated according to a visual criterion in plants with large (+) and small (−) nodules.

Species	**Nodules**	**Shoot DM**	**T**	**N_{leaf}**	**A**	**g**	**E**	**$\delta^{13}C$**	**$\delta^{15}N$**
		g	kg	gN/100gDM	µmol/m^2 s	mol/m^2 s	mmol/m^2 s	‰	
C. spectabilis	+	6.29 ± 1.16	2.11 ± 0.56	2.26 ± 0.43	6.78 ± 2.15	0.11 ± 0.06	1.70 ± 0.80	−27.75 ± 0.56	−0.45 ± 0.72
C. juncea	+/−	3.60 ± 2.61	1.48 ± 0.76	1.85 ± 0.70	5.09 ± 3.72	0.15 ± 0.09	2.21 ± 1.14	−29.46 ± 0.78	1.13 ± 2.27
	+	5.05 ± 2.72	1.87 ± 0.81	2.23 ± 0.70	7.06 ± 3.96	0.19 ± 0.10	2.72 ± 1.15	−29.23 ± 0.90	−0.74 ± 0.95
	−	1.91 ± 1.05	1.03 ± 0.39	1.40 ± 0.37	2.80 ± 1.63	0.10 ± 0.07	1.61 ± 0.87	−29.74 ± 0.55	3.30 ± 0.87
					Water-Use Efficiency				N fixation
			TE g/kg			A/g µmol/mol	A/E mmol/mol	iWUE µmol/mol	BNF %
C. spectabilis	+		3.10 ± 0.58			68.95 ± 17.86	4.25 ± 0.93	74.06 ± 6.57	92.65 ± 7.50
C. juncea	+/−		2.21 ± 0.68			33.44 ± 13.01	2.17 ± 0.89	54.15 ± 8.91	67.08 ± 22.90
	+		2.54 ± 0.74			35.22 ± 7.59	2.43 ± 0.70	57.00 ± 10.30	85.30 ± 9.27
	−		1.81 ± 0.34			31.37 ± 18.09	1.87 ± 1.06	50.83 ± 6.24	45.82 ± 8.50

Table 2. Statistical results of the Kruskal–Wallis analysis for total dry matter (Total DM), isotopic composition of ^{15}N ($\delta^{15}N$), proportion BNF (BNF), leaf stomatal conductance (g), intrinsic leaf water-use efficiency (A/g), isotopic composition of ^{13}C ($\delta^{13}C$), and intrinsic plant water-use efficiency (iWUE) in *Crotalaria spectabilis* and *C. juncea*, evaluated in plants with large (+) and small nodules (−).

	Nodules	**Shoot DM**	**$\delta^{15}N$**	**BNF**	**g**	**A/g**	**$\delta^{13}C$**	**iWUE**
					p			
Model		0.0026	<0.0001	<0.0001	NS	0.0003	0.0001	0.0001
Species				Ranks mean and Groups				
C. juncea	-	4.8A	26.50A	3.50A	-	6.67A	5.83A	5.83A
	+	14.9B	10.14B	17.4B	-	9.00A	9.29A	9.29A
C. spectabilis	+	18.9B	12.81B	19.2B	-	20.75B	20.94B	20.94B

Means with a common letter are not significantly different ($p > 0.05$), NS: not significant.

Table 3. Statistical results of an ANOVA for foliar N concentration (N_{leaf}), transpired water (T), transpiration efficiency (TE), net photosynthesis rate (A), instantaneous transpiration rate (E), and instantaneous water-use efficiency (A/E) in *Crotalaria spectabilis* and *C. juncea*, evaluated in plants with large (+) and small nodules (−).

	N_{leaf}	**T**	**TE**	**A**	**E**	**A/E**
Model			*p*			
Species	0.0352	0.0099	0.0004	NS	NS	<0.0001
Species > Fix	0.0061	0.0173	0.0339	0.0067	0.0363	NS
Contrasts			*p*			
C. juncea vs. *C. spectabilis*	0.0245	0.0071	0.0003	0.0687	NS	<0.0001
C. juncea (+) vs. *C. juncea* (−)	0.0061	0.0173	0.0339	0.0067	0.0363	NS
C juncea (+) vs. *C. spectabilis*	NS	0.4011	0.0467	NS	0.0421	0.0002

Means with a common letter are not significantly different ($p > 0.05$), NS: not significant.

On the other hand, the two *Crotalaria* species did not differ in terms of photosynthetic rate (Table 3), stomatal conductance (Table 2), and transpiration rate (Table 3). However, the transpiration and photosynthetic rate were significantly higher in *C. juncea* plants with large nodules and a higher BNF (Table 3). Moreover, the transpiration rate (E) in the *C. juncea* group with higher nodulation was significantly higher than in *C. spectabilis* (Table 3).

The mass of transpired water (T) during the plant growing cycle was higher in *C. spectabilis* than in *C. juncea* (Tables 1 and 3), and besides, T was positively correlated with the aerial biomass (Figure 2). This result was consistent with what was reported for these two same species when they grew under controlled conditions but went through a period of moderate water deficit [29]. Contrarily, no significant T difference was found when *C. spectabilis* plants were compared with *C. juncea* with larger nodules (Table 3). The T mean, however, was significantly higher in the *C. juncea* group with larger nodules and a higher BNF.

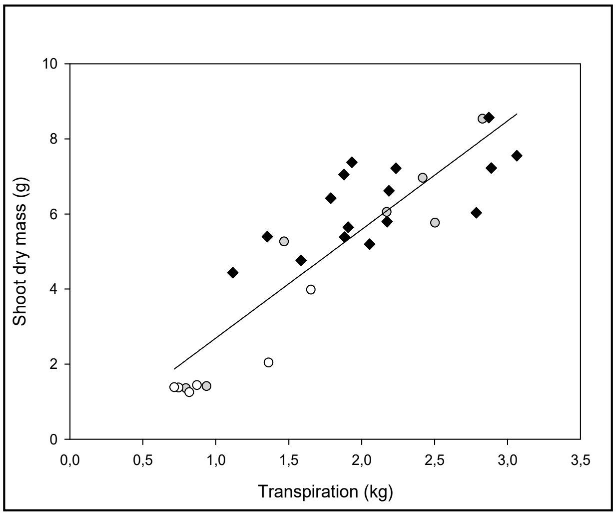


Figure 2. Relationship between shoot dry mass and water transpiration expressed for *Crotalaria spectabilis* (rhombuses) and *Crotalaria juncea* (circles). *C. juncea* was evaluated at two nodulation levels. Plants with large nodules are identified with gray circles, and those with small nodules with white circles. Regression lines: $y = 2.893x - 0.2$. $R^2 = 0.7896$ ($p < 0.0001$).

The water footprint, which corresponds to the amount of water used to generate 1 kg of dry matter, was on average 515 and 342 L water/Kg dry matter for *C. juncea* and *C. spectabilis*, respectively. Therefore, *C. juncea* was less efficient in the use of water resources than *C. spectabilis*. If the water supply of these crops in the field were only rainwater, the water footprint of both species could be classified as green [39].

The isotopic composition of ^{13}C, evaluated as δ^{13}C, was different between species and lower in *C. juncea* (Tables 1 and 2), which was due to the greater isotopic fractionation of $^{13}CO_2$ in this species [40]. As comparisons between species were made in the same environment and developmental circumstances, the δ^{13}C values are related to genetic differences [41]. In addition, the ^{13}C isotopic composition within *C. juncea* plants was not related to BNF, because there were no differences between the groups with the largest and smallest nodules; that is, plants that fixed more and less N (Table 2).

3.2. Transpiration Efficiency and Water Use Efficiency

In both species, the mean values of the different WUE indicators evaluated in this work (TE, A/E, A/g, iWUE) were consistent, and showed that *C. spectabilis* was more efficient than *C. juncea* in the use of water resources (Table 1). Interestingly, the mean TE of *C. spectabilis* was higher than that of *C. juncea*, (Table 1), regardless of the size of the nodules and the BNF values of the latter species (Table 3). Regarding A/E, A/g, and iWUE, significant differences were observed between the species, but not between *C. juncea* plants with different nodule sizes (Table 2).

When both species were grouped, positive correlations between iWUE and the other instantaneous WUE indicators, such as A/g, were found (Figure 3; Table 4). This outcome agrees which the findings of Johnson et al. [42] and Read et al. [43]; they found negative correlations between A/g and Δ^{13}C in different *Agropyron desertorum* clones, observed both under conditions without hydric limitation and under drought conditions. Overall, these results highlight the robustness of the isotopic methodology for the study of these parameters.

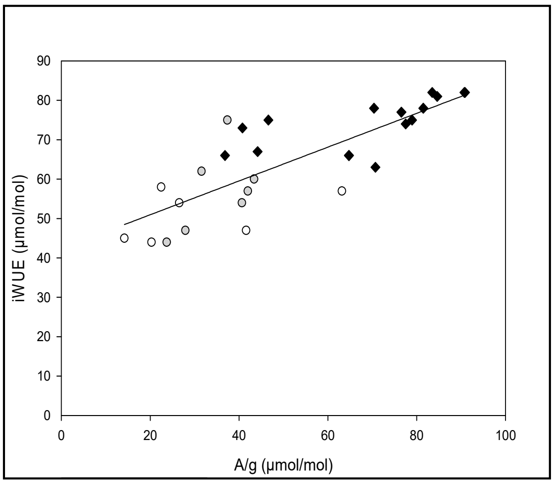


Figure 3. Relationship between the integrated intrinsic water use efficiency (iWUE) and foliar water use efficiency [quotient: photosynthesis (A) and stomatal conductance (g)] for *Crotalaria spectabilis* (rhombuses) and *Crotalaria juncea* (circles). *C. juncea* was evaluated at two nodulation levels. Plants with large nodules are identified with gray circles, and those with small nodules with white circles. Regression lines in a): $y = 0.43x + 42.2$. $R^2 = 0.66$ ($p < 0.0001$).

Table 4. Pearson's correlation matrix of transpiration efficiency (TE) in *C. spectabilis* and *C. juncea*, efficiency in the use of leaf intrinsic water (A/g), isotope composition of ^{15}N ($\delta^{15}N$), proportion of biological fixation of N (BNF), foliar N concentration (N), and efficiency in the use of intrinsic water from the entire plant (iWUE).

Variable	TE	A/g	$\delta^{15}N$	BNF	N	iWUE
TE	1					
A/g	0.49 **	1				
$\delta^{15}N$	–0.56 **	–0.36 ^{NS}	1			
BNF	0.62 ***	0.34 ^{NS}	–0.99 ***	1		
N	0.44 *	0.37 *	–0.52 **	0.54 ***	1	
iWUE	0.54 **	0.81 ***	–0.58 **	0.58 ***	0.40 *	1

*** Significant at the 0.001 level (2-tailed), ** Significant at the 0.01 level (2-tailed), * Significant at the 0.05 level (2-tailed), ^{NS}: non-significant.

A positive correlation was also established between BNF and iWUE (Table 4), as also reported by Kumarasinghe et al. [44]. These authors found a negative correlation between BNF and ^{13}C isotopic discrimination in different *Glycine max* cultivars subjected to saline stress conditions. However, Knight et al. [45], working in greenhouse conditions, reported a positive correlation between both variables. They attributed this result to the ^{13}C depletion that occurred at the leaf level, which was caused by isotopic fractionation mechanisms within N-fixing plants.

The foliar N concentration was also positively correlated with TE and iWUE (Table 4). Results obtained by Evans et al. [36] through metadata analysis of multiple plant species suggested that low $\Delta^{13}C$ values (or high $\delta^{13}C$ values) in fixing plants with high N contents were a consequence of relatively high A/g ratios.

The results indicate that *C. spectabilis* is more promising than *C. juncea* for use as a CC in this evaluation under controlled conditions. Although the results in these conditions may not be fully extrapolated to field conditions, it is important to highlight that the plants were able to nodulate with rhizobia present in soil with no history of these CCs. This is auspicious for regions where there is no commercial availability of specific rhizobia for *Crotalaria*. Similarly, the plants were harvested in the same phenological state as that used in the field to finish the CC, so it is expected that the same trends will be maintained regarding the evaluated attributes. In any case, although this first approach is necessary, field evaluation must also be carried out with the use of the same isotopic technique used in this work to determine TE, given its consistency with other forms of evaluation of this attribute and being that its main advantages are the simplicity of sampling and the precision of the results.

4. Conclusions

This study shows that under simulated conditions of high temperature and non-limiting soil water content *C. spectabilis* has advantages for use as a CC over *C. juncea* in terms of biomass production, BNF, and transpiration efficiency. Furthermore, these results suggest that the ^{13}C isotopic technique is a robust indicator to differentiate TE between these species. In *C. juncea*, the ^{13}C isotope indicator was not useful to distinguish between plants with low and high TE. In contrast, the ^{15}N isotope was useful to detect differences in TE between plants. Finally, although these results are valid only for these two species, this methodology of selecting legumes based on multiple objectives could also be applied to other species or cultivars—not only those destined to be used as CCs, but also cash crops.

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Capítulo 2

Validación del método de ^{13}C para estimar la eficiencia en el uso del agua en dos especies de *Crotalaria*

El trabajo previo demostró que las leguminosas estivales *Crotalaria juncea* y *Crotalaria spectabilis* tienen un buen potencial para ser incluidas en rotaciones tanto agrícolas como hortícolas como cultivos de cobertura, ya que estas especies aportan importantes cantidades de biomasa al suelo y N al sistema vía fijación biológica de N (FBN). Pero este aporte ecosistémico puede verse limitado en Uruguay por la existencia de frecuentes periodos de déficit hídrico estival. Para evaluar este impacto en condiciones controladas, se sembraron en macetas con suelo plantas de estas dos especies sin inocular con rizobios. Luego de 20 días de crecimiento sin limitaciones hídricas, se impusieron durante los 30 días posteriores dos tratamientos que consistieron en irrigación cuando el contenido de agua del suelo decreció por debajo de 80% y 50% de capacidad de campo, respectivamente. Durante los 40 días de crecimiento final, se restableció la condición sin limitación hídrica a ambos tratamientos. A cosecha, se determinó la producción de biomasa y el agua evapotranspirada total, determinándose por el método gravimétrico de referencia la eficiencia en el uso del agua (EUA). Además, la EUA también se determinó mediante la técnica isotópica de carbono ($\Delta^{13}C$). En ambos regímenes hídricos, la producción de biomasa de *C. spectabilis* fue 1,7 veces mayor que la de *C. juncea*, pero en ambas especies este déficit no afectó la redistribución de biomasa entre la parte aérea y la raíz. Cuando se agruparon los datos de las dos especies, existió una relación lineal negativa ($r = 0.8$; $p < 0.0001$) entre la EUA y $\Delta^{13}C$, validando de esta manera por primera vez el uso de $\Delta^{13}C$ para estimar la EUA de estas especies creciendo en un mismo ambiente. El uso de esta estrategia permitiría hacer un screening rápido y practico de especies de leguminosas con alta EUA. Los resultados se discuten en detalle en el artículo que se presenta a continuación.

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ORIGINAL PAPER

Carbon Isotope Discrimination and Water-Use Efficiency in *Crotalaria* Cover Crops under Moderate Water Deficit

Verónica Berriel[1] · Carlos Perdomo[2] · Jorge Monza[3]

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Abstract

The legume species *Crotalaria juncea* and *Crotalaria spectabilis*, used as cover crops (CC) in tropical and temperate zones, are being evaluated in Uruguay where their productive potential may be limited by periods of summer water deficit. In this study, *C. juncea* and *C. spectabilis* were sown in pots with soil as a substrate. After substantial growth (20 days), two different treatments of water (80% or 50% of field capacity) were imposed on the plants during 30 days, and later water was restored during 40 days. Afterward, the dry matter produced and total evapotranspired water was determined in all plants. Also, water-use efficiency (WUE) was determined using a gravimetric technique and by carbon isotope analysis ($\Delta^{13}C$). Dry matter production of *C. spectabilis* was 1.7 times higher than that of *C. juncea* and both legumes species tolerated a moderate water deficit, which did not affect the redistribution of dry matter between the shoot and root. A negative linear relationship ($r = 0.8$; $p < 0.0001$) was found between the WUE and $\Delta^{13}C$ in both species. In this study, the use of ^{13}C to estimate WUE in *Crotalaria spectabilis* and *Crotalaria juncea* was validated for the first time. This strategy allowed us to recommend the use of *Crotalaria spectabilis* as a cover crop in conditions of moderate water deficit in a soil with a shallow A horizon.

Keywords Water-use efficiency · Carbon isotope discrimination · *Crotalaria juncea*, *C. spectabilis*

1 Introduction

Cover crops (CC) are sown between commercial crops to reduce the risks of soil erosion and nutrients loss by leaching or runoff associated with bare or fallow land (Pinto et al. 2017; Unger and Vigil 1998; Daliparthy et al. 1994; Langdale et al. 1991). Several species of grasses and legumes, pure or in mixtures, can be used for this purpose. In general, grasses have higher potential to produce biomass, favouring C capture, while legumes contribute to maintaining the soil N balance by inputting N from the air into the soil through biological nitrogen fixation (Landriscini et al. 2019; Veloso et al. 2019).

In Uruguay, there is some information on the biomass production and N fixation capacity of winter legumes currently used as CC, but there is no similar data available for annual summer legumes such as *Crotalaria juncea* and *C. spectabilis*, which are already being utilised as CC in tropical and temperate zones (Reddy 2016; Pissinati et al. 2018). This lack of information is significant; because the productive potential of these two legumes could be limited by the prolonged periods of water deficit that frequently occur during Uruguayan summers, summed to the limited water holding capacity of most Uruguayan soils, with a shallow A horizon and a heavy-textured B horizon. Moreover, the water consumed during the CC cycle can further limit the amount of soil water left for the next crop. Therefore, it is important to consider the water-use efficiency (WUE) of the species used as CC, because this is a characteristic that partially defines the

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✉ Verónica Berriel
vberriel@gmail.com

Carlos Perdomo
chperdom@fagro.edu.uy

Jorge Monza
jmonza@fagro.edu.uy

[1] Centre for Applications of Nuclear Technology in Sustainable Agriculture (CATNAS), Soil and Water, Department, Agronomy College, University of the Republic, Av. Garzón 809, CP 12.900 Montevideo, Uruguay

[2] Soil and Water Department, Agronomy College, University of the Republic, Av. Garzón 780, CP 12.900 Montevideo, Uruguay

[3] Plant Biology Department, Agronomy College, University of the Republic, Av. Garzón 809, CP 12.900 Montevideo, Uruguay

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sustainability of this practice (Quemada and Gabriel 2016). There is, however, both locally and internationally, little or no information about the WUE of these two legume species.

Agronomically, WUE is defined as the ratio between biomass production and the amount of water consumed by a plant or crop during a certain time period. For this reason, WUE is an indicator that integrates the growth history of a plant or crop (Leakey et al. 2019; Bhattacharya 2019). Although the procedure for biomass estimation is relatively straightforward, the determination of water consumption could be difficult. The reference method involves gravimetrically registering the loss of soil water by evapotranspiration (ET). Under controlled conditions, this can be done by recording pot weight changes daily, and eventually restoring the water lost after some lower limit is passed. Under field conditions, however, this methodology can only be used if lysimeters are available, which allows for the recording of soil mass changes (Medrano et al. 2015a). Nowadays, it is also possible to use sensors to directly monitor soil moisture changes (Medrano et al. 2015a), or to estimate these changes with soil water balance models (Shen et al. 2019).

It is also possible to estimate WUE using an infrared gas analyser (Franks et al. 2015), which quantifies the gaseous concentrations of CO_2 and H_2O at the leaf level during short time intervals. In this case, WUE can be reported as instantaneous (WUE_T) or intrinsic (WUE_g). In the first case, the value is estimated as the quotient between net photosynthesis (A) and transpiration (T), while WUE_g is expressed as the quotient between A and stomatal conductance (g) (Franks et al. 2015). Both WUE_T and WUE_g have the advantage of being quick and non-destructive indices of the water-use efficiency at a given time (Medrano et al. 2010). These instantaneous measures, however, do not always reflect the WUE values of the entire plant (Flexas et al. 2010). For this reason, if this strategy is intended to be applied to one or several species, it is important to verify that WUE_T and WUE_g are correlated with WUE of the entire plant.

