Biomass Yield, Nitrogen Content and Uptake, And Nutritive Value of Alfalfa Co-Inoculated with Plant-Growth Promoting Bacteria

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Abstract

Alfalfa (Medicago sativa L.) has high forage yield potential, protein quality, palatability, and digestibility, and low seasonality. The aim of this study was to evaluate the effects of strains of Sinorhizobium meliloti and Azospirillum brasilense on the nutritive content, bromatological composition (crude protein [CP], neutral detergent fiber [NDF], acid detergent fiber [ADF], and in vitro digestibility of dry weight [IVDDW]), and shoot dry weight (SDW), relative chlorophyll index (RCI), number of tillers (NT), plant height (PH), and root dry weight (RDW) and volume (RV), of alfalfa grown in a Typic Ultisol. The experiment consisted of eight combinations of plant-growth promoting bacteria (PGPB). The treatments were as follows: T_1:non-inoculated control without N-fertilizer (NI); T_2: NI + N-fertilizer, and inoculated with T_3: Sinorhizobium (=Ensifer) meliloti SEMIA 116 + N-fertilizer; T_4: S. meliloti SEMIA 116 + A. brasilense Ab-V5 + Ab-V6 + N-fertilizer; T_5: S. meliloti SEMIA 134 + N-fertilizer; T_6: S. meliloti SEMIA 134 + co-inoculation + N-fertilizer; T_7: S. meliloti SEMIA 135 + N-fertilizer; and T_8: S. meliloti SEMIA 135 + co-inoculation + N-fertilizer. S. meliloti strains are used in commercial inoculants for the alfalfa, and A. brasilense for several non-legumes and legumes in Brazil. The experiment was performed for three successive cuts under greenhouse conditions. Application of N-fertilization increases the production cost, making alfalfa cultivation unviable. Inoculation with three strains of Sinorhizobium meliloti highly promoted alfalfa growth, considering several parameters, including PH, RCI, NT, SDW and RDW, nutritive value, and with an emphasis on RV, and total N content and total N accumulated in shoots and roots. No further increases were observed with the co-inoculation with the PGPB A. brasilense. Studies in field and greenhouse conditions are necessary to verify the benefits of the use of PGPB in the cultivation of alfalfa.

Keywords: *Medicago sativa*, biological nitrogen fixation, diazotrophic bacteria, N-total

1. INTRODUCTION

Alfalfa (*Medicago sativa* L.) has cosmopolitan importance, either based on the scope of the area explored, or because of its important properties such as high yield potential, protein quality, high palatability and digestibility, capacity for biological N_2 fixation (BNF), and low seasonality in forage yield;

additionally, it can be used directly as pasture or conserved as silage or hay [1]; [2]. It is one of the most important pastures used for feeding specialized dairy herds and animals, and can also be used in human diet, cosmetics, and the pharmaceutical industry [3].

Among the limiting factors in alfalfa production, the reduction in N availability is considered the most relevant factor, resulting in less shoot dry weight (SDW) and protein content in forage [4]. Alfalfa is able to symbiotically associate with bacteria known as rhizobia, establishing the process of BNF, which can reach up to 470 kg ha^{-1} of N in temperate conditions [5]. and twice as much in tropical and subtropical conditions, where the frequency of annual is higher [4]. is The main nitrogen-fixing symbionts of alfalfa belong to the species *Sinorhizobium meliloti* (= *Ensifer meliloti*) [6].

In addition to rhizobia, other PGPB, with an emphasis on the genus *Azospirillum,* (BPGPs), can be beneficial to increases biomass production and grain yield in a variety of non-legume and legume species [7]; [8]; [9]. BPGPs are believed to benefit plant growth through an array of mechanisms that can act simultaneously or in continuous reaction [10]; [11]; [12]. For example, this positive effect of PGPB has been verified in *Prosopis juliflora* (Sw.) DC. [13], in maize (*Zea may* L.) and wheat (*Triticum aestivium* L.) [7], in brachiarias (*Urochloa* spp.) [9], and in co-inoculation with rhizobia of soybean (*Glycine max* (L.) Merr.) and common bean (*Phaseolus vulgaris* L.) [8]. The inoculation of forage species with *Azospirillum brasilense* can increase the SDW and tillering, as well as the uptake of N of phosphorus (P) from the soil [14]; [15]; [16]; [17].

