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

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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

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:

$$TE = \frac{shoot\ dry\ mass}{T}. \quad (1)$$

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]:

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

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:

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

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]:

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

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

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

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.

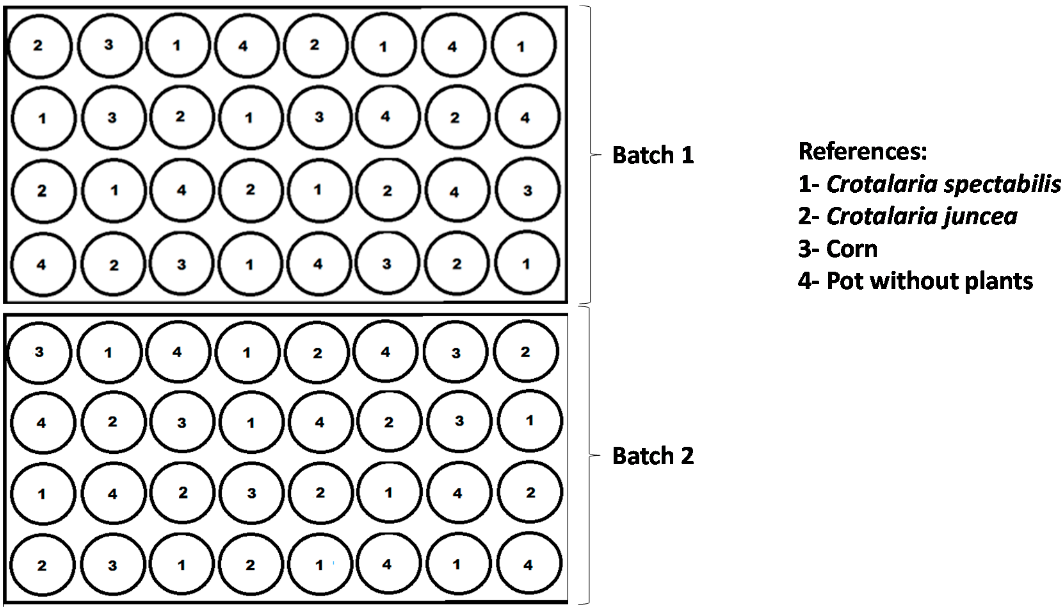

Figure 1. Design of the experiment. Circles represent the pots in the plant growth chamber.

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.

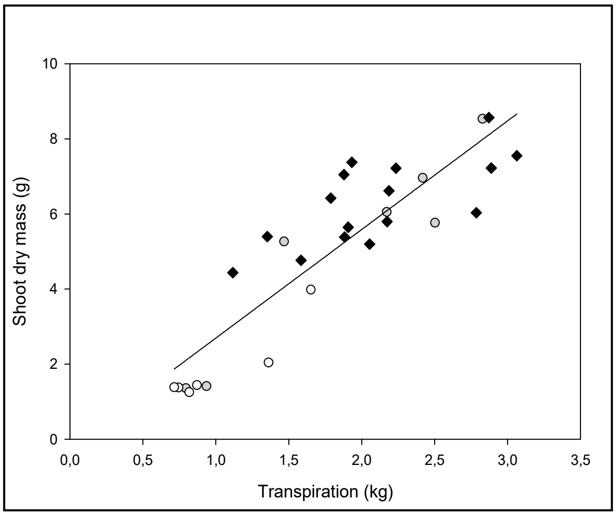

Figure 2. Relationship between shoot dry mass and water transpiration expressed for *Crotalaria spectabilis* (rhombuses) and *Crotalaria juncea* (circles). *C. juncea* was evaluated at two nodulation levels. Plants with large nodules are identified with gray circles, and those with small nodules with 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 $\delta^{13}C$, 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 $\delta^{13}C$ 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 $\Delta^{13}C$ 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.

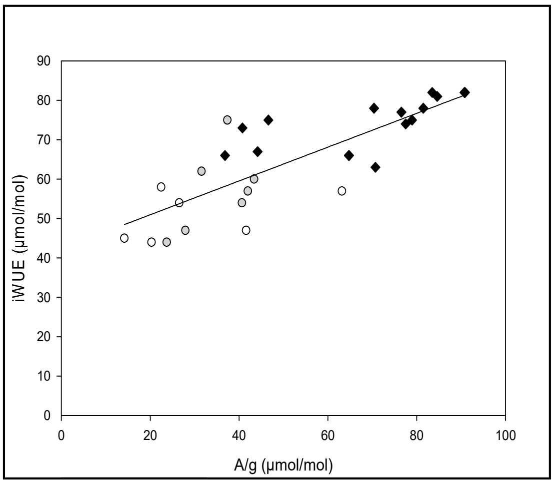

Figure 3. Relationship between the integrated intrinsic water use efficiency (iWUE) and foliar water use efficiency [quotient: photosynthesis (A) and stomatal conductance (g)] for *Crotalaria spectabilis* (rhombuses) and *Crotalaria juncea* (circles). *C. juncea* was evaluated at two nodulation levels. 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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