Another measurement at the leaf level that can be used as a *proxy* of WUE is carbon isotope discrimination ($\Delta^{13}C$) which represents the difference in the ^{13}C composition in the CO_2 between air and leaf. This methodology is based on the fact that the ratio of ^{13}C to ^{12}C isotopes in the atmosphere, in agricultural environments and during the crop cycle, is constant. However, concentrations in the leaf are variable due to the greater preference towards the use of ^{12}C by C3 plants (Farquhar et al. 1989; Pronger et al. 2019). This discrimination occurs during both stomatal and mesophyll diffusion and CO_2 assimilation by the RubisCO enzyme. In contrast to the previous instantaneous parameter, this isotopic test can integrate the temporal WUE variation that occurs during the growth cycle (Farquhar et al. 1989; Pronger et al. 2019).

Although there is ample information on the use of $\Delta^{13}C$ to quantify WUE in many species (Bhattacharya 2019), no reports have been found on the use of this methodology in *Crotalaria juncea* and *C. spectabilis*. The aim of this work was to identify through the use of isotopic techniques crotalarias with high WUE, to be used as CC in the climate scenario of moderate water deficit.

2 Materials and Methods

2.1 Growth Conditions

Seeds of *Crotalaria juncea* L. and *Crotalaria spectabilis* Roth. were sown in plastic pots using Arguidol soil (2.0% organic carbon, 24.5% sand, 48.7% silt, and 26.8% clay) as the growth media. Plants were grown in a controlled growth chamber at 30 °C, with a luminous intensity of 1500 $\mu mol\ m^{-2}\ s^{-1}$ and a 16/8 h dark-light cycle. During the first 5 days of growth (plant emergence), the relative humidity (RH) was set at 80%, and after this initial period RH remained at 40 ± 10% until the end of the experiment.

The water deficit treatment tried to simulate the typical variations of soil water content that occur during summer in southern Uruguay. In the control treatment, soil moisture remained constant at 80% (w/w) field capacity (FC) for 90 days. In the treatment with water deficit, however, soil moisture varied during the period. At the beginning, it remained at 80% of FC for 20 days, then went to 50% FC for 30 days, and finally remained at 70% FC for 40 days.

Soil water content at FC was estimated in the laboratory with a pressure cooker (Richards and Weaver 1944), and was equal to 28.5% (w/w). The amount of water needed to add to pots to reach the other thresholds were estimated daily as the difference between the target water gravimetric content and the actual soil content. The sum of these differences was considered to be the ET accumulated during the plant cycle.

2.2 Determination of Stomata Conductance

The stomata conductance (g) was determined at 20, 50, and 90 day after sowing (before, during, and after soil water limitation, respectively) in the youngest fully expanded leaf using a leaf porometer (Decagon Device, Inc., model SC-1). These determinations were made in triplicate on the abaxial side of the leaves, between 4 and 5 h after the light was turned on in the plant growth chamber.

2.3 Biomass Production

After 90 days of growth, plants were separated into leaves, stems, roots, and nodules. Each part was dried at 60 °C until a constant weight, and the dry biomass of each part was weighed and recorded.

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2.4 Determination of Water-Use Efficiency by Gravimetric Method

WUE was calculated either as the ratio of total (WUE_b) or shoot (WUE_{shoot}) biomass produced at the end of the experiment to ET (Eqs. 1 and 2, respectively).

$$WUE_b = \frac{\text{total biomass}}{ET} \tag{1}$$

$$WUE_{shoot} = \frac{\text{shoot biomass}}{ET} \tag{2}$$

2.5 Carbon Isotope Discrimination Method and N Determination

Plant organ samples were first ground with a fixed and mobile knife mill (Marconi MA-580) until the particle size was less than 2 mm and then with a rotary mill (SampleTek 200 vial Rotator). Each sample (1.5 mg) was weighed in triplicate in tin capsules and introduced in an elemental analyser (Flash EA-1112) coupled to a Thermo Finnigan DELTAplus mass spectrometer (Bremen, Germany). Data about N concentration and the natural $^{13}C/^{12}C$ isotopic ratio was obtained from this set of equipment. The isotopic $\delta^{13}C$ results were estimated according to Sulzman (2007) (Eq. 3) and calibrated against Vienna Pee Dee Belemnite (VPDB) using three reference materials (IAEA-CH6, USGS-40, and USGS-41).

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000 \tag{3}$$

Carbon isotope discrimination ($\Delta^{13}C$) was calculated with the equation of Farquhar et al. (Farquhar et al. 1989).

$$\Delta^{13}C = \left(\frac{\delta^{13}C_{atmosphere} - \delta^{13}C_{plant}}{1 + \frac{\delta^{13}C_{atmosphere}}{1000}} - 1\right) \times 1000 \tag{4}$$

2.6 Experimental Design and Statistical Analysis

Treatments consisted of combinations of two plant species and two soil water regimes (control FC and water deficit) arranged in a complete factorial design with 16 repetitions. The main effects of species and soil water status, as well as their interaction, were analysed by ANOVA using InfoStat (InfoStat®, 2009). Regression analyses were also used to study the relationships between WUE indicators.

3 Results

In this work, the WUE of two legumes of the *Crotalaria* genus was determined with the carbon isotope discrimination method, which was in turn validated against the reference gravimetric method.

The ANOVA's results for the total biomass, ET, and N analysed according to the species and soil water status are summarised in Table 1. The interaction between species x soil water status was not significant in any case; thus, soil water status produced similar trends in both species.

The two species produced different amounts of dry matter, where *C. spectabilis* produced approximately 1.7 times more biomass than *C. juncea*. There was no statistically significant effect of the water treatments on biomass production across the two species (Table 1). In turn, ET was different in both species, i.e. 1.5 times higher in *C. spectabilis* (Table 1). Although the soil water status did not significantly affect ET, under conditions of water deficit the absolute value of this parameter was lower, which can be interpreted as a trend (Table 1). Finally, in both water regimes, the amount of N accumulated in the aerial parts was higher in *C. spectabilis* than in *C. juncea* (Table 1).

During the period of soil water limitation, there were differences in stomatal conductance (g) both between species and soil water treatments (Table 2). On the contrary, before and after the period of soil water deficit, no differences were found water treatments. Thus, the decrease in soil water content induced an adaptation response in plants that lead to a decrease stomatal conductance, but after soil moisture values were restored to near optimum levels; plants were able to recover by increasing stomatal conductance.

Dry matter partition between leaf and stem varied only between species, but was not affected by soil water treatments (Fig. 1). The biomass of leaves in *C. spectabilis* was 2.2 times higher than in *C. juncea*, while that of stem was 0.4 times lower. The biomass of roots and nodules did not differ between both species (Fig. 1).

The WUE estimated from the total biomass (WUE_b, Eq. 1) was 1.3 times higher in *C. spectabilis* than in *C. juncea* (Table 3). Moreover, and in contrast to the previous results, there was a statistically significant effect of soil water status on WUE_b across both species; WUE_b was 1.4 times greater in the water deficit treatment than in the control. A similar result was observed in the case of WUE_{shoot} (Table 3). According to the $\Delta^{13}C$ analyses, a *proxy* of WUE, *C. spectabilis* was also more efficient than *C. juncea*, but both species showed higher WUE in the water deficit treatment with respect to the control condition (Table 3).

For the two species of *Crotalaria*, a positive linear relationship was found both between ET and total plant dry matter (Fig. 2a, b) and ET and shoot dry matter. Moreover, the slopes were similar between water treatments (Fig. 2), which agrees with the ANOVA's results (Table 1) which showed that there were no significant differences in biomass and ET between water treatments.

In *C. juncea*, a negative correlation was found between $\Delta^{13}C$ and total or shoot biomass (Fig. 3) under the two water conditions (Fig. 3a). In *C. spectabilis*, however, this

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Table 1 Mean, standard deviation and number of replications (inside brackets) for dry matter (DM), nitrogen concentration (N) and evapotranspiration (ET) at various combinations and aggregation levels of specie and soil water status. The statistics results are shown at the bottom

Combination			Total DM	N	ET
			g	gN/100gDM	kg H_2O
Specie-soil water status					
	C. juncea-Control		4.44±3.48 (9)	1.75±0.74 (9)	1.88±0.88 (9)
	C. juncea-water deficit		4.17±2.91 (13)	1.75±0.83 (13)	1.51±0.72 (13)
	C. spectabilis-Control		6.51±1.43 (11)	2.29±0.56 (11)	2.81±0.79 (11)
	C. spectabilis- water deficit		7.57±1.79 (15)	2.43±0.61 (15)	2.33±0.78 (15)
Specie (across soil water status)					
	C. juncea		4.28±3.07 (22)	1.75±0.788 (22)	1.66±0.79 (22)
	C spectabilis		7.12±1.70 (26)	2.37±0.58 (26)	2.53±0.81 (26)
Soil water status (across Species)					
	Control		5.58±2.70 (20)	2.05±0.69 (20)	2.39±0.94 (20)
	water deficit		5.99±2.90 (28)	2.12±0.79 (28)	1.95±0.85 (28)
Across species & soil water status					
			5.71±2.9 (48)	2.08±0.74 (48)	2.08±0.97 (48)
			Statistics		
	S. of V[1].	DF[2]	----------------*p*----------------		
	Specie	1	0.0004	0.0045	0.0005
	Soil water status	1	NS[3]	NS	NS (0.07)
	Specie* soil water status	1	NS	NS	NS

[1] Source of variation

[2] Degrees of freedom

[3] *NS* not significant ($p > 0.05$)

correlation was only found in the plants subjected to water deficit and not in the control (Fig. 3b).

In both *C. juncea* and *C. spectabilis*, a significant negative relationship was observed between WUE_b or WUE_{shoot} and $\Delta^{13}C$ (Fig. 4a, b), which indicates that the isotopic technique is a robust indicator that can be used to estimate WUE in these two legumes at least in situations of moderate water deficit.

4 Discussion

A desired trait of a CC is its ability to produce a high biomass quantity, since biomass yield is directly related to the rate at which nutrients are being recycled between plant and soil (Folorunso et al. 1992; Langdale et al. 1991). In addition, a high CC biomass production improves soil physical structure, reduces soil erosion, minimises surface runoff, and increases the soil water infiltration rate by root channel formation (Folorunso et al. 1992). In this way, CC biomass production has beneficial effects on the whole plant growth environment (Blanco-Canqui et al. 2015). However, negative effects can also occur, since CC can reduce soil water availability for the next crop (Unger and Vigil 1998), and could even compete for nutrients, although when legumes are used as CC, the net effect on terms of N would be positive. In our case, the comparison of the total biomass production between species, independently of soil water conditions, showed that *C. spectabilis* produced more biomass than *C. juncea*. Leaf N content was also higher in *C. spectabilis*, which may help to explain these differences (Table 1) (Cernusak et al. 2013).

Interestingly, soil water treatments not influenced total biomass production in any of the two species (Table 1), but the imposed soil water stress induced stomata closure (Table 2). This result allows us to infer that both *Crotalaria* species show tolerance and even were able to recover after a simulated drought similar in strength to events that commonly occur during Uruguayan's summer. Similarly, in trials under controlled conditions with *Dactylis glomerata*, *Festuca arundinacea*, and *Phalaris arundinacea*, Mårtensson et al. (2017) found no differences in biomass production between the control and the water deficit treatment, but they could establish the existence of a difference in WUE between these grasses. These WUE differences were attributed to the adaptation of these species to periods of moderate water deficit, a situation alike to that of south of Uruguay in summer. A key factor in the adaptability of legumes to drought is the higher content of leaf N in relation to other non-legume plants (Prentice et al. 2014), which reinforces the importance of using legumes as CC.

Hence, these results suggest that, in southern Uruguay, both *Crotalaria* species are capable of offering ecosystem

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Table 2 Mean, standard deviation, and number of replications (inside brackets) for leaf stomatal conductance, measuring at 20, 50, and 90 days after sowing (before, during, and after of soil water limitation period respectively) at various combinations and aggregation levels of specie and soil water status. The statistics results are shown at the bottom

Combination		Stomatal conductance mol H_2O m^{-2} s		
Specie–soil water status		Day 20	Day 50	Day 90
C. juncea–control		0.18 ± 0.04 (9)	0.15 ± 0.02 (9)	0.15 ± 0.02 (9)
C. juncea–water deficit		0.16 ± 0.03 (13)	0.10 ± 0.01 (13)	0.15 ± 0.01 (13)
C. spectabilis–control		0.12 ± 0.02 (11)	0.12 ± 0.02 (11)	0.10 ± 0.02 (11)
C. spectabilis–water deficit		0.11 ± 0.02 (15)	0.07 ± 0.01 (15)	0.09 ± 0.02 (15)
Specie (across soil water status)				
C. juncea		0.17 ± 0.03 (22)	0.12 ± 0.03 (22)	0.15 ± 0.01 (22)
C spectabilis		0.12 ± 0.02 (26)	0.09 ± 0.03 (26)	0.10 ± 0.02 (26)
Soil water status (across species)				
Control		0.15 ± 0.03 (20)	0.13 ± 0.02 (20)	0.13 ± 0.02 (20)
Water deficit		0.13 ± 0.04 (28)	0.08 ± 0.01 (28)	0.12 ± 0.03 (28)
Across species & soil water status		0.14 ± 0.04 (48)	0.11 ± 0.03 (48)	0.13 ± 0.03 (48)
Statistics				
S. of V[1].	DF[2]	*p*		
Specie	1	0.0061	0.0021	0.0002
Soil water status	1	NS[3]	0.0001	NS
Specie × soil water status	1	NS	NS	NS

[1] Source of variation

[2] Degrees of freedom

[3] *NS* not significant ($p > 0.05$)

services even when summer soil water deficits occur. Since biological N fixation is one of these important services, the threshold of soil water content at which biological N fixation rate starts to become affected should be clearly determined. It is also known that the greater the biomass production, the greater the soil coverage and less important tends to become soil water evaporation (Hatfield and Dold 2019). Therefore, in situations of high biomass production, and when there are no water and nutritional or disease limitations, the water consumption will mainly occur by transpiration (Hatfield and Dold 2019).

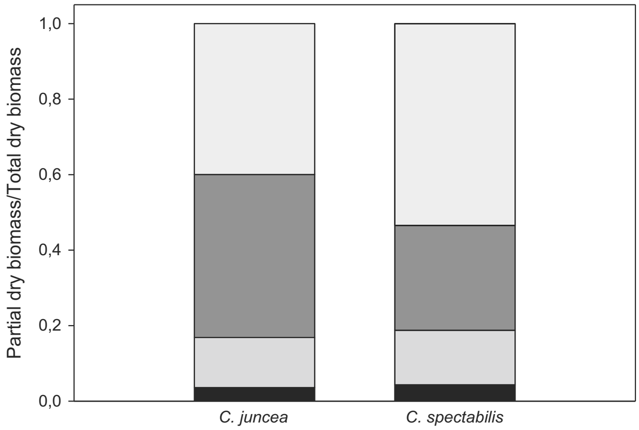

Fig. 1 Biomass distribution of leaf, stem, root, and nodule (top to bottom) within the total plant biomass of *Crotalaria juncea* ($n = 22$) and *Crotalaria spectabilis* ($n = 26$)

When water availability was not limiting, *C. spectabilis* had higher ET than *C. juncea* (Table 1), indicating the existence of a different transpiration rate between both species. In turn, ET was directly related to dry matter production, which was also higher in *C. spectabilis* than in *C. juncea* (Table 1). Although there was no difference in ET between water treatments in any specie, there was a tendency for it to decrease as water availability decreased (Table 1). There was also no difference in total biomass distribution between plant organs according to water availability (Fig. 1). In a situation of mild, moderate, or severe water limitation, it is expected that defence mechanisms against dehydration will turn on in plants. One of the first physiological responses to a water deficit is stomatal adjustment (Leakey et al. 2019), which was effectively verified in both species (Table 2).

In terms of WUE, *C. spectabilis* was more efficient than *C. juncea* under the two water conditions, and, both species increased their WUE_b during the water deficit period (Table 3). These results coincide with those reported by other authors for other legumes (Kunrath et al. 2018; Blessing et al. 2018; del Pozo et al. 2017) and grasses (Mårtensson et al. 2017; Kunrath et al. 2018).

Regarding WUE estimated by $\Delta^{13}C$, greater carbon isotope discrimination was found in *C. juncea* with respect to *C. spectabilis* (Table 3). One of the main factors that determine $\Delta^{13}C$ values in the leaf is stomatal conductance (Farquhar and Richards 1984). However, it cannot be ruled out that the differences in the rate of other post-photosynthetic

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Table 3 Mean, standard deviation, and number of replications (inside brackets) for several water-use efficiency (WUE) indexes based on total (WUE_b), shoot dry matter (WUE_{shoot}), and carbon isotope discrimination in leaves ($\Delta^{13}C$) at various combinations and aggregation levels of specie and soil water status. The statistics results are shown at the bottom

Combination		WUE_b g DM/kg H_2O	WUE_{shoot}	$\Delta^{13}C$ ‰
Specie–soil water status				
C. juncea–control		2.07 ± 0.86 (9)	1.75 ± 0.72 (9)	21.89 ± 0.58 (9)
C. juncea–water deficit		2.58 ± 0.96 (13)	2.10 ± 0.75 (13)	20.82 ± 0.67 (13)
C. spectabilis–control		2.37 ± 0.38 (11)	1.96 ± 0.36 (11)	20.41 ± 0.21 (11)
C. spectabilis–water deficit		3.47 ± 1.09 (15)	2.80 ± 0.94 (15)	19.43 ± 0.35 (15)
Specie (across soil water status)				
C. juncea		2.37 ± 0.94 (22)	1.95 ± 0.74 (22)	21.26 ± 0.82 (22)
C. spectabilis		3.00 ± 1.02 (26)	2.44 ± 0.85 (26)	19.84 ± 0.57 (26)
Soil water status (across species)				
Control		2.23 ± 0.64 (20)	1.87 ± 0.54 (20)	21.08 ± 0.86 (20)
Water deficit		3.05 ± 1.11 (28)	2.47 ± 0.91 (28)	20.08 ± 0.87 (28)
Across species & soil water status		2.71 ± 1.00 (48)	2.22 ± 0.81 (48)	20.49 ± 0.99 (48)
	Statistics			
S of V[1]	DF[2]	*p*		
Specie	1	0.0275	0.0425	< 0.0001
Soil water status	1	0.0037	0.0099	< 0.0001
Specie × soil water status	1	NS[3]	NS	NS

[1] Source of variation

[2] Degrees of freedom

[3] *NS* not significant ($p > 0.05$)

processes could also be explaining the difference in leaf $\Delta^{13}C$ between these two species (Farquhar et al. 1989). Regardless of that, the fact that the isotopic $\Delta^{13}C$ imprint remained significant after the soil water content was restored to 70% FC (Table 3), clearly indicates that the ^{13}C is a robust indicator of WUE in these species (Berriel et al. 2014).

Variations in dry matter production or crop yield as a function of ET has been studied by different authors in different species (Berriel et al. 2014; Hanks et al. 1969; Tanner and Sinclair 1983). In *C. spectabilis* and *C. juncea*, the relationships between dry matter production and ET were mostly linear, as reported in *Medicago sativa* L., and *Festuca arundinacea* Schreb. (Kunrath et al. 2018), as well as, in *Triticum vulgare*, *Sorghum vulgare*, *Panicum miliaceum*, and *Avena sativa* (Hanks et al. 1969). In our data, these relationships were also linear, but the slope in *C. juncea* was more than twice that of *C. spectabilis* (Fig. 3). Therefore, *C. juncea* would be the specie with the greater phenotypic plasticity in this attribute. This higher slope was also the cause of the greater biomass variability of *C. juncea* with respect to that of *C. spectabilis*.

