Nitrogen

Article

Effects of Soil Sucrose Application on Biological Nitrogen Fixation and Aboveground Biomass Production in Leguminous Cover Crops

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Abstract: The use of cover crops (CCs) based on tropical legumes, including *Crotalaria ochroleuca*, *Crotalaria juncea*, *Crotalaria spectabilis*, and *Cajanus cajan*, represents a pivotal aspect of agricultural rotations. These crops facilitate the incorporation of nitrogen through biological nitrogen fixation (BNF), thereby reducing the necessity for synthetic nitrogen fertilizers. Nevertheless, the capacity for the BNF of these species in Uruguay is relatively modest. To address this limitation, an approach is proposed that involves the immobilization of nitrogen in the soil using a highly energetic material, such as sucrose. The objective of this study was to examine the impact of incorporating sucrose into typical Uruguayan soil on aboveground dry matter production, nitrogen accumulation, and nitrogen fixation by legumes utilized as CCs. The experiments involved the planting of *C. ochroleuca*, *C. juncea*, *C. spectabilis*, and *C. cajan* in pots containing either soil alone or soil mixed with sucrose and the subsequent maintenance of these in a plant growth chamber for a period of 90 days. The addition of sucrose had a positive impact, with nearly double the aboveground dry matter production and nitrogen content observed. The percentage of nitrogen derived from the atmosphere (%Ndfa) increased significantly in all species, rising from an average of 83% to 96% in the sucrose-amended soil compared to the control. In the case of *C. juncea*, there was a notable threefold increase in aboveground dry matter and nitrogen accumulation across different treatments, accompanied by a 26% rise in %Ndfa and a fourfold increase in nitrogen fixation amounts. These findings indicate that *C. juncea* has the potential to significantly enhance performance and ecosystem services in typical Uruguayan soil.

Keywords: legumes; soil sugar amendment; biological nitrogen fixation

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1. Introduction

Agriculture is currently facing the critical challenge of increasing food production to meet the needs of a growing global population [1]. While intensifying agricultural practices is essential for ensuring food security, it often undermines the sustainability of agricultural systems, leading to soil degradation and water pollution, primarily due to extensive land use [2]. To mitigate these adverse effects, it is crucial to adopt strategies that focus on conserving natural resources. In this context, conservation agriculture has emerged as a key approach. A fundamental aspect of conservation agriculture is the use of cover crops, which are planted after the main crop is harvested and before the subsequent crop is sown [2,3].

Cover crops (CCs) confer a multitude of benefits, including the enhancement of biodiversity, the prevention of soil erosion [4,5], the augmentation of soil organic matter [6,7], and a reduction in reliance on chemical fertilizers [6,8]. Leguminous CCs are particularly valuable as they possess the unique ability to fix atmospheric nitrogen (N) through symbiotic relationships with diazotrophic bacteria, enabling them to thrive in N-deficient soils [9,10]. This N-fixing capacity not only diminishes the need for synthetic fertilizers but also offers substantial economic and environmental advantages [8,11].

In Uruguay, the integration of CCs into crop rotations is not only encouraged but also regulated by the Ministry of Livestock, Agriculture, and Fisheries [12]. Within this regulatory framework, CCs are recognized as essential for sustaining soil productivity across the country. Tropical legumes, employed as summer CCs in Uruguay, present considerable advantages, primarily due to their growth period coinciding with peak solar radiation [13]. This timing optimizes both photosynthesis and biological N fixation (BNF), resulting in a significant increase in dry matter production and N accumulation, as well as improved soil fertility even after the CC has been killed. The gradual release of N from these residues will benefit subsequent crops in the rotation, especially when these residues are used as green manure [14,15].

The efficiency of BNF and the symbiotic relationship between legumes and rhizobia is highly contingent upon environmental factors, including temperature, soil pH, and mineral N levels [16]. Excessive N fertilization or elevated soil mineral N concentrations can disrupt the intricate communication between rhizobia and legumes, impairing critical processes such as chemotaxis, root exudation, and nodulation [17]. Furthermore, high levels of mineral N can inhibit nitrogenase activity, thereby reducing the overall efficacy of N fixation [17,18].