Considering the potential interactions of BNF with the soil chemical attributes and the benefits attributed to different cultures by inoculation with *A. brasilense*, one can deduce that co-inoculation can improve alfalfa yield. However, although there are studies that report the benefits of co-inoculation of rhizobia and *Azospirillum* (e.g. [8]), there are still no reports on the performance of alfalfa in the tropics. Therefore, the objective of this study was to evaluate the agronomic efficiency of strains of *Sinorhizobium meliloti* and *Azospirillum brasilense* on shoot and root growth, nutritive value, and nutritional status of alfalfa grown in a Typic Ultisol.

2. MATERIALS AND METHODS

Strains characterization and growth, alfalfa cultivar and establishment and conduction of the experiment

Strains

The rhizobial strains used as inoculants are authorized for the production of commercial inoculants for alfalfa in Brazil [18]. There are three strains authorized for the pasture in Brazil: SEMIA 116 (=USDA 1088, =3DOi4, =CNPSo 933), SEMIA 134 (=CNPSo 934), and SEMIA 135 (=CNPSo 935) [18]. Despite been used in commercial inoculants, the precise origin and characterization of the rhizobial strains were not clear. According to a record of 1995, SEMIA 116 has been received from the USDA collection, while strains SEMIA 134 and SEMIA 135 were isolated from alfalfa nodules grown in the state of Rio Grande do Sul, Brazil; the three strains were evaluated and indicated for the use in commercial inoculants by the institutions Fepagro (State Agricultural Research Foundation)/UFRGS (Federal University of Rio Grande do Sul) [19]; [18]. The *Azospirillum brasilense* strains Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084)

have been selected in Brazil and used in commercial inoculants as a PGPB for both non-legumes, including brachiarias [9] and legumes as co-inoculant with rhizobia [8]. The strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection # 1054), in Londrina, State of Paraná, Brazil.

The three rhizobial strains have been previously classified as *Sinorhizobium* (=*Ensifer*) *meliloti* based on the sequencing of the16S rRNA gene [18]. As the information based only on the 16S rRNA is not precise to define species, the DNAs of the three strains were extracted using the DNeasy Blood & Tissue kit (Quiagen, Hilden, Germany), according to the manufacturer's instructions, aiming at the amplification with three housekeeping genes, *glnB* and *gyrB*, with the primers and amplification conditions described by [20] and *rpoB*, with the primers and amplification conditions described by [21]. Genes sequencing were carried out according to the conditions described by [22] on the ABI 3500XL sequencer (Applied Biosystems). The sequences were submitted to the NCBI bank database and received the accession numbers that are included in Figure 1. The sequences obtained were aligned using the MUSCLE algorithm [23] and phylogenetic analyzes were performed using the MEGA software version 6 (Molecular Evolutionary Genetics Analysis), using the maximum likelihood algorithm (ML) [24], with a bootstrap value of 1,000 replicates [25], For the Multilocus Sequencing Analysis (MLSA) the sequences of *glnB*, *gyrB* and *rpoB* genes were concatenated using the SeaView software [26] and the phylogenetic tree was built with the General Time Reversible nodel, with gamma-distributed sites.

To verify the genetic profile of each of the three rhizobial strains, DNA analysis was performed by BOX-PCR, as described by [27]. The genetic profiles were analyzed using the Bionumerics software (Applied Mathematics, Kortrijk, Belgium, v.7.6), through the construction of a dendrogram of similarity, using the UPGMA (Unweighted Pair-Group Method with Arithmetic mean) algorithm [28] and the Jaccard coefficient [29], with a 3% of tolerance.

For the experiments the inoculants were produced at the Biotechnology Laboratory of Embrapa Soybe and Center, in modified yeast-extract mannitol medium for rhizobia [30] and DYGS medium for *Azospirillum* [31].