The existence of a negative relationship between biomass and $\Delta^{13}C$ which was observed in this work, however, is not generalisable to all species and water conditions, given that

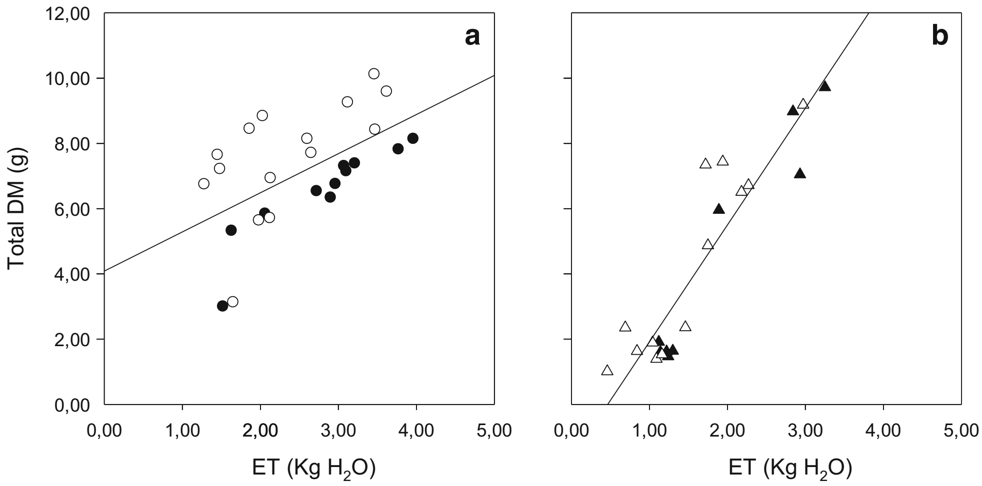

Fig. 2 Relationship between total plant biomass (total DM) and transpiration (T) for **a** *Crotalaria spectabilis* (circles) and **b** *Crotalaria juncea* (triangles), both in control conditions (black symbols) or under water deficit conditions (blank symbols). Regression lines in **a**: $y = 1.2003x + 4.0853$, $R^2 = 0.3238$ ($p = 0.0024$); and in **b**: $y = 3.5831x - 1.6649$, $R^2 = 0.8512$ ($p < 0.0001$)

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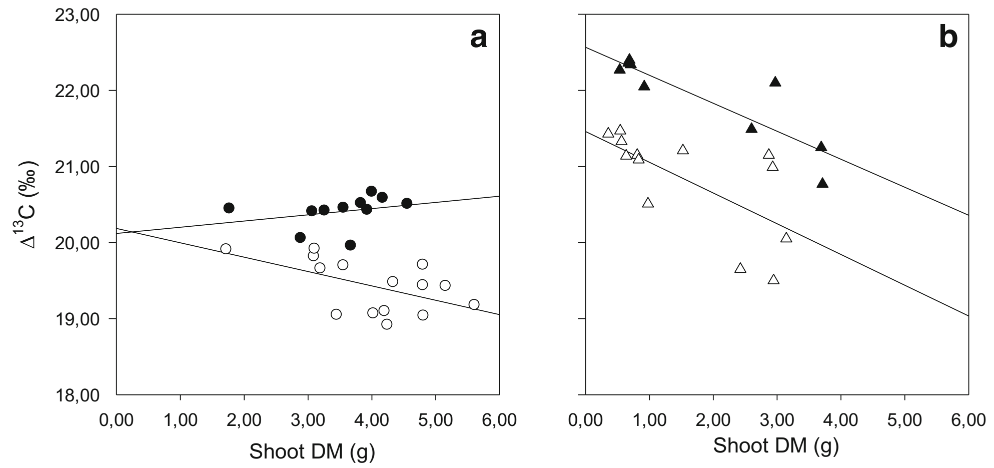


Fig. 3 Relationship between shoot biomass (shoot DM) and the carbon isotope discrimination in the leaf ($\Delta^{13}C$) for **a** *Crotalaria spectabilis* (circles) and **b** *Crotalaria juncea* (triangles), both in control conditions (black symbols) or under water deficit conditions (white symbols). Linear regression in **a** for control conditions: $y = 0.0825x + 20.118$, $R^2 = 0.0864$ (p: not significant); and for water deficit conditions: $y = -0.1896x + 20.187$, $R^2 = 0.2984$ ($p = 0.0373$); in **b** for control conditions: $y = -0.3684x + 22.568$, $R^2 = 0.7616$ ($p = 0.0008$); and for water deficit conditions: $y = -0.4057x + 21.462$, $R^2 = 0.4386$ ($p = 0.0098$)

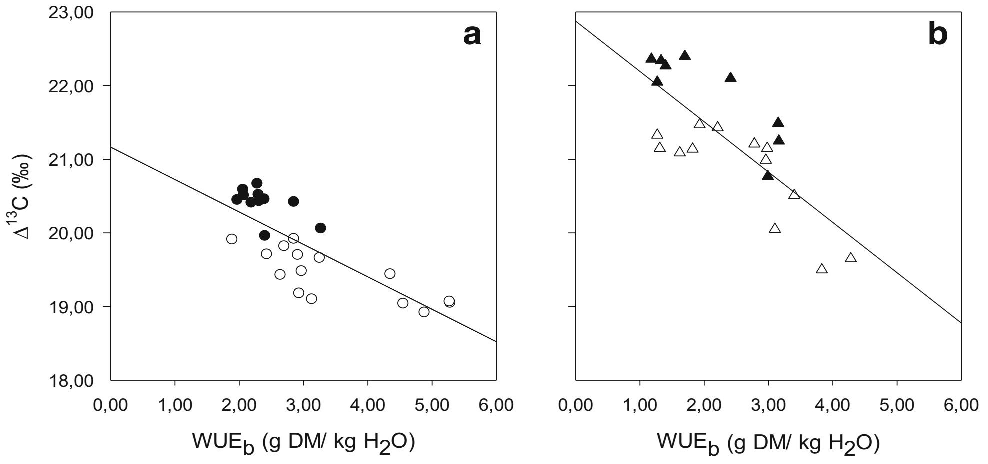
positive linear relationships between these parameters has also been reported for species such as *Medicago sativa* (Ray et al. 1998; Zhu et al. 2019) and *Triticum aestivum* L. (Condon et al. 1987), among others. In the same way, positive relationships have been reported between grain yield and $\Delta^{13}C$ in crops such as *Oryza sativa* (Kondo et al. 2004; Gao et al. 2018), and *Triticum aestivum* (Condon et al. 1987; Merah et al. 2001; Condon et al. 2004; Zhu et al. 2010). In turn, in other species such as *Beta vulgaris* L., no relationships were found between these variables (Rytter 2005).

The $\Delta^{13}C$ isotopic method is already internationally used as a *proxy* of WUE due to the simplicity of its estimation and its precision, and because it provides information that reflects the growth plant's history (Bhattacharya 2019; Pronger et al. 2019; Berriel et al. 2014). In most C3 species, there is a strong correlation between WUE and $\Delta^{13}C$, including *Phaseolus vulgaris* (White et al. 1990), *Arachis hypogea* (Wright et al. 1993), *Vigna unguiculata* (Ismail and Hall 1992), *Triticum aestivum* (Condon et al. 1990), *Hordeum vulgare* (Condon et al. 1990; Anyia et al. 2007), *Agropyron desertorum* (Ehleringer et al. 1990), *Leymus angustus* (Johnson et al. 1990), *Medicago sativa* (Johnson and Tieszen 1994), *Dactylis glomerata*, *Festuca arundinacea*, and *Phalaris arundinacea* (Mårtensson et al. 2017). However, in some species this relationship is not clear, as in *Arachis hypogea* under water deficit situations (Wright et al. 1993) or in *Beta vulgaris L.* (Rytter 2005).

The linear relationship found in our work between WUE and $\Delta^{13}C$ in *C. spectabilis* and *C. juncea* revealed that the plants kept under no water stress (control conditions) always had higher $\Delta^{13}C$ and lower WUE_b and WUE_{shoot}, values, while the opposite occurred when plants were submitted to a water deficit period (Fig. 4).

The strong correlation observed between the carbon isotope discrimination method and the reference gravimetric method in *C. juncea* and *C. spectabilis* makes it possible to

Fig. 4 Relationship between the water-use efficiency of the whole plant (WUE_b) and the carbon isotope discrimination in leaf ($\Delta^{13}C$) for **a** *Crotalaria spectabilis* (circles) and **b** *Crotalaria juncea* (triangles), both in control conditions (black symbols) or in water deficit conditions (white symbols). Linear regression in **a** $y = -0.4408x + 21.167$, $R^2 = 0.6084$ ($p < 0.0001$); and in **b** $y = -0.6855x + 22.882$, $R^2 = 0.6047$ ($p < 0.0001$)



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use either or both approaches to estimate WUE. Although these results are auspicious, it is also important to study the effect of other climatic variables in $\Delta^{13}C$ for these CC in field conditions.

5 Conclusions

In this work, the first water-use efficiency values of *Crotalaria spectabilis* and *Crotalaria juncea* were obtained, using the ^{13}C stable isotopic technique. The strong negative correlations found between the gravimetric reference method and the isotopic technique allows us to propose the use of the latter to estimate water-use efficiency in field conditions due to its simplicity. Given the lower performance of *C. juncea*, both in terms of biomass production and water-use efficiency, we recommend the use of *C. spectabilis* as summer cover crop for the shallow A-horizon soils of Southern Uruguay.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Capítulo 3

Comparación del valor B obtenido con dos metodologías que utilizan suelo como sustrato y su influencia en la estimación del porcentaje de N fijado.

Para cuantificar la proporción de nitrógeno derivado del aire (%Ndfa) en la simbiosis rizobio-leguminosa por el método de la abundancia natural de ^{15}N es necesario conocer el valor B, el cual representa el valor de $\delta^{15}N$ de una planta que solo fija N. La metodología que más se utiliza para determinar este valor se basa en crecer las plantas en soluciones nutritivas sin N. Otra metodología para la determinación de B usa suelo como sustrato, sin embargo, debe considerarse que sí la planta toma N de esta fuente sin restricción el valor B aumentará. Este incremento se debe a que la señal isotópica del suelo es mayor a la del aire; este corrimiento del valor real generará sobreestimaciones del %Ndfa, incluso con valores que superen el 100%. En este trabajo se evaluaron dos métodos para determinar el valor B de *Crotalaria juncea*, *C. spectabilis*, *C. ochroleuca* y *Cajanus cajan,*utilizando suelo como sustrato. Un método consistió en utilizar un suelo como sustrato y estimar el valor B como el promedio de los menores valores B de $\delta^{15}N$ de la parte aérea (B-mínimo). El otro consistió mezclar un suelo con sacarosa de manera de inmovilizar el nitrógeno mineral que está siendo mineralizado, tornándolo no disponible y promediar los valores de $\delta^{15}N$ de la parte aérea de todas las plantas (N-inmovilizado). En *C. juncea* y *C. cajan* los valores B fueron menores con N-inmovilizado que con B-mínimo (-2,2 y -2,9 ‰, respectivamente). Además, en *C. cajan* y *C. ochroleuca* los valores B obtenidos con N-inmovilizado fueron hasta 1 ‰ inferiores con respecto a los que figuran en la bibliografía para estas especies. Los porcentajes de N fijado también fueron menores con N-inmovilizado De acuerdo a estos resultados, la metodología de N-inmovilizado permitiría obtener de forma práctica valores B representativos de las condiciones locales y así evitar obtener valores de fijación de N irreales. En el *preprint* del artículo que se presenta a continuación se describen y discuten los resultados obtenidos.

Comparison of B values obtained by two methodologies that use soil as a substrate and their influence on the estimation of the proportion of nitrogen fixed by legumes

Abstract

The B value is required to quantify the nitrogen derived from the atmosphere (%Ndfa) in the Rhizobium-legume symbiosis using the ^{15}N natural abundance method. When the B value of a particular specie is not known, one possibility Is to use as a proxy the B value of a specie from the same genus, but this can cause the estimate of %Ndfa to be inaccurate. In this work, we compared two methodologies for determining the B value of *Crotalaria juncea*, *C. spectabilis*, *C. ochroleuca* and *Cajanus cajan,* using soil as the substrate. One method involved growing plants in soil and averaging the lowest δ^{15}N values of plant shoots (B-minimum), while the other consisted in adding sucrose to soil to immobilize the mineral nitrogen (N-immobilized), and then averaging the shoot δ^{15}N values of all plants. Results showed that B values of *C. cajan* and *C. ochroleuca* obtained using the N-immobilized method were up to 1‰ lower than those reported in the literature for these species. Therefore, we propose that, at least in these species, B values determined with the N-immobilized method should be used to estimate the %Ndfa.

Introduction

Cover crops (CCs) based on species of the genus *Crotalaria* and *Cajanus cajan* can contribute with significant amounts of nitrogen (N) to the soil through the biological fixation process (BNF) (Giller, 2001; Santana et al., 2018, Berriel et al. 2020). The high BNF of these CCs is achieved due to the efficient symbiotic association that they establish with a wide variety of rhizobia soil strains of the genus *Methylobacterium*, *Bradyrhizobium* and others (Sy et al., 2001; Zilli et al., 2020, Fossou et al., 2020).

The accurate quantification of the N fixed by these CCs is needed to balance N inputs, and this information constitutes an important input for planning sustainable agricultural rotations (Landriscini et al., 2019).

Estimation of the N proportion in plants or crops that derives from the atmosphere by BNF (%Ndfa) can be carried out using the nuclear technique proposed by Shearer and Kohl (1986), which is based on the natural differences in ^{15}N that exist between the atmosphere and the soil (Amarger et al., 1979). The determination of %Ndfa by this technique is carried out via Equation 1 (Unkovich et al., 2008).

$$\%Ndfa = \left(\frac{\delta^{15}N_{ref} - \delta^{15}N_{fix}}{\delta^{15}N_{ref} - B}\right) \times 100$$ ……………………………………………………………….. ..Eq. 1

where $\delta^{15}N_{ref}$ is the ^{15}N abundance determined in a non-fixing reference plant, $\delta^{15}N_{fix}$ is the ^{15}N abundance of the legume that has grown and developed in the site and/or soil of interest, and B is the ^{15}N abundance of the legume whose only N source is the atmospheric N.

A key component in Eq. [1] is the B value, which in some cases could be obtained from the literature. However, it should be noted that this value depends on the plant species (Unkovich et al., 2008) and the rhizobium strain with which symbioses were established (Guimarães et al., 2008; Pauferro et al., 2010).

Usually, the B value is determined by growing plants inoculated with a rhizobium strain on inert substrates (sand, vermiculite, hydroponic solutions, etc.), providing the necessary requirements for plant growth, except mineral N. Although the growth may be limited under these conditions, this ensures that the only N source available of for the plant is the atmospheric N (Unkovich et al., 2008).

Another strategy to estimate the B value is to perform a sampling of a site where the species of interest is growing and estimate as value B an average of the lowest. Here, the mean of the most negative $\delta^{15}N$ shoot values is used as the B estimate (B-minimum), assuming that in these cases the plant's N came exclusively from the BNF (Peoples et al., 2002). A different approach to determine

B is force the immobilization of mineral N from the soil (N-immobilized) by adding a substrate with a high C/N ratio, in such a way that the plant does not have N from the soil for its growth and that this mineral N does not inhibit BNF (Ferguson et al., 2019). A material highly energetic or with a high C/N ratio promotes net N immobilization (Mori et al., 2012; Romero et al., 2015), because soil microorganisms will require more N to metabolize the added C (Chen et al., 2020). Therefore, soil nitrate and ammonium will be converted into organic forms and incorporated into cellular components, such as proteins, thus leaving the soil devoid of mineral N (Cao et al., 2020).

Moreover, these two approaches can also be used with uninoculated seeds, which allows estimating the B value of the rhizobia strains already present and adapted to this particular soil. Thus, this B value will be representative of a specific soil-rhizobia-legume symbioses system.

Our aim was to compare the B value obtained with these two methods that use soil as the substrate not only between them, but also with values reported in the literature for the same species, and to assess the impact of this variations in the %Ndfa estimations.

Materials and Methods

Materials and methods

Plant material and growing conditions

Plants of *Crotalaria juncea*, *Crotalaria spectabilis*, *Crotolaria ochroleuca* and *Cajanus cajan* were cultivated in pots containing samples of an agricultural soil (4kg pot^{-1}) which has never been planted with these legume species. Before planting, seeds *were* superficially sterilized (Okito et al., 2004), and sown at a rate of one seed per pot. The soil was an Argiudol from southern Uruguay (organic carbon = 11.6 g/kg; sand = 24.5; silt = 48.7%; clay = 26.8%; NO_3^-: 3.6 mg/kg; NH_4^+: 7.1 mg/kg).

Plants were grown for 90 days in a growth chamber at 30ºC, with a variable relative humidity of between 30 and 50% and a light intensity of 500 µmol m^{-2}. s^{-1} with a 16/8 h light/dark cycle and irrigated with deionized water.

To determine the B value according to the minimum B method, plants of each specie were grown in 40 pots containing the original soil.

To determinate the B value with the N-immobilized method, soil was mixed with sucrose (in ratio 1 kg soil : 5 g sucrose) and then incubated at 30ºC for 20 days, after which the soil mixture was divided among sixteen pots, four pots for each plant species.

Biomass production and analytical measurements

After harvesting, samples of leaves, stems, roots, and nodules from each plant were dried separately at 60 °C until constant weight and then the dry mass of each part was determined. All samples were first ground with a fixed and mobile blade mill (Marconi MA-580) until reaching a particle size of less than 2 mm, and then with a rotary mill (SampleTek 200 vial Rotator) until reaching the required granulometric size for isotopic analysis. Samples were weighed into tin capsules, and their total N concentration and ^{15}N natural abundance were then determined in a Thermo Finnigan DELTAplus mass spectrometer (Bremen, Germany) coupled to a Flash EA 1112 elemental analyzer through a ConFloIII interface.

The isotopic ratio was expressed in delta notation (δ) in parts per thousand (‰) using Equation 2 (Sulzman, 2007):

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000 \quad \text{……………………………………………………….Eq. 2}$$

where R is the ratio of intensities (measured in the mass spectrometer) of the least abundant to the most abundant isotope.

Equations 3, 4 and 5, respectively, were used to determine the δ^{15}N values for the shoot, radicular system, and the whole plant of each replication:

$$\delta^{15}N_{shoot} = \frac{\delta^{15}N_{leaf} x N_{leaf} + \delta^{15}N_{stem} x N_{stem}}{N_{leaf} + N_{stem}}$$ ....

..Eq. 3

$$\delta^{15}N_{radicular\ system} = \frac{\delta^{15}N_{root} x N_{root} + \delta^{15}N_{nodule} x N_{nodule}}{N_{root} + N_{nodule}}$$ ……

…..Eq. 4

$$\delta^{15}N_{plant} = \frac{\delta^{15}N_{leaf} x N_{leaf} + \delta^{15}N_{stem} x N_{stem} + \delta^{15}N_{root} x N_{root} + \delta^{15}N_{nodule} x N_{nodule}}{N_{leaf} + N_{stem} + N_{root} + N_{nodule}}$$.

Eq. 5

where N-leaf, N-steam, N- and N-nodule are the amounts in grams of N in the leaf, stem, root and nodule, respectively.

The δ^{15}N shoot value (B value) for the minimum method was obtained as the average of the four lowest δ^{15}N shoot values, while that for the N-immobilized method as the average of all δ^{15}N values.

To estimate the %Ndfa seeds of each specie were sowed in six additional pots under the same conditions as in the B-minimum method assay. The mean δ^{15}N value from these 6 pots was used as the value of δ^{15}N-fix in Equation 1. These estimations were made not only with the two B values determined in this work, but also with those reported in the literature. Reference plants was maize with δ^{15}N = 9.7 ‰.

Experimental design and statistical analysis

We studied the effect of two factors (method of determination of B value, and species) on the N mass and ^{15}N isotopic composition of each plant part (leaf, stem, root, nodules) and their partial totals (part area , root system) or the total plant, with a completely randomized statistical design.

The species factor had four levels (*C. juncea*, C. *spectabilis*, *C. ochroleuca* and *C. cajan*) and the method had 2 levels (B-minimum, N-immobilized). The main effects (method and species), as well as their interaction was analyzed by Anova, but when the interaction was significant, the effect of the method was compared by Anova only within each species. The means were compared by LSD, and the existence of significant differences was assumed when $p \leq 5\%$.

Results

Total N mass in different plant parts

In all species, the highest N mass in the shoot was found in leaves (approximately 83%), while in the root system each of its components contributed differently depending on the species. In *C. spectabilis* and *C. ochroleuca* the contribution from the nodule and the root to the N mass entire root system was 40 and 60%, respectively; while in *C. juncea* these contributions were 60 and 40%, respectively. On the other hand, in *C. cajan* nodule and root contributed 20 and 80% of the total N mass of the root system, respectively.

For the N mass of leaves, no interaction was found between the main factors species x method, and significant differences were found between both species and methods (Fig. 1 A). The specie *C. cajan* accumulated more N than the *Crotalaria* species. Regarding the method, more N was accumulated when immobilized-N method was used, compared to minimum-B method, except in *C. ochroleuca*, where there were no differences neither for leaf nor for aerial part. In stems, on the other hand, the N mass was influenced by the interaction between the species and the methods, for which the method effect was analyzed separately for each species (Fig. 1B). As expected, in all species the total aerial part-followed the same trend as the leaf, given the greater contribution of the N mass in leaves to this total Fig. 1 C).