Agricultural research has identified *Crotalaria* spp. and *Cajanus cajan* as promising CCs due to their capacity to produce over 12 Mg/ha of biomass [13,19,20]. Also, these species offer a range of ecosystem services, including soil coverage, enhancement of soil quality, suppression of pests and weeds, improved water retention, and increased biodiversity [21]. Despite these benefits, a noted deficiency in their N-fixing capacity exists [13]. Specifically, the BNF of *C. cajan*, *C. spectabilis*, *C. juncea*, and *C. ochroleuca* in symbiosis with native soil rhizobia ranges from 47% to 73% in Uruguay [13]. This variability underscores the need to optimize N fixation in these species, particularly considering reports that suggest potential BNF values exceeding 90% [22]. Therefore, it is crucial to investigate agronomic practices that can enhance BNF in *Crotalaria* spp. and *C. cajan* under the specific edaphoclimatic conditions of Uruguay.

A promising strategy for stimulating BNF in plants is to limit the availability of mineral N in the soil [23] in order to prevent the inhibition of this process. A reduction in mineral N levels in the soil can be achieved through N immobilization. This process is facilitated by the addition of readily metabolizable carbohydrates, such as sucrose or glucose, or materials with a high carbon-to-nitrogen (C/N) ratio, such as vegetative residues, to the soil [24–29]. However, the impact of mineral nitrogen immobilization in the soil on BNF in *Crotalaria* spp. and *Cajanus cajan* remains unclear, as does the potential benefit of sugar amendments in this context.

This study aimed to evaluate the impact of sucrose as a carbon amendment on soil BNF and aboveground dry matter accumulation in *Cajanus cajan*, *Crotalaria juncea*, *Crotalaria spectabilis*, and *Crotalaria ochroleuca*. The primary objective was to evaluate whether sucrose application to the soil can synergistically enhance BNF and dry matter production in various legume species utilized as CCs. The study aims to determine if sucrose amendments can increase C and N inputs into the agricultural system both during the CC cycle and after its termination, thereby improving soil fertility. The results are expected to provide new insights into how carbon amendments can refine agricultural management practices and promote more sustainable farming systems.

2. Materials and Methods

2.1. Plant Material and Growing Conditions

For this study, soil was collected from an agricultural site in southern Uruguay (sand = 24.5%; silt = 48.7%; clay = 26.8%; organic carbon = 11.4 g/kg; nitrogen organic = 1.1 g/kg; N-NO_3^-: 3.6 mg/kg; and N-NH_4^+: 7.1 mg/kg). A portion of this soil was mixed and homogenized with sucrose (at a ratio of 1 kg:5 g). One week after preparing the mixture, the soil mixed with sucrose was distributed into pots with a diameter of 15 cm and a depth of 18 cm. Simultaneously, other pots of the same dimensions were filled with soil without sucrose.

Seeds of *Crotalaria juncea* L., *Crotalaria spectabilis* Roth, *Crotalaria ochroleuca* G. Don., and *Cajanus cajan* L. cv IAPAR 43 were sown at a rate of one seed per pot, with ten pots per treatment. The seeds of tropical legumes were procured from the BrSeeds Company in Brazil (https://www.brseeds.com), as they are not accessible in Uruguay.

Plants were grown for 90 days in a growth chamber at 28 °C, with a variable relative humidity of between 30 and 50% and a light intensity of 500 µmol $m^{-2} \cdot s^{-1}$ with a 16/8 h light/dark cycle, and they were irrigated with deionized water. The trial was replicated twice.

2.2. Production of Aboveground Biomass and Nodule Dry Matter, and Analytical Measurements

The aboveground biomass and nodules of each plant were harvest and dried at 60 °C until a constant weight was achieved, and then the dry mass was determined by gravimetry. After that, all shoot dry samples were first ground with a fixed and mobile blade mill (Marconi MA-580) until a particle size of less than 2 mm was reached, and then with a rotary mill (SampleTek 200 vial Rotator) until the required granulometric size for isotopic analysis was reached.