Experiment establishing and conduction, harvests and analyses

The experiment was conducted under greenhouse conditions at the São Paulo State University (UNESP), Campus of Araçatuba, São Paulo State, Brazil with an average daytime temperature of 26 to 36 °C and night mean time temperature of 20 °C, 415 m altitude. The soil used was characterized as a Typic Ultisol with the following chemical attributes at the 0 to 20 cm layer [32]: pH in $CaCl_2$ = 5.2 (1:2.5 soil/solution), soil organic matter (SOM) = 26 g kg^{-1}, phosphorus (P - resin) = 23.0 mg kg^{-1}, sulfur (S) = 19.0 mg kg^{-1}, potassium (K^+) = 2.9 $mmol_c$ kg^{-1}, calcium (Ca^{2+}) = 25.0 $mmol_c$ kg^{-1}, magnesium (Mg^{2+}) = 17 $mmol_c$ kg^{-1}, aluminum (Al^{3+} = 28.0 $mmol_c$ kg^{-1}, potential acidity (H+Al) = 44.9 $mmol_c$ kg^{-1}, cation exchange capacity (CEC) = 72.9 $mmol_c$ kg^{-1}, boron (B) = 0.6 mg kg^{-1}, copper (Cu) = 1.2 mg kg^{-1}, iron (Fe) = 111.0 mg kg^{-1}, manganese (Mn) = 9.9 mg kg^{-1}, zinc (Zn) = 3.5 mg kg^{-1}, clay = 155 g kg^{-1}, and sand = 735 g kg^{-1}. Before sowing, base saturation was increased to 80% [33]; [6] with $CaCO_3$ and $MgCO_3$ application in a 3:1 ratio, and the soil was incubated for 30 days, keeping the humidity close to 80% of field capacity (FC).

The experiment was performed in plastic pots containing 5.0 kg of soil. After the incubation period, basic fertilization was performed according to [34], with the application of 200 mg kg^{-1} of P [$Ca(H_2PO_4)_2$], 150 mg kg^{-1} of K, 61.5 mg kg^{-1} of S (K_2SO_4), 0.5 mg kg^{-1} of B (H_3BO_3), 1.0 mg kg^{-1} of Cu (CuSO4), 0.1 mg kg^{-1} of Mo (H_2MoO_4), 3.0 mg kg^{-1} of Mn ($MnSO_4$), and 2.0 mg kg^{-1} of Zn ($ZnSO_4$). NH_4NO_3 (50.0 mg kg^{-1} of N) 14 days after emergence.

The population of rhizobia symbionts of alfalfa was evaluated by the method of the most probable number (MPN) in alfalfa plants of cultivar "Crioula", while the population of diazotrophic microorganisms in soils was evaluated by the MPN method in semi-solid NFb medium, [35]. The soil populations were estimated at 1.4×10^2 cells of rhizobia g^{-1} and 1.2×10^4 cells of diazotrophic bacteria g^{-1} of soil.

The experiment was performed with a completely randomized block design with eight treatments and five replicates with repeated measures over the time. The treatments were as follows: T_1:non-inoculated control without N-fertilizer (NI); T_2: NI + N-fertilizer, and inoculated with T_3: Si*norhizobium* (=*Ensifer*) *meliloti* SEMIA 116 + N-fertilizer; T_4: *S. meliloti* SEMIA 116 + *A. brasilense* Ab-V5 + Ab-V6 + N-fertilizer; T_5: *S. meliloti* SEMIA 134 + N-fertilizer; T_6: *S. meliloti* SEMIA 134 + co-inoculation + N-fertilizer; T_7: *S. meliloti* SEMIA 135 + N-fertilizer; and T_8: *S. meliloti* SEMIA 135 + co-inoculation + N-fertilizer. The bacterial concentration of the inoculants was adjusted to 2×10^9 cells per mL for rhizobia and 2×10^8 cells per mL for *Azospirillum*. The inoculant dose of 15 mL k^{-1} of seed was used. Fifteen seeds were sown, and after thinning, five uniform plants remained per pot.

Three cuts at 10 cm above the ground were made when the plants had an onset of 10% flowering to evaluate plant height (PH), number of tillers (NT), relative chlorophyll index (RCI), and shoot dry weight (SDW). The chlorophyll content was determined indirectly before collection using the SPAD-502 Plus Chlorophyll Meter (SPAD - Soil and Plant Analysis Development) in the average third of five plants per pot. After each cut, the material was dried in an oven with forced air circulation at a temperature of 65 °C for 72 h. Subsequently, the material was weighed and ground for chemical analysis. The neutral detergent fiber (NDF), acid detergent fiber (ADF) as described by [36], N content (NC) in shoot and root and *in vitro* digestibility of dry weight (IVDDW), were determined as described by [37]. The crude protein (CP) concentration was calculated by multiplying the NC by a factor of 6.25. The total N accumulation was calculated by multiplying the NC by SDW. At the end of the experiment, root dry weight yield (RDW) and root volume (RV) of alfalfa were determined.

Statistical analysis

Data were tested for error normality and homogeneity of variances and PH, NT, RCI, SDW, RDW, RV, and chemical composition (NDF, ADF, NC and IVDWD) were evaluated statistically. The results were assessed using analysis of variance (ANOVA), F test ($p \leq 0.05$) and compared using the t test (LSD) with a 5% probability using SAS (Statistical Analysis System, version 8.2) [38].