As in the aerial part, the N mass of nodules and roots was influenced by the species-by-method interaction; thus, the method effect was analyzed separately for each species. In nodules, only in *C. juncea* the N mass was different between methods in (Fig. 1 D), while in roots there were differences in both *C. juncea* and *C. spectabilis* (Fig. 1 E), but the total root system followed a trend that was more similar to that observed in nodules (Fig. 1 F). As occurred for leaves and shoot, when there were significant differences between methods, in all species the highest N mass accumulation was registered with the immobilized-N method.

For the root system, the species with similar trends were grouped together; one group was formed with *C. cajan* and *C. ochroleuca*, and the other with *C. juncea* and *C. spectabilis*, but in none of these groups the method-by-species interaction was significant. In addition, within both groups, the results were maintained, since the method was only significant in the first group and the higher values were obtained with the immobilized-N method.

In the whole plant, the N mass followed a similar same trend as in the leaf, because this part had contributed with most N to the shoot, and in turn, the N in shoot constituted on average 78% of the total. As it had happened in leaf, the species and method effects were statistically significant, but the species-by-method interaction was not. The mass of N accumulated in the whole plant was higher in *C. cajan* compared to the *Crotalaria* species, and again, the highest N mass was obtained with the minimum-B method (Table 1).

δ^{15}N

For both the total of the aerial part and the root system, the δ^{15}N value was estimated from the weighted addition of its parts (leaf and stem on one side and nodules and root on the other), as was indicated in Eqs. 4 and 5. In all species, leaves had δ^{15}N values that were closer to zero (less negative) than in stems, and since leaves contained more N mass, shoot δ^{15}N values were similar to those of leaves. The δ^{15}N values of the root system, on the other hand, were always positive and between those of nodules and roots, since the N mass of both parts was similar and their δ^{15}N values were also positive, although more positive in nodules than in roots.

In the case of the δ^{15}N values of leaves and stem, the shoot components, a significant species-by-method interaction was found (Fig. 2A and 2B, respectively). Thus, separate ANOVAS for each species revealed that only in leaves of *C. juncea* and *C. cajan* did the δ^{15}N values vary between methods (Fig. 2A). In stems, on the other hand, a similar result was observed in *C. juncea* and *C. spectabilis* (Fig. 2B). In both cases, when the results were statistically significant, the most negative values were always recorded for the immobilized-N method.

The δ^{15}N values of shoots followed the same trend as its constituent parts, resulting in a significant method-by-species interaction of the ANOVA (Fig. 2C). This analysis is, however, of special importance, because the δ^{15}N value of the shoot also represents the B value of the plant. The results of the separate ANOVAs for each species showed in *C. cajan* and *C. juncea* that B values differed between methods, being also more negative when the immobilized-N method was used. In the other two species (*C. ochroleuca* and *C. spectabilis*), instead, the method did not significantly affect the B value.

Due to this interaction between method and species, the B value differences between species were evaluated only within each method. For the minimum-B method, significant differences ($p=0.0033$) were detected; and mean comparisons showed that *C. juncea* (-0.18 ‰) differed from the rest of the other species, which in turn did not differ among themselves (-2.06; -1.77 and -1.46 ‰, for *C. ochroleuca*, *C. cajan* and *C. spectabilis*, respectively). On the other hand, for the immobilized-N method, there were also significant differences between the B values of the species ($p<.0001$). Mean comparisons showed that B values of *C. juncea* and *C. ochroleuca* were statistically equal (-2.21 and -1.83‰, respectively), but they were different from those of *C. cajan* (-2.94‰).) and *C. spectabilis* (0.94‰), which in turn also were different from each other.

In nodule and root, there was no significant interaction between method and species (Fig. 2D and 2E). In nodules, statistical differences were found both between both species and methods (Fig. 2D). In root, the ^{15}N isotopic composition was different between methods only in *C. juncea*, with less positive values in the immobilized-N method (Fig. 2E). The root system showed a different tendency to its parts, since differences were only found at the species level (Fig. 2F). This result was obviously the consequence of the mass balance between the sum of the parts of nodules and roots.

In the whole plant, the ^{15}N isotopic composition of all species followed a similar trend as that of the leaf, due to the greater contribution of the N mass of this part to the total plant. There was no significant interaction between species and method, but the main effects of species and method were significant. In terms of species, the $\delta^{15}N$ values were more negative in *C. cajan* than in the other species, while, in terms of method, the $\delta^{15}N$ values were more negative with immobilized-N (Table 1).

Proportion of N derived from atmosphere

Estimations of %Ndfa obtained with Eq. 3 by substituting with the B values obtained with the minimum-B or immobilized-N methods, revealed that the greatest difference were obtained in *C. juncea* (13%) and *C. cajan* (8%); in these cases, the lower %Ndfa values were obtained with immobilized-N method. In addition, %Ndfa values obtained with previously published B values were also higher than those obtained with minimum-B in *C. cajan*, *C. juncea* and *C. ochroleuca* by 14; 8.6 and 8.5%, respectively (Table 2).

Discussion

N mass derived from atmosphere

The two methods for B value determination produced different levels of N mass derived from fixation because the BNF process was enhanced when plants grew on soil with sucrose addition. This result was expected, since the incorporation to the soil of plant residues with a high C/N ratio, such as corn or rice straw, has already been used to enhance soil mineral N immobilization and increase BNF (Mori et al., 2012; Salgado et al., 2021), and sucrose is an energetic material with no N in its chemical structure.

The inverse relationship between the Ndfa mass in the evaluated legumes and the mineral N availability in the substrate was consistent with the fact that these species are native to tropical areas with limited N availability and legumes evolved to depend on their BNF potential to acquire N (Trytsman et al., 2019; Jaiswal and Dakora, 2019). Thus, these species are particularly suitable to be used as CC in environments with low N availability (dos Santos Nascimento et al., 2021; Chu et al., 2004; Fan et al., 2006). To this respect, *C. cajan* was in this study the species with the highest BNF capacity, even without specific rhizobia inoculation, depending only in the native soil strains.

The distribution pattern of $\delta^{15}N$ among the different plant parts was similar in all the evaluated species, being the shoot depleted while the root enriched in ^{15}N. This pattern was consistent with reports from other species that grew on substrates without mineral N inputs (Gathumbi et al., 2002; Okito et al., 2004; Woldekirstos et al., 2014). In *Crotalarias*, instead, the $\delta^{15}N$ composition of the entire plant was close to zero, indicating that although isotopic fractionation occurred within plant parts, it did not happen during the BNF process itself (Unkovich, 2013). Conversely, the ^{15}N isotopic composition of *C. cajan* at the whole plant level was negative, a result that had also been reported for other tropical legume species (Okito et al. 2004; Unkovich, 2008; Woldekirstos et al., 2014).

With respect to whole plant $\delta^{15}N$ negatives, Unkovich (2013) interpreted that they could be the result of error accumulations, spanning from culture conditions to analytical processing. Chalk and Croswell (2018), in turn, disagreed with at least part of this interpretation, since the $\delta^{15}N$ variability of interlaboratory rounds is generally low. Although it should be noted that if these negative $\delta^{15}N$ values were just the result of randomness, positive values should also have been reported, but instead they fail to show up in the literature. At least in our study, the whole-plant negative $\delta^{15}N$ values found in *C. cajan* but not in *Crotolarias* would not have been the result of different growing conditions or analytical processing, since all evaluated species were grown and processed similarly. Therefore, it would be possible that there were real ^{15}N isotopic fractionation differences during BNF between these two groups.

Influence of B value determination methods

The minimum-B methodology was originally proposed to estimate the %Ndfa in white clover and ryegrass pastures grazed by cattle at open-air, receiving, thus, animal depositions (Hansen and Vinther, 2001). In these situations, the $\delta^{15}N$ values of the mixed pasture (grasses and legumes) were more negative than the B values determined in sand-vermiculite media, resulting in the estimation of negative %Ndfa values. This reduction of the isotopic values was due to the absorption by plants of N from the urine, which was $\delta^{15}N$ depleted with respect to that of the original mixed pasture This depletion occurred within animal metabolism, with $\delta^{15}N$ variations that ranged from -1.7‰ in the original pasture to -2.8 ‰ in the urine (Steele & Daniel, 1978).

Some of this deposited N is then volatilized as NH_3, which is further depleted in ^{15}N. The absorption of this N by leaves would explain the $\delta^{15}N$ decreases of grasses from positive values to -7‰, cited by Eriksen and Høgh-Jensen (1998). On the other hand, the nitrogenous compounds remaining in the soil become ^{15}N enriched (Robinson, 2001). To this regard, Tonn et al. (2019) applied urine ($\delta^{15}N$ =2‰) to a mixed pasture of *L. perenne* and *T. repens*, and found that *L. perenne* leaves were initially rapidly depleted in ^{15}N compared to a control without this application ($\delta^{15}N$ 0.1 vs. 5.8‰, respectively). The leaves of *T. repens*, on the other hand, did not change their isotopic composition. Subsequently, foliar $\delta^{15}N$ values increased (*T. repens* to 4.5‰ and *L. perenne* to 5.9‰), presumably due to the absorption of these remaining inorganic soil compounds enriched in ^{15}N. At the end of the experiment, δ^{15}N changes were higher in grasses than in legumes, in part because the ammonia-N absorption rate by leaves tend to be inversely proportional to its foliar N concentration, which is lower in grasses (Tonn et al., 2019). In addition, this result could be partly a consequence of the BNF process, which tends to imprint in legumes the δ^{15}N value of air.

Based on the cited results from other authors, as well as those found in this study, we consider that prior to making %Ndfa estimations, it would be advisable to compare the B values to be used with the ^{15}N signature of the plant material, especially for legumes under grazing. Mori et al. (2012) evaluated different methodologies to determine B values for *Lotus corniculatus*, *Trifolium repens*, and *Trifolium pretense*, including, among others, the immobilized-N and minimum-B methods. These authors observed that the B values obtained with the immobilized-N method were always more negative than those obtained with the minimum-B method, although they were not statistically different from each other. In other words, this reported trend was similar to that found in our study for *C. cajan* and *C. juncea*, although in the case of *C. spectabilis* and *C. ochroleuca*, the B values identified from these two methods were similar (Fig. 2C).

To estimate the %Ndfa in *C. spectabilis* and *C. ochroleuca*, some authors have used the B values reported for other legumes of the same genus, because there were no published B values for these two species (Resende et al., 2002; Ojiem et al., 2007). In this work, we have determined specific B values for the symbiotic association between these two species and native soil rhizobia. In the case of *C. juncea* and *C. cajan*, on the contrary, several B values had already been proposed (Boddey et al., 2000; Gathumbi et al., 2002). In spite of that, our results suggest that even for these species it would be preferable to use specific B values locally estimated. Because as suggested by Chalk et al. (2017) and Woldekirstos et al. (2014) B values would tend to vary with the native or inoculated *Rhizobium* strain and with the legume genotype or cultivar.

When comparing the %Ndfa calculated with the B values estimated with the immobilized-N method obtained in this work with those from the bibliography, differences were only relevant in *C. cajan* and *C. ochroleuca*, the species with the highest %Ndfa values. In other words, the importance of using specific B values increases as plants acquire a greater proportion of their N from the atmosphere. This explains why no practical %Ndfa differences were found in *C. juncea* when B values estimated with these two methodologies were used. Overall, we would recommend the immobilized-N method for determining local B values, since this methodology constitutes a simple and practical way to integrate into the B value the local effects of growing conditions and rhizobium strains.

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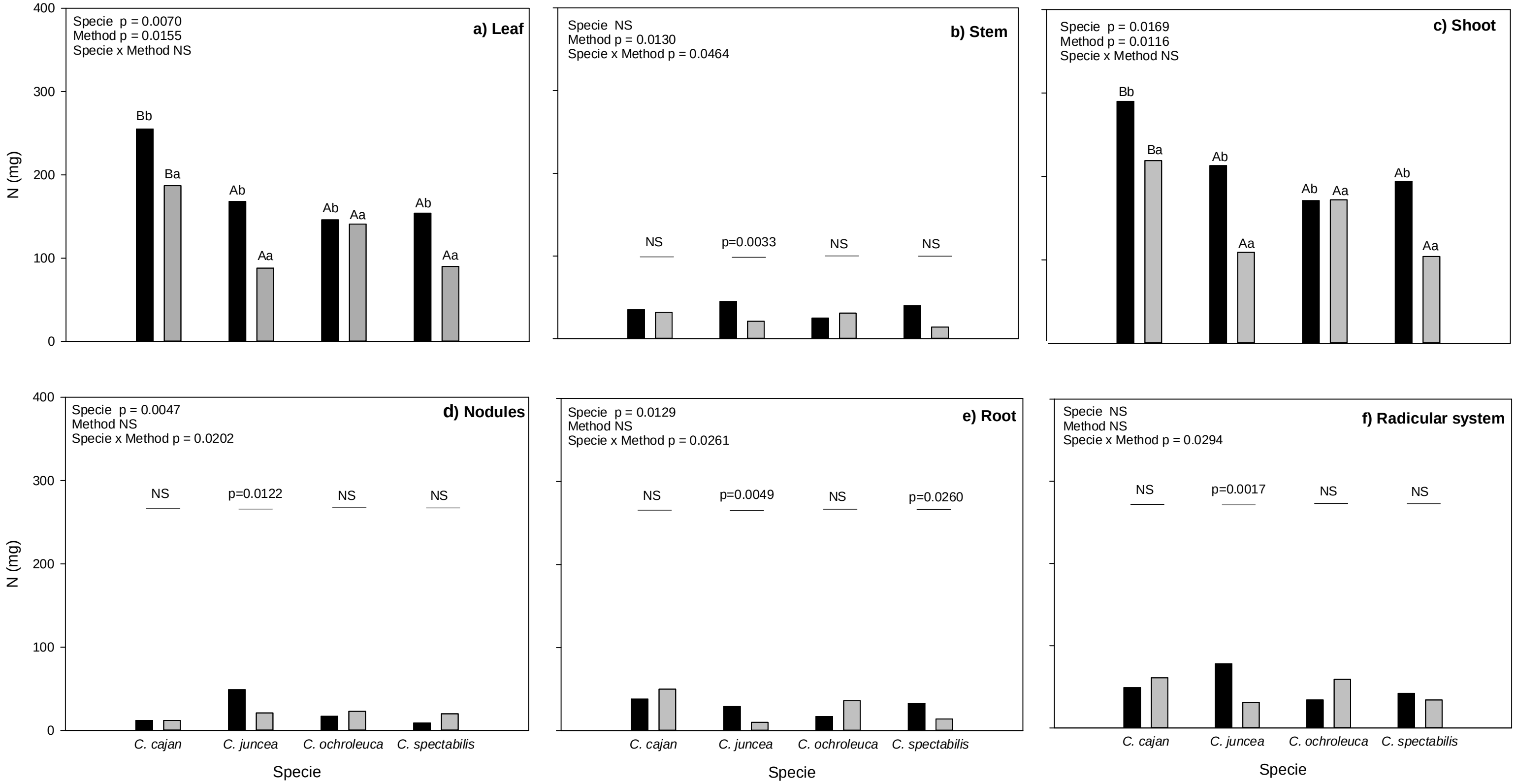

Fig. 1. Masa de N en diferentes partes de las plantas que fueron cultivadas sobre distinto sustrato o métodos para determinar el valor B (N-inmovilizado y B-mínimo). Las barras negras y grises indican la masa de N obtenida con el método de N-inmovilizado y B-mínimo respectivamente. Las letras mayúsculas y minúsculas indican las diferencias entre especies y métodos respectivamente (p<0,05).

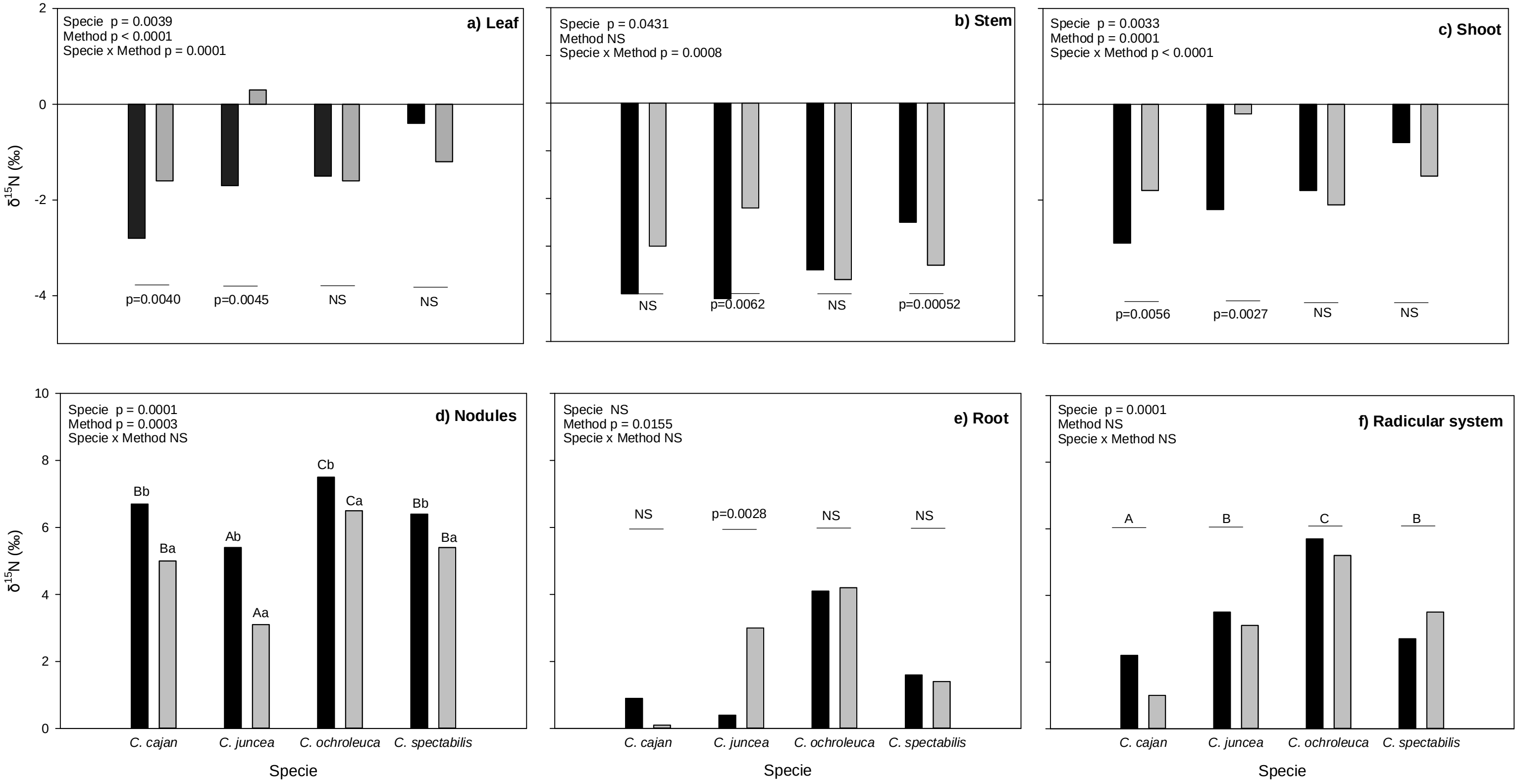

Fig. 2. Valores de $\delta^{15}N$ en diferentes partes de las plantas que fueron cultivadas sobre distinto sustrato o métodos para determinar el valor B (N-inmovilizado y B-mínimo). Las barras negras y grises indican la masa de N obtenida con el método de N-inmovilizado y B-mínimo respectivamente. Las letras mayúsculas y minúsculas indican las diferencias entre especies y métodos respectivamente (p<0,05).

Cuadro 1. Valores medios de la masa de N (expresada en mg) y de δ^{15}N (expresada en ‰) en la planta entera en cuatro especies de leguminosas obtenidos con dos métodos N-inmovilizado (N-inm) and B-mínimo (B-min). Las letras mayúsculas y minúsculas indican las diferencias entre especies y métodos respectivamente (p<0,05).

Species	N mass			δ^{15}N		
	N_inm	B_min	Mean	N_inm	B_min	Mean
C. juncea	291 Ab	140 Aa	216	-0,70 Ab	0,60 Aa	-0,05
C.spectabilis	236 Ab	138 Aa	187	-0,16 Ab	0,75 Aa	0,30
C.chroleuca	205 Ab	231 Aa	218	-0,54 Ab	-0,40 Aa	-0,47
C. cajan	339 Bb	280 Ba	310	-2,17 Bb	-1,16 Ba	-1,67
Mean	268	197	233	-2,17	-0,05	-0,47
Factor	Statistics					
Specie	0,0424			0,0056		
Method	0,0301			0,0332		
Specie x Method	NS			NS		

Cuadro 2. Proporción de nitrógeno fijado del aire (%Ndfa) usando valores B determinados en este estudio y otros de la literatura.