Samples were weighed into tin capsules, and their total N concentration (in percentage weight–weight) and isotopic ratio of N 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 the delta notation (δ) in parts per thousand (‰) using Equation (2):

$$\delta^{15}_{7}\mathrm{N} = \left(\frac{\mathrm{R_{sample}}}{\mathrm{R_{standard}}} - 1\right) \times 1000 \quad (1)$$

The proportion of N derived from the atmosphere (%Ndfa) in legumes was estimated as described by Shearer and Kohl [30]:

$$\%\mathrm{Ndfa} = \left(\frac{\delta^{15}_{7}\mathrm{Nref} - \delta 1^{15}_{7}\mathrm{Nfix}}{^{15}_{7}\mathrm{Nref} - \mathrm{B}}\right) \times 100 \quad (2)$$

where $\delta^{15}N_{ref}$ is the ^{15}N delta value determined in a non-fixing reference plant, $\delta^{15}N_{fix}$ is the ^{15}N delta value determined in the legumes, and B is the ^{15}N delta value determined in legumes grown without N mineral and whose only N source is the atmospheric N. The B value was obtained as described by Berriel and Perdomo [31], being the B values of *C. cajan*, *C. spectabilis*, *C. juncea*, and *C. ochroleuca*. The reference plant was maize with $\delta^{15}N$ = 9.7‰.

The total N amount of the aboveground dry matter of the legumes was calculated by multiplying the nitrogen concentration and the aboveground dry matter. To determine the amount of fixed N, the %Ndfa was multiplied by the total N amount [32]. The amount of soil N uptake was then obtained by subtracting the N fixed from the total plant N amount.

2.3. Experimental Design and Statistical Analysis

The experimental design utilized was a completely randomized design with two factors: the substrate and the species. The species factor comprised four levels (*Crotalaria juncea*, *Crotalaria spectabilis*, *Crotolaria ochroleuca*, and *Cajanus cajan*), while the substrate factor had two levels (soil with or without sucrose). The variables analyzed included the shoot dry matter, the nodule dry matter, the shoot N concentration (%N), the shoot nitrogen amount, the percentage of nitrogen derived from the atmosphere (%Ndfa), and the amount of nitrogen derived from the atmosphere (Ndfa) and from soil (Ndfs).

The Bliss transformation technique was employed to convert percentages into decimal values. Before performing a two-way ANOVA, checks were conducted to validate that the data met assumptions of normality, homogeneity of variances, and independence. Once these criteria were confirmed, the main factors—the substrate and the species—along with their interaction, underwent ANOVA analysis. Following this, a post hoc analysis using Tukey's test was performed to compare means, with the statistical significance set at $p \leq 0.05$.

3. Results

3.1. Aboveground Dry Matter, Nitrogen Concentration, and Nitrogen Amount across Different Species and Treatment

No significant interaction was detected between the species (*C. cajan*, *C. juncea*, *C. spectabilis*, and *C. ochroleuca*) and the treatments (soil with or without sucrose addition) concerning the aboveground dry matter and N amount, according to the ANOVA analysis. Nevertheless, a noteworthy interaction was identified in the variable nitrogen concentration.

Differences in aboveground dry matter were identified across species and treatments (Table 1). Between species, *C. cajan* exhibited higher aboveground dry matter production compared to *C. ochroleuca*, while *C. spectabilis* and *C. juncea* showed no statistically significant differences relative to the two aforementioned species. It is noteworthy that plants sowed in soil with sucrose displayed doubled aboveground dry matter production compared to those nurtured in soil without sucrose. This increase was particularly pronounced in *C. juncea*, revealing an increase exceeding threefold.