3. RESULTS AND DISCUSSION

Strains characterization

The analysis of the 16S rRNA represents the backbone of the taxonomy of prokaryotes; however,

nowadays, the great majority of the data has shown that this singles analysis is not capable of distinguish species, only genus. The analysis of other housekeeping genes, in the MLSA (multiloccus sequencing analysis) has been used as a much more powerful technique for species classification (e.g. [20]; [30]; [21]. The phylogenetic trees obtained for each single gene (data not shown), as well as the tree obtained when the three housekeeping genes ———*glnB*, *gyrB*, *rpoB*——— were concatenated and analysed (Figure 1) confirmed the results obtained with the 16S ribosomal gene [18], indicating that the three rhizobial strains used in commercial inoculants in Brazil, SEMIA 116 (=USDA 1088, =CNPSo 933), SEMIA 134 (=CNPSo 934), and SEMIA 135 (=CNPSo 935) belong to the species *Sinorhizobium* (=*Ensifer*) *meliloti*.

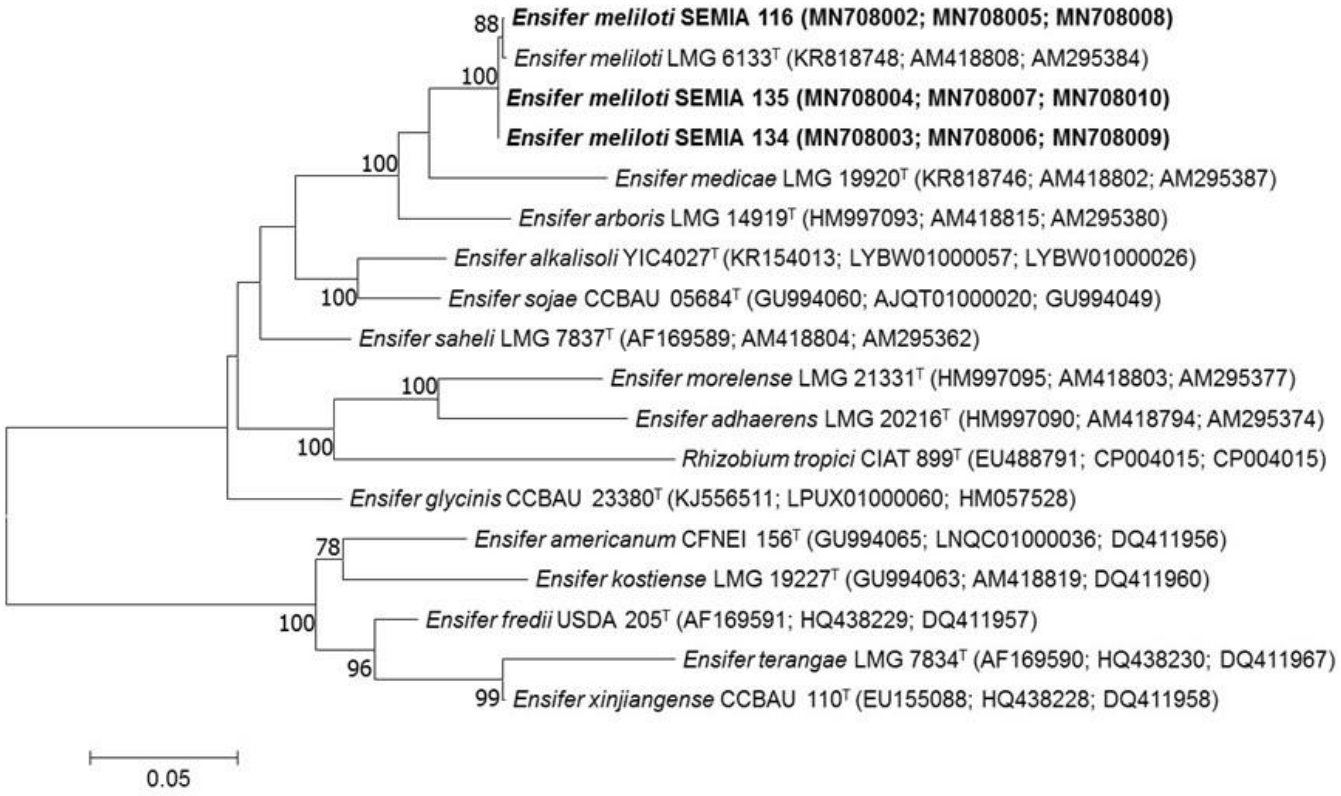

Figure 1. Maximum likelihood phylogeny based on the concatenated gene sequences of *glnB* + *gyrB* + *rpoB* of *Sinorhizobium* (=*Ensifer*) species. Bootstrap values >70 % are indicated at the nodes. The model used was the General Time Reversible, with gamma-distributed sites (G). Bar indicates five substitutions per 100 nucleotide positions.