	Species												
	C. cajan			*C.spectabilis*			*C. ochroleuca*			*C. juncea*			
$\delta^{15}N_{shoot}$	-0,17±0,98			0,49±1,16			-0,33±0,41			2,08±1,87			
	Ninm	Bmin	B*	Ninm	Bmin	B**	Ninm	Bmin	B*	Ninm	Bmin	B**	B*
B-value	-2,9	-1,8	-1,0	-0,94	-1,5	-1,0	-1,8	-2,0	-1,0	-2,2	-0,2	-1,0**	-0,6*
Ndfa (%)	78	86	91	86	82	86	86	87	94	64	77	67	70

*Gathumbi et al. 2002

**Unkovich, 2008

Capítulo 4

Índices de performance para leguminosas estivales que crecen en condiciones de déficit hídrico

En el capítulo 2 de esta tesis se mostró que *Crotalaria juncea* y *Crotalaria spectabilis* serían buenas candidatas para ser usadas como cultivos de cobertura (CC) basándose en la fijación biológica de N (FBN) y en la eficiencia transpiratoria (ET) que es la biomasa producida por unidad de agua transpirada. Otras leguminosas potencialmente aptas serían *Crotalaria ochroleuca* y *Cajanus cajan,* ya que según la literatura estas especies tienen una alta capacidad de FBN, sin embargo, hasta el momento no se dispone de información sobre el ranking de ET. En este capítulo se comparó en condiciones de cámara de crecimiento la performance de estas cuatro especies a través de estimaciones directas de la masa de FBN acumulada y del agua transpirada (T), o a través de aproximaciones de esta última (Δ^{13}C y Δ^{18}O). Estas comparaciones se realizaron en dos condiciones hídricas con diferentes umbrales de reposición de agua por riego (50 y 80% de capacidad de campo). Los resultados mostraron que todas estas especies serian buenas candidatas para ser usadas como CC, ya que tuvieron buena productividad, ET y FBN, pero *Cajanus cajan* mostró una mejor performance en todos estos índices. Además, el índice Δ^{18}O aumentó bajo déficit hídrico moderado y mostró una relación inversamente proporcional con la cantidad de agua transpirada, lo que sustenta el uso de este indicador isotópico como *proxy* de T y de la conductancia estomática. Para ninguno de los parámetros isotópicos se encontró interacción entre los factores régimen hídrico y especie, lo que evidencia la robustez de los mismos. Basados en estos resultados se propone utilizar un índice de performance compuesto (masa de FBN/Δ^{13}C) que permite indexar especies de leguminosas en base al input de N a igualdad de agua consumida. Los resultados y su implicancia se comunican en el artículo que se presenta a continuación.

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Crop Performance Indexes Applied to Legume Used as Summer Cover Crops under Water Deficit Conditions

Verónica Berriel [1], Carlos H. Perdomo [1], Santiago Signorelli [2,3] and Jorge Monza [2,*]

[1] Soil and Water Department, School of Agronomy, Universidad de la República, Montevideo 11000, Uruguay; vberriel@gmail.com (V.B.); chperdomo@gmail.com (C.H.P.)
[2] Plant Biology Department, School of Agronomy, Universidad de la República, Montevideo 11000, Uruguay; ssignorelli@fagro.edu.uy
[3] The School of Molecular Sciences, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia
* Correspondence: jmonza@fagro.edu.uy

Abstract: Summer legume cover crops (CC) such as *Crotalaria juncea*, *Crotalaria spectabilis*, *Crotalaria ochroleuca*, and *Cajanus cajan* could offer diverse advantages for the environment and productive cropping systems. A low transpiration efficiency (TE) of CC can induce soil water content to levels that present a challenge for the subsequent crop. In a 75-day growth chamber experiment, using the natural abundance of ^{13}C, ^{18}O, and ^{15}N we evaluated the TE and BNF under two soil water conditions. Our results showed that the four species tested are good candidates for their use as CC because they showed good results in terms of productivity parameters, TE, and BFN. *Cajanus cajan* had the highest TE, a high shoot dry matter production, and accumulated more N from BFN in the shoot than *C. spectabilis*, *C. juncea*, and *C. ochroleuca*. $\Delta^{18}O$ increased under moderate water deficit and showed an inversely proportional relationship with the amount of transpired water, supporting the use of this isotopic indicator as a proxy for transpiration and stomatal conductance. For the isotopic parameters no interaction between the factors water regimen and species were found. We propose the mass ratio of nitrogen fixed by the volume of transpired water and the isotopic discrimination of ^{13}C as useful indicators of drought fixing legumes tolerance.

Keywords: legumes; cover crops; drought; biological nitrogen fixation; water use efficiency; nitrogen use efficiency; stable isotopes

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1. Introduction

The annual summer legumes *Crotalaria juncea*, *C. spectabilis*, *C. ochroleuca*, and *Cajanus cajan*, are species that are characterized by their high biomass production and ability for biological nitrogen fixation (BNF) [1–3]. Due to these characteristics, they are commonly used as cover crops (CC) in cropping agriculture rotations in tropical and temperate zones [4,5]. Cover crops could protect the soil surface temporarily or permanently between two commercial crops [6], and their use is a strategy to improve soil quality and reduce nutrient losses, including water shortages. In the context of drought, a species' ability to accumulate dry matter (DM) production and N must be balanced with its water consumption [7].

Transpiration efficiency (TE) also called water-use efficiency (WUE) is defined as the amount of DM produced by water transpired [8] and can be determined on different scales of time (instantaneous or time-integrated measurements) and space (at leaf, shoot or whole plant level). The selection of species based on TE is a key strategy of plants in the acclimation to drought [9]. The reference method to determine TE, at whole plant level and long term is through lysimeters with gravimetric determinations [10], which in practice present some limitations due to labour requirements. Instantaneous WUE or TE can be also determined by measuring the concentration of CO_2 and H_2O vapour, which can be applied at the leaf level and faster than gravimetric determination using lysimeters [11]; however, the robustness of this methodology is more limited than TE determination. In addition, the instantaneous WUE can be estimated by the relationship between the photosynthesis rate (A) and transpiration (T) and the intrinsic WUE by the relationship between A and stomatal conductance (gs) [12].

Another alternative to determine long-term TE, extrapolated to whole plant level that does not require measuring transpiration or DM production, is through isotopic discrimination of ^{13}C (Δ^{13}C) [13,14]. This isotopic indicator is highly heritable in C3 plants and has a low genotype by environment interaction [15,16].

The plant Δ^{13}C depends on its water status [17] and, therefore, on soil moisture [18]. Δ^{13}C has a strong negative correlation with rainfall [19] and soil moisture [20]. Moreover, physiological factors such as stomatal conductance and photosynthetic rate also determine Δ^{13}C [21,22].

Although Δ^{13}C is widely used as an indicator or proxy for TE, it is not possible to distinguish if under water limiting conditions its variation is due to decreases in stomatal conductance or photosynthetic rate. To differentiate between them, the isotopic discrimination of ^{18}O (Δ^{18}O) can be used, because it does not depend on the photosynthetic rate but on the stomatal conductivity [23]. Thus, the determination of Δ^{13}C and Δ^{18}O allows a quick and reliable measurement of TE and the stomatal conductance, respectively [23,24].

The natural abundance of ^{15}N can be used to estimate the BNF. The different composition of nitrogen isotopes in plants grown in the same condition can be attributed to the fact that ^{15}N abundance in the air is lower than in the soil [25]. Therefore, the determination of N isotopes in plants allows estimation of the amount of N obtained through BNF.

Moderated water restrictions have been shown to increase the natural abundance of ^{13}C in plant tissues, including *C. juncea* and *C. spectabilis,* during their growth that allows determining their TE [26]. However, that study was limited to two species and the fixated nitrogen and its relation with the WUE was not evaluated. Therefore, the objective of our work is to evaluate the performance of four species of tropical legumes based on different desirable attributes in CC under water-limiting conditions and also to propose new index parameters related to TE and BNF under water-limiting conditions.

2. Materials and Methods

2.1. Plant Material and Growing Conditions

Seeds of *Crotalaria juncea* L. (Sunnhemp), *C. spectabilis* Roth (Showy rattlepod), *C. ochroleuca* (Slender leaf rattlebox), and *Cajanus cajan* (Pigeon pea) cv. IAPAR 43 were purchased from BRSEEDS Company (Araçatuba, SP, Brazil). Seeds were sown at the rate of one per pot with 4 kg of a typical soil from southern Uruguay (carbon = 11.6 g/kg; clay = 268 g/kg; silt = 487 g/kg; sand = 245 g/kg). The plants were grown in a growth chamber at 30 °C with a relative humidity of approximately 50% and a light intensity of 500 mmol m^{-2} s^{-1} with a 16/8 h light-dark cycle.

The experimental design used was a randomized complete block with two factors, legume species and water regime. Plants from each species, six replicates each, were firstly grown at 80% of field capacity (FC) for 30 days. From day 30, a moderate water deficit was imposed by subjecting the plants to 50% FC for 45 days whereas other plants were kept at 80% FC as control treatment. The daily water volumes of 50% and 80% FC were estimated by gravimetric determination and were calculated considering that the water content at FC (θf, on the basis of mass) was 28.5% (m/m). The amount of transpired water was determined according to Berriel et al. [14], and the TE was calculated at the end of the test as: TE = shoot DM produced/transpired water.

2.2. Isotopic Ratio Mass Spectrometry Determination

To determine the produced shoot biomass expressed as DM, leaves and stems were dried at 60 °C until a constant weight was reached. Plant samples were ground in a fixed and mobile knife mill (Marconi MA-580, Piracicaba, Brazil), achieving a particle size of less than 2 mm and then with a rotary mill (SampleTek 200 vial Rotator, Lawrenceburg, KY, US) until reaching the required granulometric size for isotopic analysis. One and a half mg of each sample was weighed into tin capsules. The natural abundance of ^{13}C and ^{15}N was determined in a Flash EA 1112 elemental analyser (Milan, Italy) coupled to a Thermo Finnigan DELTAplus mass spectrometer (Bremen, Germany) at the Centre of Nuclear Application in Sustainability Agricultural of School of Agronomy, Uruguay. The isotopic ratio was expressed in delta notation (δ) in parts per thousand or ‰ using the following equation [27]:

$$\delta^{13}\mathrm{C} = \left(\frac{\mathrm{R_{sample}}}{\mathrm{R_{standard}}} - 1\right) \times 1000$$

Carbon isotope discrimination ($\Delta^{13}C$) was calculated using the following equation [28]:

$$\Delta^{13}\mathrm{C} = \left(\frac{\delta^{13}\mathrm{C_{air}} - \delta^{13}\mathrm{C_{plant}}}{1 + \delta^{13}\mathrm{C_{air}}/1000} - 1\right) \times 1000$$

The proportion of N fixed from the air (% Ndfa) used the formula of Shearer and Khol [29]:

$$\%\mathrm{Ndfa} = \left(\frac{\delta^{15}\mathrm{N_{ref}} - \delta^{15}\mathrm{N_{fix}}}{\delta^{15}\mathrm{N_{ref}} - \mathrm{B}}\right) \times 100$$

with % Ndfa the proportion of plant N derived from BNF; Δ^{15}Nref, the $\delta^{15}N$ value of the reference plant (not fixing); $\delta^{15}N_{fix}$, the $\delta^{15}N$ value of the fixing plant; and B, the $\delta^{15}N$ value of a fixing plant growing in a medium without N.

As a reference plant, corn was used, with a value of +9.7‰ of $\delta^{15}N$, determined under the same conditions.

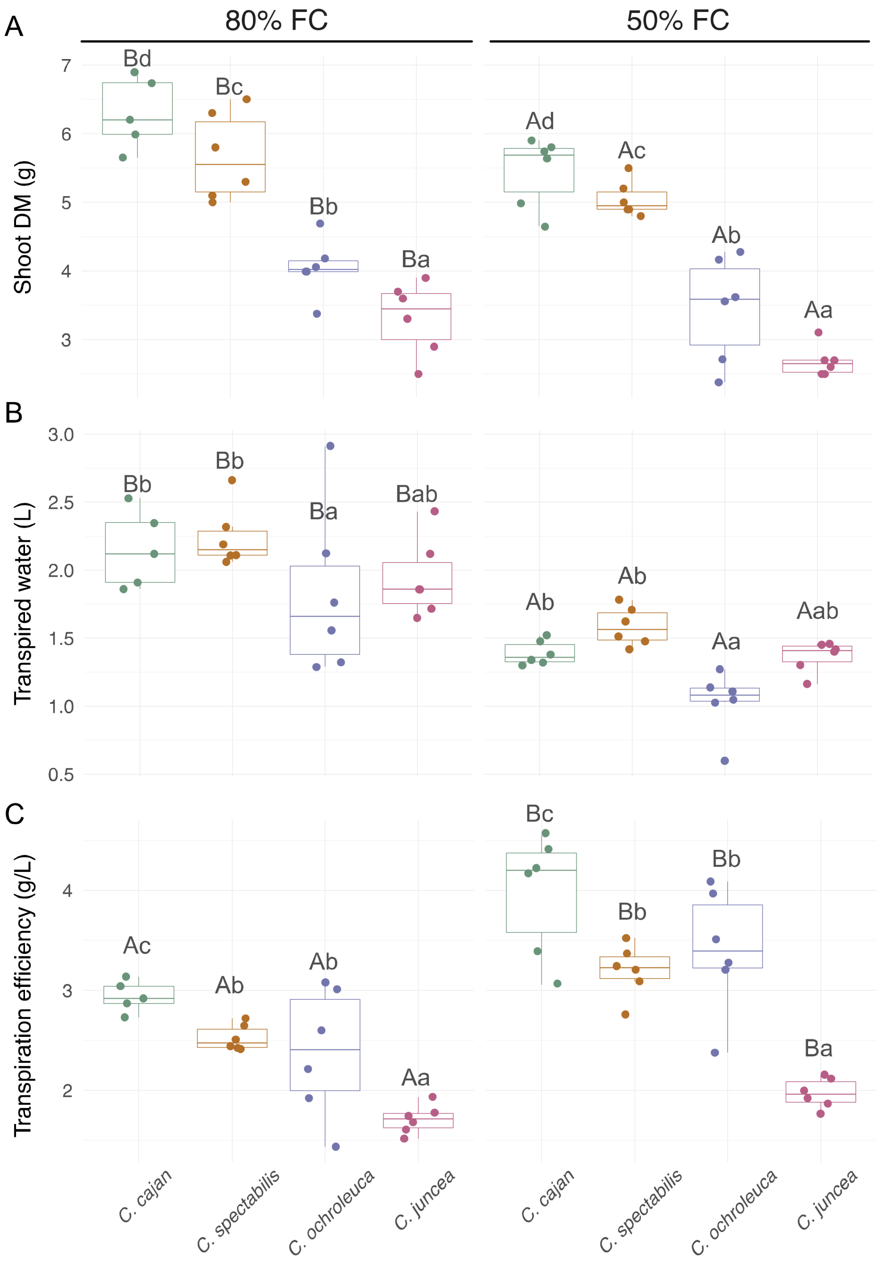
The $^{18}O/^{16}O$ isotopic ratio was determined on the DM of leaves, and the analytical determination was carried out in a Thermo Scientific Delta V mass spectrometer (Bremen GmbH, Germany) with a Conflo IV interface connected to a Costech 4010 elemental analyser (EA) (Milan, Italy) and a high-temperature conversion elemental analyser (CSI laboratory of the University of New Mexico).

2.3. Statistical Analysis

The experimental design consisted of completely randomized blocks with 6 repetitions each. Factors consisted of combinations of four plant species and two soil water regimes (80% FC and 50% FC). The main effects of species and soil water status, as well as their interaction, were analysed by ANOVA and the mean separations were performed with the Tukey's HSD (honestly significant difference) at the 5% significance level using the statistic software InfoStat® version 2020 (Universidad Nacional de Córdoba, Córdoba, Argentina) [30]. The correlation between the variables studied was analysed using the Pearson correlation matrix also using the InfoStat® [30].

3. Results

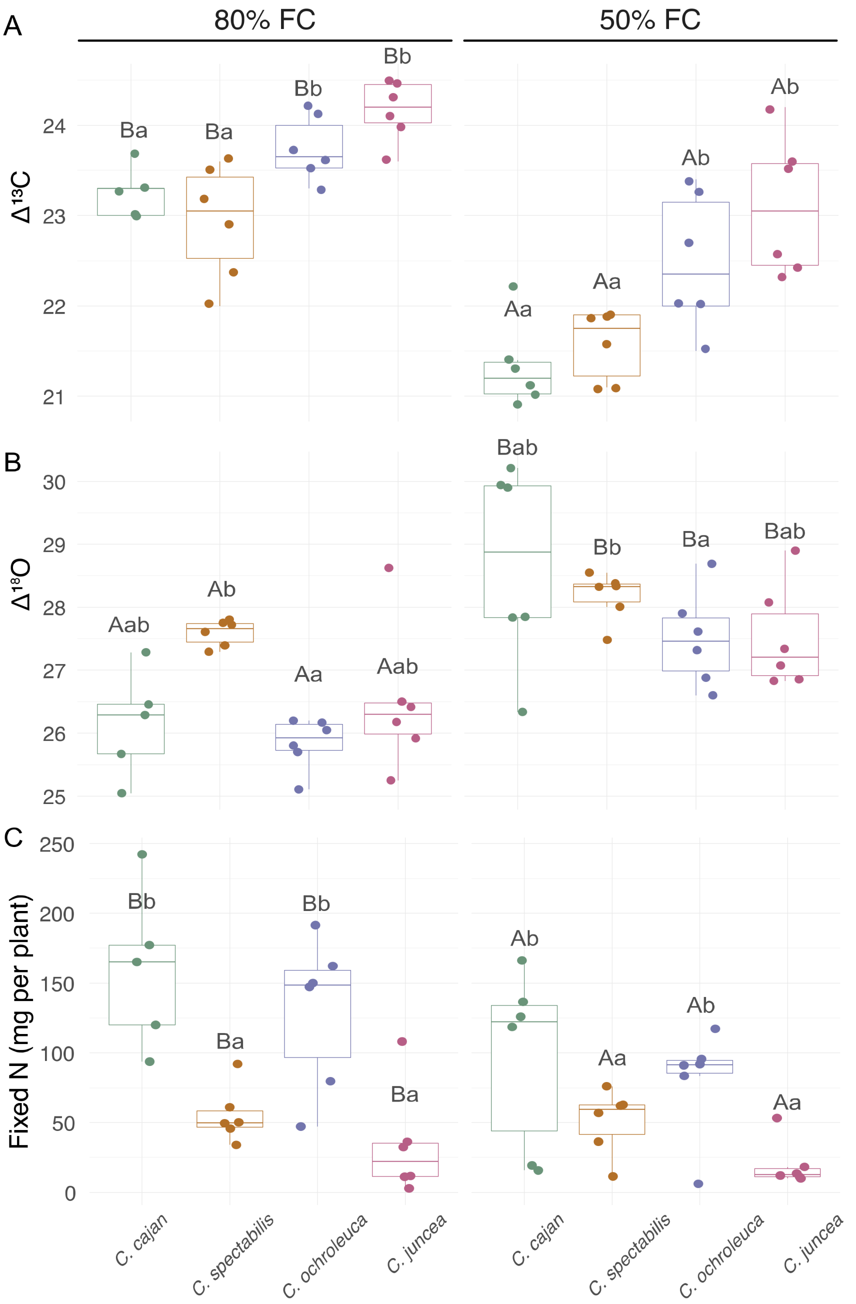
The legume species *C. juncea*, *C. spectabilis*, *C. ochroleuca,* and *Cajanus cajan* were evaluated according to their shoot DM production, transpired water, TE, and isotopic parameters in two water regimes, moderate water deficit (50% FC) and well-watered (80% FC). For these variables, no interaction between the factors water regimen and species were found, therefore the response of each species to the water regime followed a similar pattern. The main effects of water regime and species were observed on shoot dry matter, transpired water, and TE (Figures 1 and 2).