Table 1. The mean and standard deviation values of the shoot dry matter, shoot nitrogen concentration, and shoot nitrogen amount for *Cajanus cajan*, *Crotalaria juncea*, *Crotalaria ochroleuca*, and *Crotalaria spectabilis* cultivated in soil with and without sucrose amendment. Different letters indicate statistical significance between species (lowercase) and treatments (capital letters) in a Tukey's Honestly Significant Difference (HSD) post hoc test at a significance level of 0.05.

Species	Dry Matter (g/plant)			N Concentration (%)			N Amount (mg/plant)		
	Soil	Soil + sucrose	Mean	Soil	Soil + sucrose	Mean	Soil	Soil + sucrose	Mean
C. cajan	7.4 ± 3.6	13.5 ± 3.0	10.4 B	2.9 ± 0.8 b	2.1 ± 0.1 a	2.5	190.2 ± 68.1	290.0 ± 73.7	240.1 B
C. juncea	4.2 ± 2.6	12.4 ± 1.7	8.3 AB	1.6 ± 0.9 a	1.7 ± 0.2 b	1.7	70.3 ± 52.0	212.8 ± 8.2	141.6 A
C. ochroleuca	5.2 ± 2.7	7.4 ± 1.7	6.3 A	3.5 ± 1.7 NS	2.3 ± 0.5 NS	2.9	163.4 ± 95.9	171.3 ± 64.8	167.3 A
C. spectabilis	6.4 ± 2.9	10.4 ± 3.9	8.4 AB	1.4 ± 0.4 NS [1]	1.9 ± 0.1 NS	1.6	89.6 ± 38.2	193.8 ± 71.0	141.7 A
Mean	5.8 a	11.0 b		2.4	2.0		123.6 a	217.0 b	
S of V [1]					*p*				
Species (S)		0.0212			0.0001 [3]			0.0019	
Treatment (T)		<0.0001			0.00134 [3]			<0.0001	
Interaction SxT		NS [2]			0.0366 [3]			NS [2]	

[1] SV: Source of variation. [2] NS: not significant ($p > 0.05$). [3] Statistical differences detected after Bliss transformation.

Regarding nitrogen concentration, expressed as a weight percentage, variability emerged across species and treatments following Bliss transformation, as indicated by the ANOVA test (Table 1). However, due to the significant interaction between both factors, a univariate ANOVA was conducted for each species individually. This analysis revealed a distinct pattern across species in response to the treatment. Therefore, further scrutiny was focused on the nitrogen amount variable to extract more meaningful insights.

The nitrogen amount accumulated in the dry matter, which combines aboveground dry matter and nitrogen concentration, explains the similar trend observed in the aboveground dry matter variable. Regarding the nitrogen amount variable, differences were found across species and treatments. The three *Crotalaria* species exhibited similar nitrogen amounts, which were, on average, 1.6 times lower than that of *C. cajan*. Regarding the treatment, plants grown in sucrose-amended soil accumulated more nitrogen, showing a 1.8-fold increase in nitrogen compared to those grown in soil without sucrose. The most significant increase in this variable was observed in *C. juncea*, reaching three times the nitrogen amount compared to plants grown in soil without sucrose.

3.2. Nodule Dry Matter

No significant interaction between species (*C. cajan*, *C. juncea*, *C. spectabilis*, and *C. ochroleuca*) and treatments (soil with or without sucrose addition) was observed regarding nodule dry matter, as indicated by the ANOVA analysis. However, significant differences in nodule dry weight were detected across species and treatments (Figure 1). Specifically, significant differences were observed only between the means of *C. ochroleuca* and *C. juncea*, with *C. ochroleuca* showing a higher nodule dry matter compared to *C. juncea*. No significant differences were found between *C. cajan* and *C. spectabilis* compared to the other species. Notably, plants grown in soil with sucrose generally exhibited greater nodule dry matter compared to those grown in soil without sucrose (Figure 1).