D

Factors	*p*-values		
	Shoot dry matter	Transpired water	TE
Specie	<0.0001	0.0021	<0.0001
Water status	<0.0001	<0.0001	<0.0001
Specie x Water status	NS	NS	NS

Figure 1. Shoot dry matter (DM), transpired water and transpiration efficiency (TE) of legumes used as CC compared with no water deficit (80%) to moderate water deficit (50%). (**A**). DM production expressed in g per plant. (**B**). Transpired water as L of water per plant. (**C**). TE determined as DM produced per transpired water (g of DM/L of water). (**D**). *p*-values of the ANOVA for the species and water status effects and the species × water status interaction. The box plots represent the means, each dot represents independent replicates, and the vertical lines represent the standard deviation. Different letters indicate statistical significance between species (lowercase) and water regimes (capital letters) in a Tukey's HSD (honestly significant difference) post hoc test at 0.05 *p*-value.



D

Factors	*p*-values		
	Δ13C	Δ18O	Fixed N
Specie	<0.0001	0.0096	<0.0001
Water status	<0.0001	<0.0001	0.0119
Specie x Water status	NS	NS	NS

Figure 2. $\Delta^{13}C$, $\Delta^{18}O$, and fixed N in legumes used as CC during moderate water deficit (50% FC) and in the absence of water deficit (80% FC). (**A**). Carbon-13 isotope discrimination ($\Delta^{13}C$) (**B**). Oxygen-18 isotope discrimination ($\Delta^{18}O$). (**C**). Fixed nitrogen (mg per plant). (**D**). *p*-values of the ANOVA for the species and water status effects, and the species × water status interaction. The box plots represent the means, each dot represents independent replicates, and the vertical lines represent the standard deviation. Different letters indicate statistical significance between species (lowercase) and water regimes (capital letters) in a Tukey's HDS post hoc test at 0.05 *p*-value.

However, the N fixation did not show differences between species (Figures 1 and 2). In particular, the DM production and transpired water were lower during moderate water deficit (Figure 1). *Cajanus cajan* produced the highest DM and transpiration rate, followed by *C. spectabilis*, *C. ochroleuca*, and *C. juncea* (Figures 1 and 2). In addition, the TE increased in all species under moderate water deficit, being *Cajanus cajan* the most efficient, *C. juncea* the least efficient, and *C. ochroleuca* and *C. spectabilis* showed an intermediate efficiency (Figure 1).

The two factors tested, water regime and species, influenced the isotopic parameters but not their interaction. In general, Δ^{13}C was lower under moderate water deficit, whereas the Δ^{18}O increased under moderate water deficit compared to the control condition (Figure 2). Among the species, *Cajanus cajan* and *C. spectabilis* showed a lower Δ^{13}C than *C. ochroleuca* and *C. juncea* in both control and moderate water deficit (Figure 2). In terms of Δ^{18}O under moderate water deficit, *only C. ochroleuca* and *C. spectabilis* exhibited differences, being higher for *C. spectabilis* (Figure 2). The correlation analysis, considering all species and water soil content, showed a negative correlation between the variables TE and Δ^{13}C, Δ^{13}C and Δ^{18}O, and Δ^{18}O and transpired water (Table 1).

Table 1. Pearson correlation coefficients for the correlations between shoot dry matter (DM), transpired water (T), transpiration efficiency (TE),^{13}C and ^{18}O isotope discrimination (Δ^{13}C, Δ^{18}O respectively) from the values obtained for *C. cajan*, *C. spectabilis*, *C. ochroleuca,* and *C. juncea* taken together.

	DM	**T**	**TE**	**Δ^{13}C**	**Δ^{18}O**
DM	1				
T	0.50 ***	1			
TE	0.49 ***	−0.47 ***	1		
Δ^{13}C	−0.32 ***	0.44 ***	−0.77 ***	1	
Δ^{18}O	0.13 *	−0.44 ***	0.56 ***	−0.69 ***	1

p: * 0.05; *** 0.001.

The %Ndfa was lower under moderate water deficit relative to the control condition, and no differences between the species were found (Figure 3). However, the DM production was different between these species (Table 1) explaining the differences in the amount of N derived from BNF ($p < 0.0001$) in the shoot. The water regime did not affect the amount of total N; thus, when total N was considered irrespectively of the water condition, *C. juncea* and *C. spectabilis* had lower N content (43 and 72 mg, respectively) than *C. ochroleuca* and *Cajanus cajan* (132 and 177 mg, respectively).

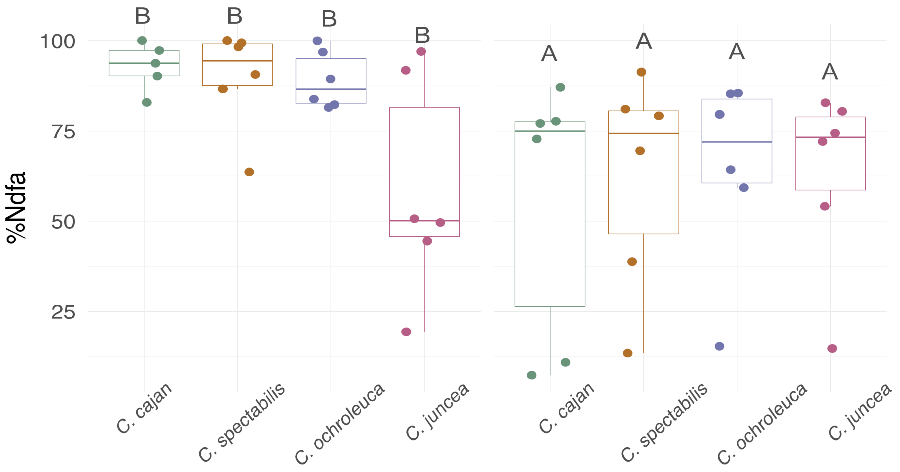


Figure 3. Proportion of nitrogen derived from the air (%Ndfa). The box plots represent the means, each dot represents independent replicates, and the vertical lines represent the standard deviation. The statistical analysis revealed no species effect but a treatment effect. Different letters indicate statistical significance between treatments in a Tukey's HDS post hoc test at 0.05 *p*-value.

Fixed N was affected by the water regime and also by different species, but no statistically interaction was found between water regime and species (Figure 2). In both water conditions, *Cajanus cajan* and *C. ochroleuca* fixed more N than *C. spectabilis* and *C. juncea* (Figure 2). Moreover, in both water regimes *Cajanus cajan* and *C. ochroleuca* had the highest ratio N_{fix}/T and $Nfix/\Delta^{18}O$, while *C. juncea* and *C. spectabilis* had the lowest (Table 2).

Table 2. Performance indexes for legumes that were subjected to moderate water deficit (50% FC) and control (80% FC) conditions. The evaluated indexes related the amount of fixed N from air with the transpired water, $\Delta^{18}O$ and $\Delta^{13}C$ (N_{fix}/T, $N_{fix}/\Delta^{18}O$, and $N_{fix}/\Delta^{13}C$). Different letters indicate statistical significance between species (lowercase) and water regimes (capital letters) in a Tukey's HDS post hoc test at 0.05 *p*-value.

Species	**N_{fix}/T**		**$N_{fix}/\Delta^{13}C$**		**$N_{fix}/\Delta^{18}O$**	
	80% FC	**50% FC**	**80% FC**	**50% FC**	**80% FC**	**50% FC**
Cajanus cajan	75^{a}	69^{a}	6.87^{Aa}	4.52^{Ba}	6.7^{a}	6.3^{a}
Crotalaria spectabilis	25^{b}	33^{b}	2.43^{Ab}	2.37^{Bb}	2.2^{b}	2.3^{b}
Crotalaria ochroleuca	77^{a}	83^{a}	5.49^{Aa}	3.60^{Ba}	5.7^{a}	4.3^{a}
Crotalaria juncea	16^{b}	14^{b}	1.39^{Ab}	0.84^{Bb}	1.8^{b}	1.4^{b}
Factor			*p*-value			
Specie	<0.0001		<0.0001		<0.0001	
Water status	NS		0.0325		NS	
Specie × Water status	NS		NS		NS	

In legumes in which $\Delta^{13}C$ correlated more with transpired water than with photosynthetic rate, the $N_{fix}/\Delta^{13}C$ index can be used as an index informing about the nitrogen fixation in relation to water transpired. The moderate water deficit condition implicated a decrease in this index in all four species tested and followed the same trend as the amount of fixed N (Table 2). Finally, strong positive correlations were found between the N_{fix}/T, $N_{fix}/\Delta^{18}O$, and $N_{fix}/\Delta^{13}C$ indexes and between them and the TE when the species and water conditions were grouped (Table 3).

Table 3. Pearson correlation coefficients for the correlation between different indicators of performance, transpiration efficiency (TE), mg of fixed N per L of water (N_{fix}/T), mg of fixed N per ^{13}C isotope discrimination ($N_{fix}/\Delta^{13}C$), and mg of fixed N per ^{18}O isotope discrimination ($N_{fix}/\Delta^{18}O$) from the values obtained for *C. cajan*, *C. spectabilis*, *C. ochroleuca,* and *C. juncea* taken together.

	TE	**N_{fix}/T**	**$N_{fix}/\Delta^{13}C$**	**$N_{fix}/\Delta^{18}O$**
TE	1			
WUEfix	0.45 ***	1		
$N_{fix}/\Delta^{13}C$	0.29 *	0.87 ***	1	
$N_{fix}/\Delta^{18}O$	0.43 ***	0.73 ***	0.85 ***	1

p: * 0.05; *** 0.001.

4. Discussion

The legumes *C. spectabilis*, *C. juncea*, *C. ochroleuca,* and *Cajanus cajan* may be good candidates for cover cropping because of their high ability to fix N [31]. Besides these attributes, high WUE and TE are desirable characteristics for CC in water-limited environments. In this study, we determined these parameters in these four species under different water regimes.

In both water regimes, *Cajanus cajan* had the greatest DM production, TE, and amount of fixed N among the four species tested. Moreover, *Cajanus cajan* had the greatest DM production in relation to transpired water. This shows its potential as a CC in water-limited environments. *Cajanus cajan* can combine these desirable attributes, maximizing the DM produced in relation to water consumed or transpired. These findings show that *Cajanus cajan* has a potential to be used as CC in soils where water restrictions are common.

Most climate change scenarios foresee rainfalls to be decreased around the world and its pattern to be more erratic [32]. Therefore, the redesign of agricultural crop rotations seems to be imminent to mitigate the effect of climate change on natural resources [33–35]. High WUE and TE in drought conditions are desired features that crops, varieties, or genotypes must have to be considered in this redesign [36].

In moderate water deficit, *C. spectabilis, C. juncea*, *C. ochroleuca*, and *Cajanus cajan* were tolerant to a moderate water deficit, in agreement with previous reports that evaluated the performance of these species under field conditions [37,38]. The strong decrease in transpired water in response to a moderate water deficit, together with a slight decrease in DM production, resulted in an increased TE (Figure 1), which explains their tolerance to water restrictions. Berriel et al. [25] found that moderate drought also negatively impacted DM production and transpired water of *C. spectabilis* and *C. juncea* when plants were studied at whole plant level and in longer term. This suggests that our findings may be translatable for all four species when studied in longer periods of moderate water deficit and no matter the methodological approach used (i.e., whole plant or shoot only).

The decrease in DM production can be a consequence of the reduction in photosynthetic rate, which often relates to a decrease in stomatal conductance. One of the few studies quantifying the impact of water deficit on the variables determining the water–carbon balance in leaves, was carried out in *C. cajan* in which moderate water deficit caused a decrease in transpired water, stomatal conductance, and photosynthetic rate, leading to an increase in instantaneous WUE [39].

In all species, $\Delta^{13}C$ decreased in water-limited condition relative to the control condition (Figure 2). Regardless of the water regime, the least discrimination was exhibited by *Cajanus cajan*, the greatest by *C. juncea*, and intermediate discrimination was found in *C. ochroleuca* and *C. spectabilis*. In turn, regardless of the water conditions position (Figure 2). Berriel et al. [25] observed the same pattern for *C. spectabilis* and *C. juncea*, in terms of $\Delta^{13}C$, during water deficit and after rehydration. In our study, when the water deficit prevailed, the decrease in $\Delta^{13}C$ indicates that either the decrease in stomatal conductance or the transpiration rate led to a decrease in both transpired water and DM production (Figure 1).

$\Delta^{13}C$ was negatively related to TE in all four evaluated species (Figure 1; Table 1). The use of $\Delta^{13}C$ as a proxy for TE has been confirmed in different C3 species [40–42] but not in many grain legumes [43]. The relationships we found between $\Delta^{13}C$ and TE and those reported by Berriel et al. [14,25], supports the strength of ^{13}C as an isotopic indicator of TE in *Cajanus cajan*, *C. spectabilis*, *C. juncea*, and *C. ochroleuca*.

The variation in $\Delta^{18}O$ by the water regimes (Figure 2; Table 1) and the strong inversely proportional relationship between this and transpired water, support the use of this isotopic indicator as a proxy for transpiration and stomatal conductance [44,45]. Determining $\Delta^{18}O$ is advantageous compared to determining transpiration and stomatal conductance because it evaluates the transpiration rate in a longer time scale [46].

The negative correlation between $\Delta^{18}O$ and $\Delta^{13}C$ helped to interpret the differences in TE estimated through the $\Delta^{13}C$ proxy (Table 1), indicating that the increase in TE is mainly determined by the decrease in stomatal conductance than by the decrease in photosynthetic rate. This interpretation is consistent with the greater decrease in transpired water than in DM production (Figure 1).

In this study, we defined indexes relating the amount of fixed N (N_{fix}) and transpired water, determined directly, as $\Delta^{13}C$ and $\Delta^{18}O$. Based on the relationships established between fixed N and transpired water, we propose three sustainability indexes focused on the amount of water used to fix N and applicable to legumes: N_{fix}/T, $N_{fix}/\Delta^{13}C$, and $N_{fix}/\Delta^{18}O$. All these indexes showed a strong positive correlation among them (Table 2). Methodologically, the $N_{fix}/\Delta^{13}C$ index is the preferred one, given its simplicity to be determined. According to these indexes, *C. cajan* and *C. ochroleuca* are the most promising species as CC in conditions of moderate water deficit. It is worth bearing in mind that the application of rhizobia inoculants would increase the BNF in any of the species, something not tested here, which can cause differences in the ranking produced here. Regardless of this, the information generated in this work, as well as the use of the indexes defined here, can contribute to the study and design of agricultural rotations that allow the generation of ecosystem services and mitigate the impact of climate change on farms. Since this study was completed in controlled conditions, more research is necessary to evaluate the significance of these indexes in field conditions.

Author Contributions: V.B. conceived the study; V.B. performed all the experiments and data analysis; S.S. prepared the figures; C.H.P. and J.M. supervised the study; all the authors contributed to the data interpretation and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Aislamiento y caracterización de cepas de rizobios aislados de *Crotalaria juncea*, *C. spectabilis*, *C. ochroleuca* y *Cajanus cajan*: generación de una colección

Introducción

Las leguminosas anuales de los géneros *Crotalaria* y *Cajanus* se usan en rotaciones agrícolas como cultivos cobertura (CC) para implementar sistemas agrícolas sustentables que permitan reducir la aplicación de fertilizantes nitrogenados, con beneficios para el productor y el medio ambiente (Jaiswal y Dakora, 2019). Los CC basados en *Crotalaria* y *C. cajan* logran estos beneficios debido a la alta producción de biomasa, a la fijación de nitrógeno y a la capacidad de recuperar la estructura del suelo, degradada en sistemas intensivos (Pacheco et al., 2015).

De todas formas, hay relativamente poca información sobre los rizobios que nodulan especies del género *Crotalaria,* leguminosas promiscuas (Jaiswal y Dakora, 2019) que establecen simbiosis efectivas con distintas especies de *Bradyrhizobium* (Gao et al., 1994; Camarillo-Castillo y Mangan 2020), *Mesorhizobium* sp. (Rocha, 2011), *Ensifer* sp. (Sankhla et al., 2015) y *Burkoldheria* sp. (Liu et al., 2007; Lemaire et al., 2016) y *Methylobacterium nodulans* (Sy et al., 2001; Jourand et al., 2004).

Cajanus cajan, otra leguminosa promiscua, si bien establece simbiosis eficientes mayormente con cepas de *Bradyrhizobium* sp. (Dudeja y Khurana 1988; Araujo et al., 2020), también lo hace con *Rhizobium* sp. (Pandya et al., 1999; Datta et al., 2000; Ganava et al., 2020) y *Ensifer* sp. (Dubey et al., 2010).

En Sudamérica el uso de *Crotalaria* sp. y *C. cajan* como CC tiene cada vez más interés (Díaz et al., 2015; Hernández et al., 2020) pero el desarrollo de inoculantes es incipiente, por lo que la práctica de inocularlas no es común y nodulan con rizobios presentes en los suelos donde se las siembra.

Una consideración de carácter general respecto a la introducción de leguminosas a nuevos entornos, es que requieren de la selección de inoculantes rizobianos apropiados (Howieson y Ballard, 2004). Para esto es necesario colectar y seleccionar cepas según su eficiencia simbiótica y competitividad (Irisarri et al. 2019). El primer paso en la selección de cepas es su identificación de manera precisa y sencilla. Una técnica ampliamente usada con este fin es la amplificación por PCR de elementos repetitivos (rep-PCR) intercalados en el genoma de bacterias y altamente conservados en rizobios como REP (*Repetitive Extragenic Palindromic*), ERIC (*Enterobacterial Repetitive Intergenic Consensus*) (De Bruijn et al., 1992) y BOX (*Enterobacterial Repetitive Sequences*) (Versalovic et al., 1991). Esta estrategia se ha usado en estudios de diversidad y filogenia de rizobios que nodulan trébol (Batista et al. 2015; Tartaglia et al. 2019) y lotus (Sotelo et al. 2011; Batista et a. 2013) con el fin de desarrollar inoculantes para Uruguay (Irisarri et al. 2019).

En predios dedicados a la producción comercial de grano, la secuencia de rotaciones que incorporan CC contribuyen a mitigar la extracción que hacen al suelo los cultivos renta, y a evitar el incremento del uso de fertilizantes nitrogenados. En este contexto, y con el fin de desarrollar inoculantes rizobianos en un futuro próximo, nos planteamos identificar y caracterizar cepas aisladas de nódulos de *Crotalaria juncea*, *C. spectabilis*, *C. ochroleuca* y *Cajanus cajan* de un suelo de Uruguay, y comenzar a generar una colección.

Materiales y Métodos

Bacterias y condiciones de crecimiento

Se evaluaron cepas usadas como inoculantes comerciales para trébol, soja y lotus, y aislamientos de nódulos de *Crotalaria juncea, C. spectabilis, C. ochroleuca y Cajanus cajan* crecidas en un suelo Argiudol típico del Departamento de San José (Cuadro 1). Las bacterias se crecieron en medio YEM con agitación a 150 rpm o en YEM agar (Vincent, 1970) a 27ºC hasta una DO_{620} = 0.7 – 0.8. Las cepas se conservaron a 4ºC para su uso rutinario. La colección se conservó a -80ºC según (Howieson y Dilworth, 2016).

Cuadro 1. Cepas y aislamientos de rizobios utilizados como inoculantes.

Cepas y aislamientos	**Hospedero/s**	**Procedencia**
Rhizobium leguminosarum U204	*Trifolium repens* *T. pratense*	Colección MGAP
Mesorhizobium huakui U526	*Lotus corniculatus* *L. tenuis*	Colección MGAP
Bradyrhizobium sp. (Lotus) U510	*Lotus uliginosus*	Colección MGAP
Bradyrhizobium sp. U1301 y U1302	*Glicine max*	Colección MGAP
Oc3, Oc5, Oc8, Oc14, Oc16	*Crotalaria ochroleuca*	Aislamientos .#
Sp4, Sp8	*Crotalaria spectabilis*	Aislamientos .#
J4, J19, J20, J26	*Crotalaria juncea*	Aislamientos .#
Cj2, Cj3, Cj4, Cj9, CjI4, CjI5, CjI8, CjI9	*Cajanus cajan*	Aislamientos .#

de plantas trampa utilizando un suelo de aptitud agrícola del sur de Uruguay realizados en el marco de esta tesis.

Material vegetal y condiciones de crecimiento

Como leguminosas hospedadoras se usaron *Crotalaria juncea, C. spectabilis, C. ochroleuca y Cajanus cajan*. Las semillas se esterilizaron superficialmente según Batista et al. (2015).

Los ensayos de nodulación con las cepas usadas como inoculante de trébol, lotus y soja y el de inoculación cruzada con una cepa de cada especie de *Crotalaria* y de *Cajanus cajans* se realizaron en frascos de 500 mL que contenían 200 mL de de medio R & P (Rigaud y Puppo, 1975) agarizado (1% m/v) estéril, y en macetas con arena-vermiculita (1:1) regadas con solución de R & P. En ambos casos se sembraron 3 semillas por unidad de crecimiento y se inocularon con 200 µL por semilla con cultivos frescos de cada cepa pura. Para cada tratamiento, que se realizó por triplicado, se incluyeron controles sin inocular.