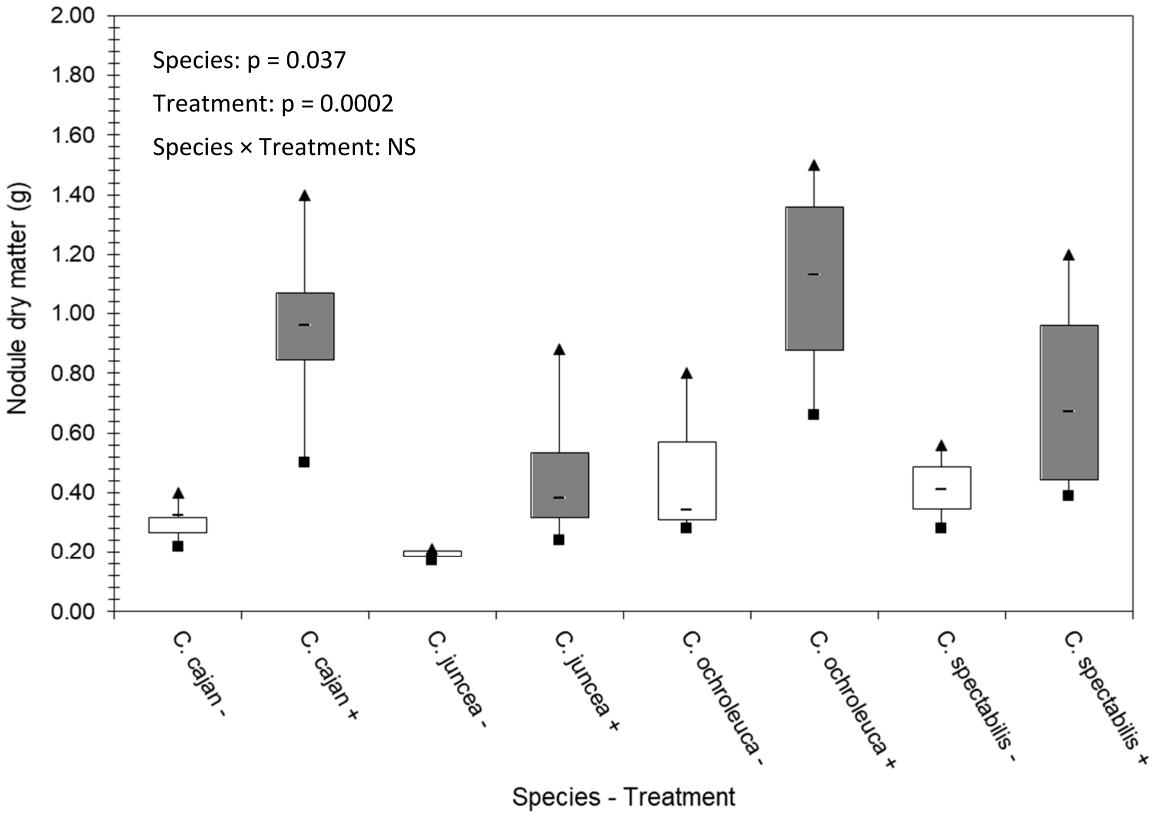

Figure 1. The nodule dry matter of *Cajanus cajan*, *Crotalaria spectabilis*, *Crotalaria ochroleuca*, and *Crotalaria juncea* cultivated in soil with (+) and without (-) sucrose, represented with gray and white boxes respectively. Minimum and maximum values are represented by squares and triangles, respectively, while the median is indicated by a line.

3.3. Percentage of Nitrogen Derived from Air and Nitrogen Amount Derived from Air and Soil

No significant interaction between species and treatment was observed for %Ndfa and the amount of Ndfa and Ndfs in aboveground matter, as indicated by the ANOVA results (Table 2).

After applying the Bliss transformation, the ANOVA test revealed no differences across species regarding %Ndfa, but significant distinctions emerged across treatments (Table 2). On average, %Ndfa ranged from 84% to 93% between species. Regarding treatments, %Ndfa was observed to be 13% higher in plants grown in soil with sucrose compared to those in soil without sucrose amendment, averaging 96% in the amended soil.