Las plantas crecieron en una cámara de crecimiento a 27°C a 350 µM/cm.s durante 30 días.

Aislamiento de rizobios de nódulos

Los nódulos se desinfectaron superficialmente mediante lavado con abundante agua, 1 min en etanol 70 % (v/v), enjuague con agua estéril y 3 min en una solución de NaClO 4 % (v/v) seguida de enjuagues con agua estéril. Los nódulos se conservaron a -20 °C en una solución de glicerol 20 % en NaCl 0,85 % (m/v). Los nódulos desinfectados se aplastaron entre dos portaobjetos estériles con 20 µL de agua estéril. Con un ansa se sembró por estría en placa con medio YEM y se hicieron sucesivos repiques hasta la obtención de un cultivo puro (Howieson y Dilworth, 2016).

Extracción y amplificación de ADN de rizobios

La extracción de ADN a partir de cultivos puros se realizó siguiendo en general el procedimiento usado por Rivas et al. (2001). Para ello se centrifugó 1 mL de cultivo a 12.000 rpm 2 min y se extrajo el sobrenadante. Las células se resuspendieron en 100 µL de NaOH 0,05 M y se incubó 4 min a 100 ºC seguido de 2 min en hielo. Se agregaron 500 µL de agua MiliQ estéril, se centrifugó a 12.000 rpm 2 min y se recogieron 100 µL del sobrenadante que se conservó -20 ºC en microtubos, no más de 5 días.

La amplificación del ADN se realizó con los cebadores ERIC.

Cebadores ERIC (De Bruijn, 1992):

ERIC1 5’ ATGTAAGCTCCTGGGGATTCAC 3’ y

ERIC2 5’ AAGTAAGTGACTGGGGTGAGCG 3’

La amplificación con cebadores ERIC se realizó según Agius et al. (1997) en un volumen final de 25 µL: 2,5 µL de buffer NH_4SO_4 (10X), 2 µL de $MgCl_2$ (25 mM), 0,5 µL de dNTPs (10 mM), 2 µL del cebador ERIC1 y ERIC2 (25 µM), 0,2µL de Taq ADN polimerasa y 5 µL de ADN genómico. El programa utilizado fue: 1 ciclo de 5 min a 95 ºC, 30 ciclos de 1 min a 54 ºC, 6 min a 65 ºC y una fase final de elongación de 16 min a 65 ºC.

Los productos de amplificación se resolvieron en geles de agarosa 1,2% (m/v) en buffer TAE pH8,2 con 2µL SyBrSafe cada 100 mL. En cada pocillo se sembró 6 µL compuestos por 5µL del producto de amplificación y 1µL de buffer de carga ó 5µL de marcador de peso molecular (GeneRuler 1 Kb Plus ADN Ladder, Fermentas). La electroforesis se llevó a cabo a 100 volts (10vol/cm) durante 1 h. El gel se visualizó en transiluminador UV y se registró con un equipo Kodak MI SE.

Amplificación y secuenciación del gen 16SrRNA

La amplificación del gen 16SrRNA sobre los aislamientos con perfiles ERIC diferentes, se realizó según Herrera-Cervera et al. (1999) con los cebadores:

27f 5′ AGAGTTTGATCMTGGCTCAG 3′ y

1525r 5′ AAGGAGGTGATCCAGCC 3′.

El volumen final de la amplificación fue de 25 µL: 2,5 µL de buffer NH_4SO_4 (10 X), 1,5 µL de $MgCl_2$ (25 µM), 0,4 µL de dNTPs (10 mM), 0,5 µL de cada cebador 27f (10 µM) y del 1525r (10 µM), 0,2 µL de Taq ADN polimerasa, y 5 µL de ADN genómico. El programa de amplificación fue: 1 ciclo de 3 min a 94°C; 35 ciclos de 18 s a 94°C, 1 min a 58°C, 1 min a 72°C; y un ciclo final de 10 min a 72°C.

Los productos de amplificación se separaron en geles de agarosa según se describe en el apartado anterior. El ADN del gel se purificó con el kit QIAquick Gel Extraction (Quiagen). La secuenciación se realizó en Macrogen (Korea). La búsqueda de secuencias se realizó en la base de datos disponibles en el NCBI (Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/genbank).

Resultados y Discusión

Identificación de aislamientos de nódulos de *C. juncea, C. spectabilis*, *C. ochroleuca* y *C. cajan* por perfiles ERIC

Los aislamientos de *C. juncea* J4, J19, J20 y J26 presentaron perfiles ERIC que no permitieron diferenciarlos entre sí, por lo que se los consideró como una única cepa denominada J4 (Figura 1 A). En relación a los aislamientos de *C. ochroleuca*, Oc3, Oc5, Oc8, Oc14 presentaron el mismo perfil ERIC por lo que se los consideró una cepa, que se denominó Oc8, diferente al perfil de Oc16 (Figura 1 B).

Los dos aislamientos de *C. spectabilis* presentaron perfiles diferentes y se los denominó Sp4 y Sp8 (Figura 1 C).

De los aislamientos de *C. cajan* se identificaron tres perfiles diferentes, uno que incluyó a los aislamientos Cj2 y Cj4, que consideramos como una cepa a la se denominó Cj2, otro al que pertenecen los aislamientos Cj3, Cj9, Cj14, Cj18 y Cj19 que consideramos como otra cepa que denominamos Cj9 y otro corresponde al aislamiento Cj15 (Figura 1 D).

De los 19 aislamientos obtenidos de las cuatro especies en estudio, con el criterio utilizado se identificaron 8 perfiles ERIC diferentes, a los que nos referiremos como cepas. Esto evidencia que en el suelo donde se realizó el muestreo existe una diversidad interesante de rizobios capaces de nodular a estas leguminosas introducidas.

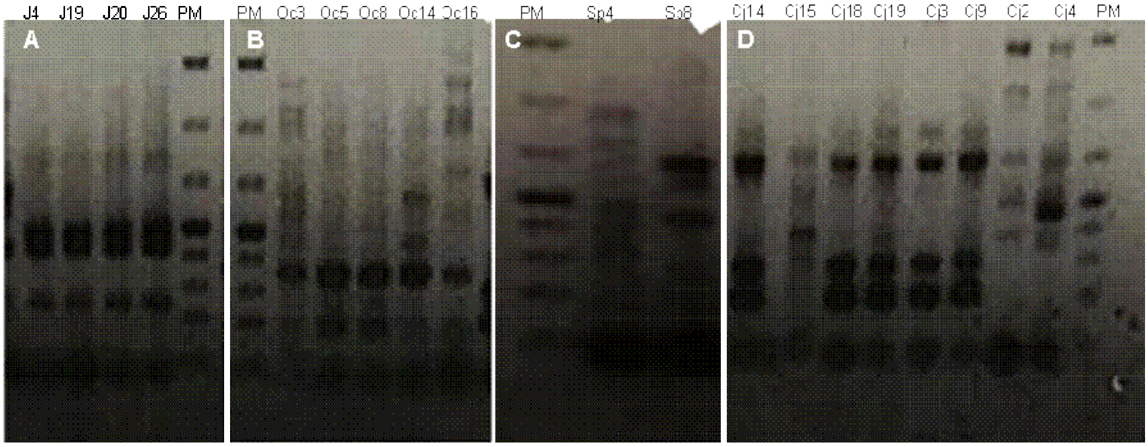

Figura 1. Perfiles ERIC de los diferentes aislamientos obtenidos de A) *C. juncea,* B) *C. ochroleuca,* C) *C. spectabilis*, D) *C. cajan*. PM: marcador de peso molecular 1kb Plus.

Análisis de la secuencia del gen 16SrRNA

Cuando se compararon secuencias parciales de 1.497 nt del gen 16rRNA (Figura 2), se observó que los aislamientos de *C. juncea* (J4, J19, J20 y J26) forman un grupo junto a *R. lusitanum,* que nodula *P. vulgaris* (Valverde et al., 2006) y que los aislamientos de *C. ochroleuca* (Oc8, Oc14 y Oc16) agrupan próximos a *B. ganzhouense*, que nodula *Acacia melanoxylon* (Lu *et al*. 2014).

Los aislamientos de *C. cajan* forman dos grupos, uno que incluye a Cj9, Cj14 y Cj15 próximos a *R. alamii* aislado de la rizosfera de *Arabidopsis thaliana* (Berge et al. 2013) y otro a Cj2 y Cj4 que agrupan con *Paenibacillus riograndensi* aislado de rizósfera de *Triticum aestivum* (Beneduzi et al. 2010).

Los aislamientos de *C. spectabilis* Sp4 y Sp8 están en diferentes clusters, mientras Sp4 agrupa con *Burkholderia* cepacia, un patógeno (Rojas – Rojas et al. 2018) la cepa Sp8 está claramente separada (Fig. 2).

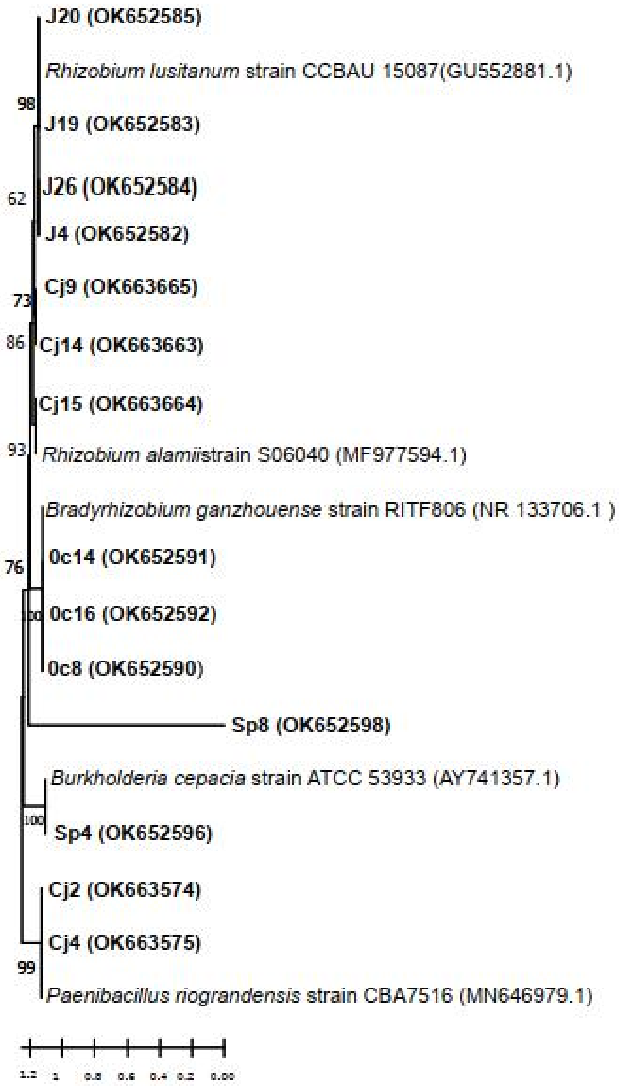

Figura 2. Árbol de máxima verosimilitud de alineamientos de secuencias parciales del 16SrRNA (1497 nt). Con letras en negrita se indican las cepas aisladas de suelos de Uruguay. Los valores de *bootstrap* superiores a 60 (con 1000 repeticiones) se indican en los puntos de ramificación.

Nodulación de *Crotalaria spectabilis*, *C. juncea, C. ochroleuca* y *Cajanus cajan* por cepas usadas como inoculantes de trébol, lotus y soja

Las cepas usadas como inoculantes comerciales para trébol, lotus y soja (U204, U510, U526 y U1301+U1302 respectivamente) no indujeron nódulos en las raíces de las especies de *Crotalaria* sp. ni de *C. cajan*. Si bien la información disponible indica que estas especies son promiscuas y que mayormente nodulan con rizobios de crecimiento lento (Huang et al., 2018; Sankhla et al., 2018), no nodularon con las cepas de *Bradyrhizobium* sp. usadas como inoculantes comerciales *Lotus uliginosus* (U510) ni para soja (U1301 y U1302), como tampoco con *Rhizobium leguminosarum* sv. trifolii cepa U204.

Inoculación cruzada de *Crotalaria spectabilis*, *C. juncea, C. ochroleuca* y *C. cajan*

En cuanto a la inoculación cruzada los resultados se resumen en el Cuadro 3. En este ensayo la cepa Oc8 se caracterizó por inducir una abundante nodulación en las cuatro especies de leguminosas e inducir el desarrollo de nódulos de gran tamaño. Un comportamiento similar tuvo el aislamiento Cj14, que formó nódulos grandes y abundantes en *C. spectabilis* y *C. ochroleuca*.

Lo llamativo es que no se logró, en las condiciones usadas, inducir nódulos en *C. juncea* con las cepas aisladas de esos hospederos (Cuadro 3), pero esto puede deberse a las condiciones *in-vitro* en que se realizó el ensayo.

Cuadro 3. Inoculación cruzada de cepas aisladas de *C. juncea*, *C. spectabilis*, *C. ochroleuca* y *C. cajan*.

Cepa	***C. juncea***	***C. spectabilis***	***C. ochroleuca***	***C. cajan***
J4	-	-	-	-
Sp8	-	+	+	-
Oc8	+	+	+	+
CjI4	+	+	+	+
Cj2	-	-	-	-

Dado que la cepa Oc8 noduló a los cuatro hospederos, con abundante nodulación y tamaño de nódulos esta cepa se seleccionó para secuenciar su genoma, y los resultados se presentan en el capítulo siguiente.

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Capítulo 5

Caracterización y generación de una colección de cepas de rizobios aislados de *Crotalaria juncea*, C*. spectabilis*, *C. ochroleuca* y *Cajanus cajan*

A partir de nódulos de plantas de tres especies de *Crotalaria* y de *Cajanus cajan,* colectadas en un predio agrícola del Departamento de San José, se obtuvieron un total 19 aislamientos de rizobios. A partir de los perfiles ERIC se identificaron 8 cepas diferentes. Posteriormente, se secuenció el gen 16SrRNAs de todas las cepas, a través de la comparación de estas secuencias se puso en evidencia la gran diversidad de especies que son capaces de nodular a estas leguminosas tropicales. En la prueba de inoculación cruzada se destacó una cepa aislada de *C. ochroleuca,* que fue capaz de inducir en las cuatro especies nódulos de gran tamaño y eficientes. Esta cepa, denominada Oc8, resultó ser promisoria para ser usada como inoculante de las cuatro especies. En el manuscrito en formato artículo que se presenta a continuación se presentan los resultados más relevantes.

Capítulo 6

Secuencia borrador del genoma de *Bradyrhizobium* sp. cepa Oc8 aislada del nódulo de *Crotalaria ochroleuca*

En este capítulo se informa la secuencia borrador del genoma de *Bradyrhizobium* sp. cepa Oc8, aislada de *Crotalaria ochroleuca* y que generó nódulos eficientes en *C. ochroleuca*, *C. juncea*, *C. spectabilis* y *Cajanus cajan*. La secuenciación del genoma completo (Novaseq-Illumina) se realizó en Macrogen (Corea). El borrador generado comprende 46 scaffolds, 8.283.342 pb y el contenido GC estimado es del 63,27%. Los análisis comparativos con *B. icense, B. jicamae, B. japonicum, B. centrolabi, B. embrapense, B. elkanii, B. arachidis, B. diazoefficiens* y *B. lablabi* mostraron valores de ANIb y ANIm inferiores al 85,9 y al 88,6 %, respectivamente. Estos resultados sugieren que la cepa Oc8 no pertenece a esas especies cuyos genomas fueron comparados. El artículo que se presenta a continuación resume los principales resultados obtenidos.

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Draft genome sequence of *Bradyrhizobium* sp. strain Oc8 isolated from *Crotalaria ochroleuca* nodule

Verónica Berriel[a], María A. Morel[b,c], Carla V. Filippi[b], Jorge Monza[b,*]

[a] Centro de Aplicaciones de Tecnología Nuclear en Agricultura Sostenible, Departamento de Suelos y Aguas, Facultad de Agronomía, Universidad de la República, Av. Garzón 809, Montevideo PC 12.900, Uruguay
[b] Departamento de Biología Vegetal, Laboratorio de Bioquímica, Universidad de la República, Av. Garzón 809, Montevideo, PC 12.900, Uruguay
[c] Laboratorio de Microbiología del Suelo (LMS), Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo, PC 11.400, Uruguay

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ABSTRACT

In this study, we report the draft genome sequence of *Bradyrhizobium* sp. strain Oc8, a rhizobium isolated from *Crotalaria ochroleuca,*efficient in *C. ochroleuca, C. juncea, C. spectabilis*, and *Cajanus cajan*. The whole genome of the strain Oc8 contains 46 scaffolds, 8,283,342 bp, and 63.27% of GC content. *Bradyrhizobium* sp. Oc8 is an effective nitrogen-fixing bacterium with potential use as an inoculant for legumes used as cover crops and green manures.

Rhizobia are Gram-negative bacteria belonging to alpha and beta-proteobacteria that establish nitrogen-fixing symbiosis with legumes. This association makes legumes self-sufficient in nitrogen (N) and important in ecological and economic terms (Lorite et al., 2018). The use of legumes as cover crops offers advantages for the environment and agriculture since they contribute N to the ecosystem through biological fixation (Berriel et al., 2020), increasing soil productivity and the yield of the subsequent cash crops (Mahama et al., 2016). *Crotalaria ochroleuca, C. juncea, C. spectabilis* and *Cajanus cajan*, used as cover crops associated with specific rhizobia have a potential to fix N (Oliveira et al., 2007; Pereira et al., 2016; Berriel et al., 2020). These tropical forage legumes are nodulated by a relatively large group of rhizobia (Jorrin et al., 2021), and so their agronomic evaluation should include the rhizobia present in the soil.

In this study, we report the draft genome of *Bradyrhizobium* sp. Oc8 strain, isolated from a nodule of *C. ochroleuca* grown in soil of Uruguay (34.6 S, 55.6 W). Rhizobia isolation was carried out using the nodule squash technique (Gaunt et al., 2004) after surface sterilization according to Batista et al. (2015). A drop of the resulting suspension was subsequently spread onto YEM agar medium (Vincent, 1970) and incubated at 28 °C for 4–5 days. Strain Oc8 was obtained by picking a single colony from the agar plate. The isolated strain was checked for its ability to nodulate its host plant *C. ochroleuca, C. juncea, C. spectabilis* and *C. cajan* as described by Batista et al. (2015).

Oc8 strain was grown in a liquid YEM medium with 180 rpm orbital shaking for 24 h at 27 °C. Subsequently, genomic DNA was extracted using QIAamp DNA Micro Kit (QIAGEN, Germany). Whole-genome sequencing (Novaseq-Illumina, paired-end, PE, 2 × 151 bp) was performed at Macrogen (Korea). Sequencing quality was visually inspected using FastQC (Andrews, 2010) and Trimmomatic (v0.36) (Bolger et al., 2014) was used to discard/trim low-quality reads, keeping 94.35% of the initial PE (i.e., 10,379,129 PE reads). Unicycler (v0.4.7) was used for de novo contig assembly (Wick et al., 2017) yielding 63 contigs. After that, SSPACE (v2.1) was used for scaffolding (Boetzer et al., 2011). Assembly statistics, for both contig and scaffold level assemblies, were obtained using QUAST (v5.0.20) (Gurevich et al., 2013). While scaffolding generated a significant fragmentation reduction, it had no impact on main assembly metrics as the largest contig length, N50 or L50. Thus, the generated draft genome comprises 46 scaffolds, covering 8283,342 bp (largest contig: 1882,916 bp; N50: 537,804 bp; L50: 5; N's per 100,000 bp: 1.03). The GC content was estimated at 63.27%. Blastn (v2.5.0, Altschul et al., 1990) was locally run, with the NCBI RefSeq virus database (v5), in order to check for potential viral (phage) contamination. In addition, PlasmidFinder (v2.0.1, default parameters, Carattoli et al., 2014) and plasmidSPAdes (v3.13.1, Bankevich et al., 2012) were used to discard plasmid contamination.

Benchmarking Universal Single-Copy Orthologs (BUSCO, v5.1.2) was used to assess the completeness of the assembly (dataset: bacteria_odb10) (Simão et al., 2015). Of 124 BUSCOs, 123 were complete (123/124, 99.2%), while one was fragmented (shorter than expected).

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* Corresponding author.
E-mail address: monzajorge@gmail.com (J. Monza).

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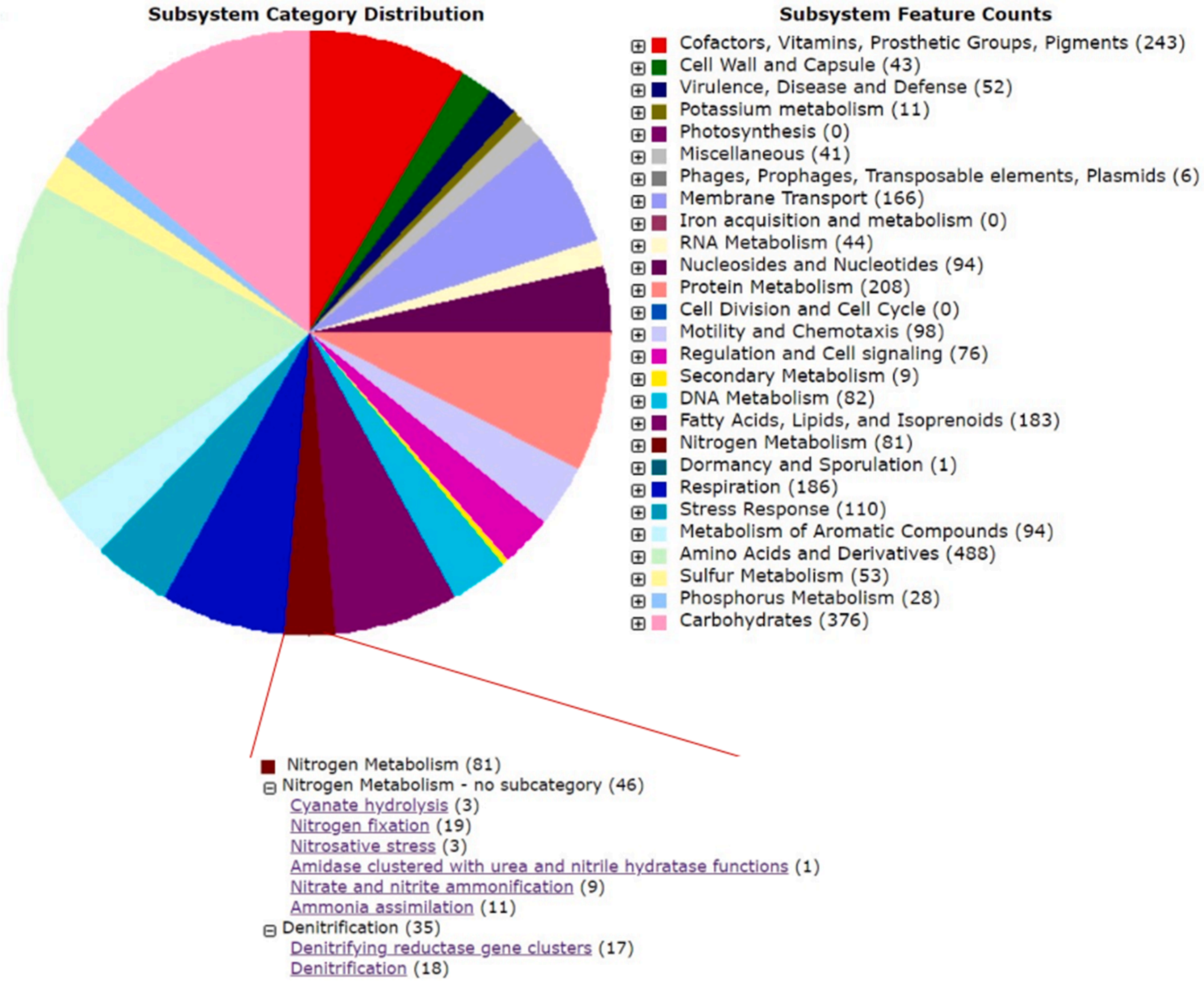

Fig. 1. Bacterial genome representation showing subsystem category distribution of coding sequences (CDS) from strain Oc8, generated through RASTtk pipeline. The number of CDS in the subsystem is shown in brackets.

From the 123 complete BUSCOs 121 were single-copy and two were duplicated. Finally, prokka (v1.12) was used for genome annotation (Seemann, 2014), obtaining 7776 predicted coding sequencing (CDS), 3 rRNA and 52 tRNA. Genome annotation was also carried out through Rapid Annotation Using Subsystem Technology (RAST) server (v2.0) (Overbeek et al., 2013). The most abundant subsystem was Amino acids and derivatives, followed by Carbohydrates. CDS related to N metabolism stood out among genes of agricultural importance (Fig. 1). A complete view of the genome was generated using the CGView Server (Fig. 2) (Petkau et al., 2010).

The 16S rRNA gene sequence was extracted from Oc8 genome using RNAmmer (Lagesen and Hallin, 2007) and it was BLASTed (Camacho et al., 2009) against the 16S rRNA gene sequence of each of the currently type strains available in Type Genome Server (TYGS) database (Meier-Kolthoff and Göker, 2019). Additionally, an extended 16S rRNA gene analysis, performed to detect not yet genome-sequenced type strains relevant to the study, was performed via the Genome-to-Genome Distance Calculator (GGDC) web server (Meier-Kolthoff et al., 2013). For maximum likelihood (ML) tree inference, rapid bootstrapping in conjunction with the autoMRE bootstopping criterion (Pattengale et al., 2010) and subsequent search for the best tree was used. For maximum parsimony (MP) tree inference 1000 bootstrapping replicates were used in conjunction with tree-bisection-and-reconnection branch swapping and ten random sequence addition replicates. Since 16S rRNA gene sequences are conserved in *Bradyrhizobium* (Willems et al., 2001), phylogenetic analysis based on two housekeeping genes, *recA* and *ftsA* (encoding for an actin-like protein involved in prokaryotic cell division) were also conducted, as recommended by Ormeño-Orrillo and Martinez (2019) and Kalita and Malek (2019), respectively. The *recA* and *ftsA* sequences obtained from the genome were compared with *recA* and *ftsA* sequences available in GenBank. Alignment and MP trees were constructed with the MEGA 7 software (Kumar et al., 2016).

The extended 16S rRNA gene-based analysis indicated that the isolate is a *Bradyrhizobium* sp. (Fig. 3.). The ML bootstrapping converged after 950 replicates; the average support was 53.70%. MP analysis yielded the best score of 185 (consistency index 0.56, retention index 0.84) and 50 best trees. The MP bootstrapping average support was 49.35%. Gene comparisons of *recA* and *ftsA* sequences of Oc8 versus publicly available sequences showed lower than 94% and 96.67% of identity percentages, respectively, either with *B. guangzhouense, B. guangdongense, B. diazoefficiens*, and several others *Bradyrhizobium* sp. According to Ormeño-Orrillo and Martinez (2019), nucleotide identities of 98.2% for *recA* could be used as cutoff values to discriminate between described bradyrhizobial species. Kalita and Malek (2019) reported that the *ftsA* sequence similarity range from 80 to 97.4% between *Bradyrhizobium* species (Fig. 4). Based on those reports, the strain Oc8 of *Bradyrhizobium* does not show a close genetic relationship with any *Bradyrhizobium* species.

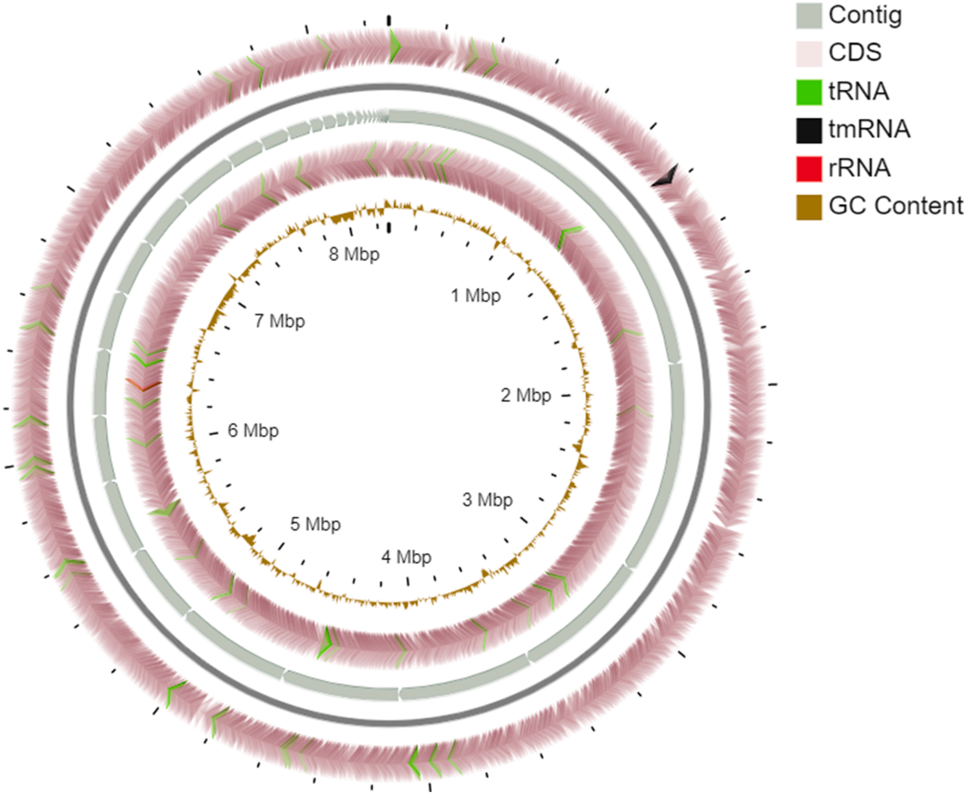

Fig. 2. Circular bacterial genome containing coding sequences (CDS), tRNAs, rRNAs, and GC content skew. The map was generated using the CGView Server beta online software.

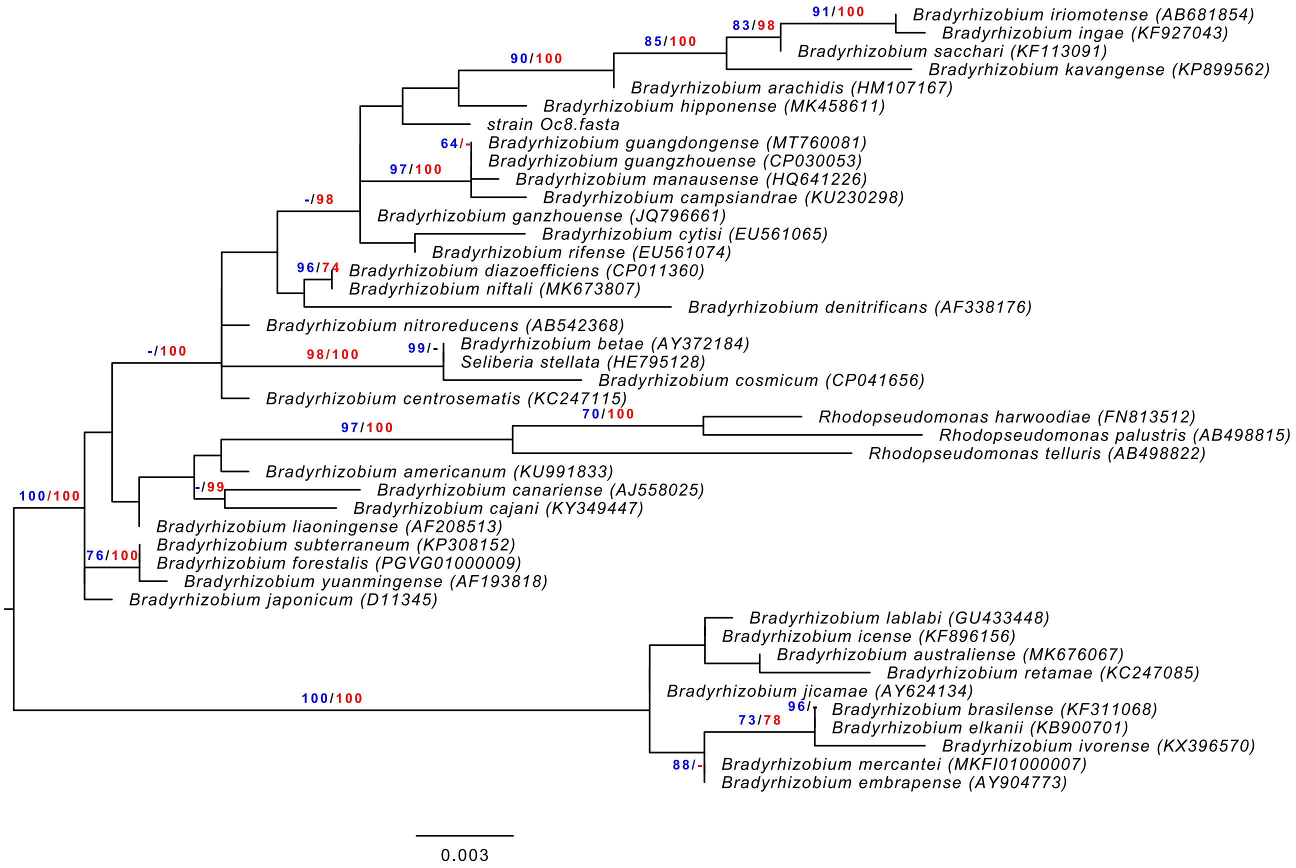

Fig. 3. ML tree inferred from sequences alignments of 16S rRNA sequences under the GTR+GAMMA model and rooted by midpoint-rooting performed via the GGDC web server (Meier-Kolthoff et al., 2013). The branches are scaled in terms of the expected number of substitutions per site. The numbers above the branches are support values when larger than 60% from ML (blue) and MP (red) bootstrapping.

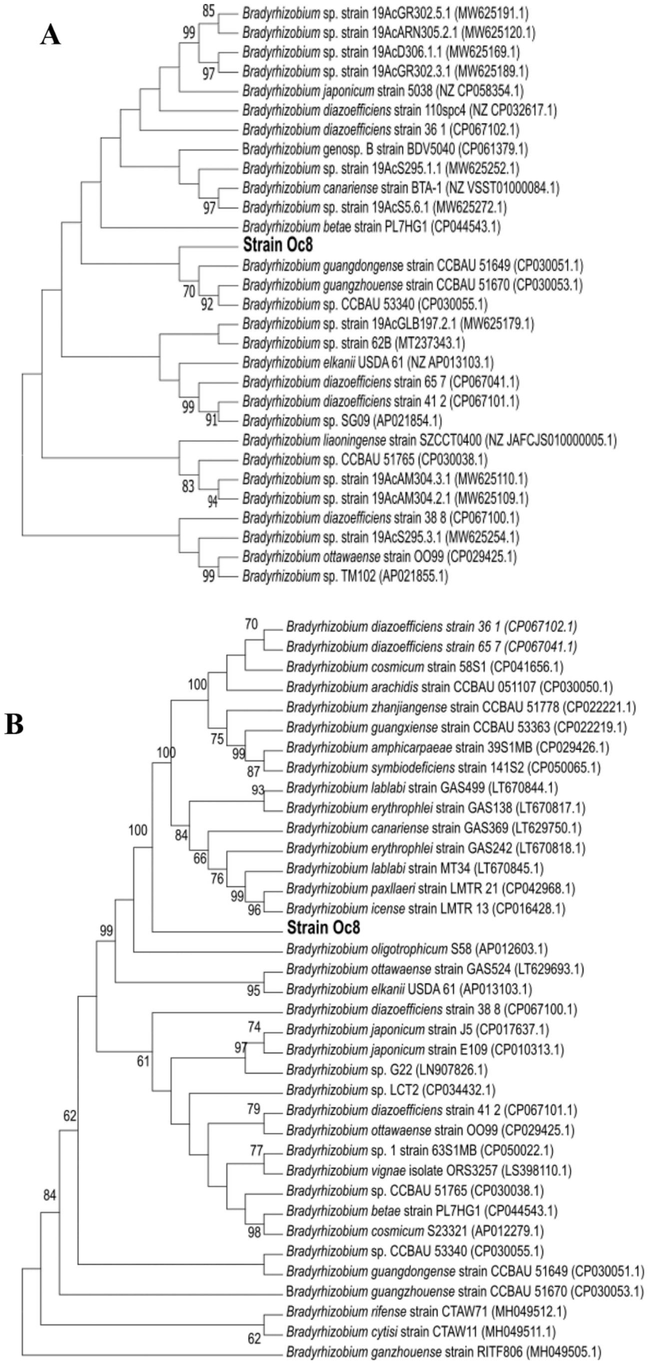

Fig. 4. MP trees inferred from sequences alignments of *recA* (A), and *ftsA* (B) genes using gene sequences selected among the first 100 hit sequences from Blast search and comparison. The numbers in each branch represent bootstrap support values of >60% from 1000 replications.

Although our results showed that the phylogenies of *ftsA* and *recA* were congruent with a possible new species of *Bradyrhizobium*, additional analysis should be performed to verify the taxonomic affiliation of strain Oc8. Next, we will determine measures of nucleotide-level genomic similarity, multilocus phylogenetic analysis, and characterization of biochemical and metabolic attributes.

Data availability

The draft genome sequences have been deposited in GenBank under the BioProject accession number PRJNA752993. The version described in this paper is the first version.

Author contributions

Conceptualization, V.B, and J.M.; writing—original draft preparation and visualization, M.A. M., and C.V.F.; writing—review and editing were developed by all authors; supervision, J.M; project administration, V.B. and J.M.; funding acquisition, V.B. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conclusiones

La identificación de especies a ser usadas como cultivos de cobertura (CC) debe estar basada en criterios de sustentabilidad ambiental y productiva. En consonancia con ese objetivo es necesario disminuir la dependencia del aporte de N de origen industrial e incrementar la entrada de N proveniente de la fijación biológica en las rotaciones agrícolas u hortícolas. Para ello, en el diseño de rotaciones es necesario incluir especies de leguminosas con alta capacidad de fijación. En particular, las especies de leguminosas de origen tropical tienen un gran potencial para fijar N en cortos períodos de tiempo, lo cual podría suplir la ausencia del aporte de N desde las pasturas que han tendido a desaparecer de las rotaciones actuales.

En esta tesis se cuantificó la tasa de fijación biológica de N (FBN) en cuatro especies de leguminosas estivales, crecidas en condiciones controladas en dos niveles de disponibilidad hídrica uno limitante y otro no limitante. *Cajanus cajan* se identificó como la especie más promisoria en ambos escenarios de disponibilidad hídrica. Previo a la estimación de la FBN fue necesario realizar el ajuste de la metodología de determinación del valor B, para obtener resultados robustos. Debido a que los valores B son propios de cada especie y ambiente, el ajuste metodológico evitó la aparición de valores sesgados hacía arriba e incluso superiores a 100%, lo cual es frecuente cuando se utilizan los valores B de bibliografía.

La factibilidad de aporte de N de estas especies de leguminosas estaría además respaldada por otro resultado encontrado en esta tesis, de que los suelos bajo uso agrícola de Uruguay poseen una diversidad de especies de rizobios capaces de establecer nódulos efectivos con estas leguminosas. Una de estas cepas rizobianas aisladas y caracterizadas fue *Bradyrhizobium* Oc8, que de acuerdo con los resultados obtenidos sería una nueva especie reportada dentro del género. Es de resaltar la excelente capacidad de nodulación de esta cepa, la cual la hace una fuerte candidata para iniciar otras pruebas con la finalidad de desarrollo de un inoculante comercial.

Una desventaja del uso de CC es el consumo de agua del suelo que estos cultivos realizan durante su ciclo productivo, con respecto a mantener el suelo en barbecho. Por esto, otra característica relevante y deseable de las especies utilizadas para este fin es que mantengan una alta eficiencia de uso de agua (EUA). En esta tesis se identificó que *Cajanus cajan* fue también en ambos escenarios de contenido hídrico del suelo la especie que sobresalió en esta característica. Previo a esto, se validó el uso de la metodología de discriminación isotópica de ^{13}C ($\Delta^{13}C$) para estimar EUA, que resulta mucho más práctica y rápida que la engorrosa metodología gravimétrica de referencia. A partir de esta información se propone un nuevo índice integrado (masa de N fijado/$\Delta^{13}C$) que permite indexar distintas especies de leguminosas tropicales en función de la masa de N fijada a igualdad de agua transpirada.

Estos resultados deben sin embargo ser validados en experimentos de campo, los cuales ya se realizaron, pero no se integraron a esta tesis debido a que el análisis de esta información aún no está finalizada al vencimiento de los plazos para la finalización del doctorado.