Differences were observed across species and treatments regarding the amount of Ndfa accumulated in the aboveground dry matter, as indicated by the ANOVA test (Table 2). *Cajanus cajan* exhibited the highest amount of Ndfa, surpassing the *Crotalaria* species on average by 1.6 times. The three *Crotalaria* species did not differ from each other in this variable. Regarding the treatment, plants cultivated in soil with sucrose amendment experienced an almost twofold increase in the amount of Ndfa compared to plants grown in soil without amendment.

Table 2. The mean and standard deviation values of nitrogen derived from air expressed in percentages (Ndfa, %), the shoot nitrogen derived from air (Ndfa, (mg/plant), and the shoot nitrogen derived from soil for *Cajanus cajan*, *Crotalaria juncea, Crotalaria ochroleuca,* and *Crotalaria spectabilis* cultivated in soil with and without sucrose. Different letters indicate the statistical significance between species (lowercase) and soil with or without sucrose amendment (capital letters) in a Tukey's Honestly Significant Difference (HSD) post hoc test at a significance level of 0.05.

Species	Ndfa (%)			Ndfa (mg/plant)			Ndds (mg/plant)		
	Soil	Soil + sucrose	Mean	Soil	Soil + sucrose	Mean	Soil	Soil + sucrose	Mean
C. cajan	85.2 ± 8.3	99.2 ± 0.6	92.2	164.5 ± 68.1	289.4 ± 74.0	227.1 B	25.7 ± 19.4	0.3 ± 0.6	13.0
C. juncea	70.9 ± 16.0	96.4 ± 2.5	83.6	51.4 ± 42.5	210.8 ± 10.5	131.1 A	18.9 ± 17.8	2.2 ± 2.4	10.5
C. ochroleuca	88.3 ± 8.0	92.6 ± 3.2	90.4	143.4 ± 84.8	167.9 ± 64.6	155.6 A	20.4 ± 19.0	3.4 ± 3.1	11.7
C. spectabilis	89.2 ± 11.0	97.4 ± 2.3	93.3	80.4 ± 36.8	188.5 ± 72.5	134.5 A	9.1 ± 11.4	5.3 ± 5.6	7.2
Mean	83.4 a	96.4 b		110.0 a	214.2 b		18.4 b	2.8 a	
S of V [1]				*p*					
Species (S)		NS [2,3]			0.0009			NS [3]	
Treatment (T)		<0.0001 [2]			<0.0001			0.0016	
SxT		NS [2,3]			NS [3]			NS [3]	

[1] S of V: Source of variation. [2] Difference detected after Bliss transformation. [3] NS: not significant ($p > 0.05$).

No statistically significant differences were detected in the amount of Ndfs in the aboveground dry matter across species, but differences were found across treatments (Table 2). A higher quantity of Ndfs, exceeding 6.6 times, was observed for plants grown in soil without sucrose compared to plants grown in soil with sucrose.

4. Discussion

Sustainable food production management requires strategies that ensure high crop yields while preserving soil quality [33]. In this regard, the inclusion of legumes as CCs in rotation schemes is crucial, as these plants have the capability to fix atmospheric N through their symbiotic relationship with rhizobacteria [34]. However, the successful integration of legumes into such rotations is significantly dependent on the effective interaction between the plants and the soil microbiome [35].

The addition of easily degradable sugars, such as glucose and sucrose, to soil has been demonstrated to significantly enhance microbial N immobilization [36,37]. Likewise, other materials, such as plant residues with a high N content, can also contribute to N immobilization in the soil. The magnitude of this immobilization process is closely correlated with both the quality and quantity of the carbon source or amendment applied [38], as well as the C/N ratio of the material used [39]. Prior research has indicated that amendments promoting N immobilization typically result in a temporary depletion of mineral N availability, with these effects generally diminishing within a year [40]. This reduction in soil mineral N has been associated with significant decreases in aboveground dry matter production and foliage growth in non-leguminous plants [41].

Our study demonstrates that the addition of sucrose to the soil significantly increased aboveground dry matter in all examined legume species, which effectively formed symbiotic relationships with native *Rhizobium* strains. Among these species, *Crotalaria juncea* exhibited the most pronounced increase in aboveground dry matter production when grown in sucrose-amended soil compared to those grown in control soils. This finding underscores the potential benefits of soil sucrose amendment when cultivating legumes, as it not only enhances short-term carbon input into the agricultural system but also significantly contributes to soil organic matter following the incorporation of CCs. The greater aboveground dry matter observed in *Crotalaria* or *C. cajan* plants grown in soil amended with sucrose compared to those grown in control soil can be explained by the genotypic adaptation of these species to marginal soils, which has allowed them to reduce their dependence on soil mineral N [42]. It is well established that low levels of mineral N can inhibit BNF, as plants tend to minimize metabolic energy expenditure when sufficient mineral N is available in the soil [43]. Our results align with those of Gannett et al. [44], who found that amending soil with materials that have a high C/N ratio, such as sawdust and sucrose, did not decrease soybean biomass production, similar to that observed in plants grown in unamended soil. In contrast, non-leguminous weeds showed a reduction in dry matter when grown in soil amended with carbon compared to plants grown in control soil [44].

The amendment of soil with sucrose or other carbon-rich materials stimulates the BNF process in legumes by immobilizing soil mineral N [45]. Our findings support this hypothesis, as the addition of sucrose significantly promoted BNF, evidenced by an increase in %Ndfa in legumes grown in sucrose-amended soils compared to those grown in control soils. Notably, *C. juncea* exhibited the greatest increase, with a difference in %Ndfa between treatments reaching 26%. These results are consistent with those reported by Wagner and Zapata [46], who observed a substantial increase in %Ndfa in soybeans grown in sucrose-amended soils.

The higher N content and %Ndfa in the above-ground dry matter of all species cultivated in soils with added sucrose, relative to the control group, provide compelling evidence of the positive impact of sucrose amendment on BNF in legumes. *Cajanus cajan* exhibited the highest %Ndfa accumulation, while *C. juncea* demonstrated an almost fourfold increase compared to its control. These results suggest that sucrose induces a synergistic loop effect of enhanced BNF, N accumulation, and dry matter production in the species evaluated in this study.

Recent research has highlighted the underutilization of soil microbial activity as a tool in sustainable agricultural management [33]. These researchers have proposed revisiting an old concept termed "reverse fertilization" [47]. The aim of "reverse fertilization" is to immobilize N and other nutrients by promoting microbial growth through the addition of carbon. This strategy involves applying soil amendments with a high C/N ratio to control weeds in disturbed environments, a problem and approach investigated by Gannet et al. [48]. Our study aligns with the principles of "reverse fertilization" but extends the application to the promotion of BNF in legumes used as CCs in crop rotations.

Despite our promising findings, further research is needed to elucidate the impact of sucrose and other high C/N ratio amendments, such as plant residues with C/N ratios exceeding 23:1, on the growth, development, and BNF of legume CCs in symbiosis with rhizobia under field conditions. Generating this knowledge is essential for the development of sustainable agricultural practices that optimize plant–microbiome interactions and maximize agronomic benefits.

5. Final Remarks

This study elucidates that the incorporation of sucrose into soil improves the performance of leguminous CCs by enhancing BNF, increasing N content, and augmenting both nodule and aboveground dry matter production. This enhancement is attributed to the immobilization of soil mineral N induced by sucrose, which consequently drives the activation of the BNF process. The use of carbon-rich amendments for N immobilization, recently revisited by various researchers to manage weed development, promotes more sustainable and environmentally friendly agricultural practices. Although the results are promising, further research is needed, particularly to evaluate other carbon amendments with potential comparable to sucrose under field conditions. Considering the role of N immobilization as a critical regulator of plant growth, it should be thoroughly integrated into the development of cover crop management strategies within agroecosystems.

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