Previously, strains were isolated from effective nodules, as it was the case of SEMIA 134 and SEMIA 135, but there were no techniques available to distinguish, what has changed with the molecular biology. The DNA profile obtained by the BOX-PCR technique has proven to be a reliable technique to distinguish strains [40], such that it has been included as the official method for confirming the identity of strains in Brazilian inoculants [41]. Interestingly, the analysis of the DNA profile by BOX-PCR indicated identical profiles for the strains SEMIA 135 and SEMIA 136 (Figure 2), indicating that they could be the same strains. As these two strains were isolated from efficient alfalfa nodules in Rio Grande do Sul soil, they can indicate an efficient strain adapted to the region's edaphoclimatic conditions, which stood out in the selection process. As the genetic characterization of the strains was not carried out at the time of selection,

it was not possible to identify that they were the same strain. The profiles of SEMIA 135 and SEMIA 135 were distinct from the North American strain SEMIA 116, with less than 60% of similarity (Figure 2), indicating that SEMIA 135 and SEMIA 136 may be indigenous strains, of some native legume, which have adapted to the alfalfa growing in southern Brazil.

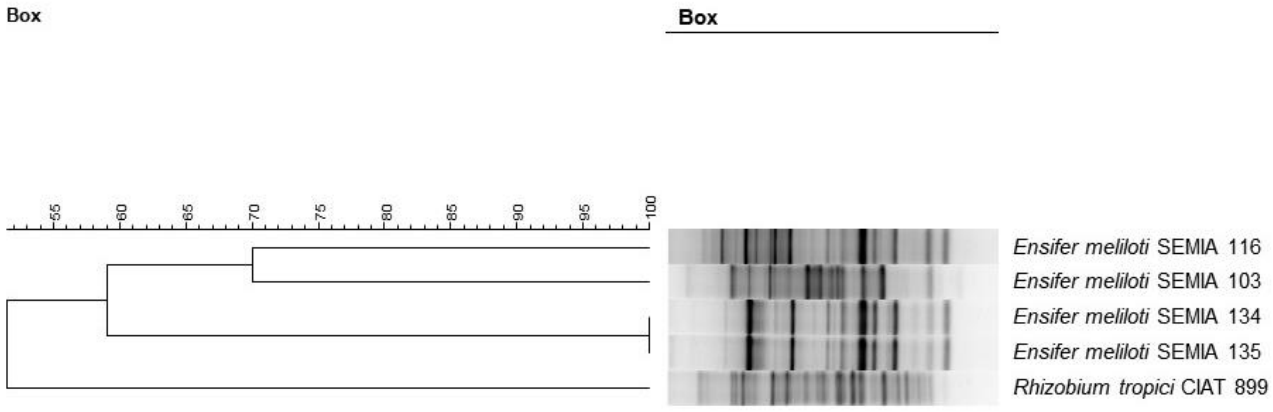

Figure 2. Dendrogram of alfalfa strains SEMIA 116, SEMIA 134 and SEMIA 135 used in commercial inoculants for the forage in Brazil based on cluster analysis of BOX-PCR products using the UPGMA algorithm and the Jaccard index, with 3% of tolerance. SEMIA 103 is also a symbiont of alfalfa, while CIAT 899 is a symbiont of common bean.

Plant performance with inoculation of PGPB

The analysis of variance indicated that the plant height (PH) was influenced by the interaction of treatments and cuts (Figure 3a). The height of the plants varied in the average of the three cuts from 37.9 to 53.3 cm, with the absolute control (non-inoculated without N-fertilizer, NI) having the lowest PH in relation to the other treatments. It should be noted that even in the absence of significance between treatments with PGPB, PH in the co-inoculation treatment with *S. meliloti* SEMIA 134 + *A. brasilense* (Ab-V5 + Ab-V6) was 12.7% higher than that in NI + N treatment (non-inoculated + N-fertilizer). In the third cut, both inoculated and co-inoculated treatments, except for those co-inoculated with SEMIA 135, were statistically superior in relation to both the NI and the NI + N treatments, emphasizing the benefits of the nitrogen fixing symbiosis with rhizobia even after successive cuts [42]; [43].

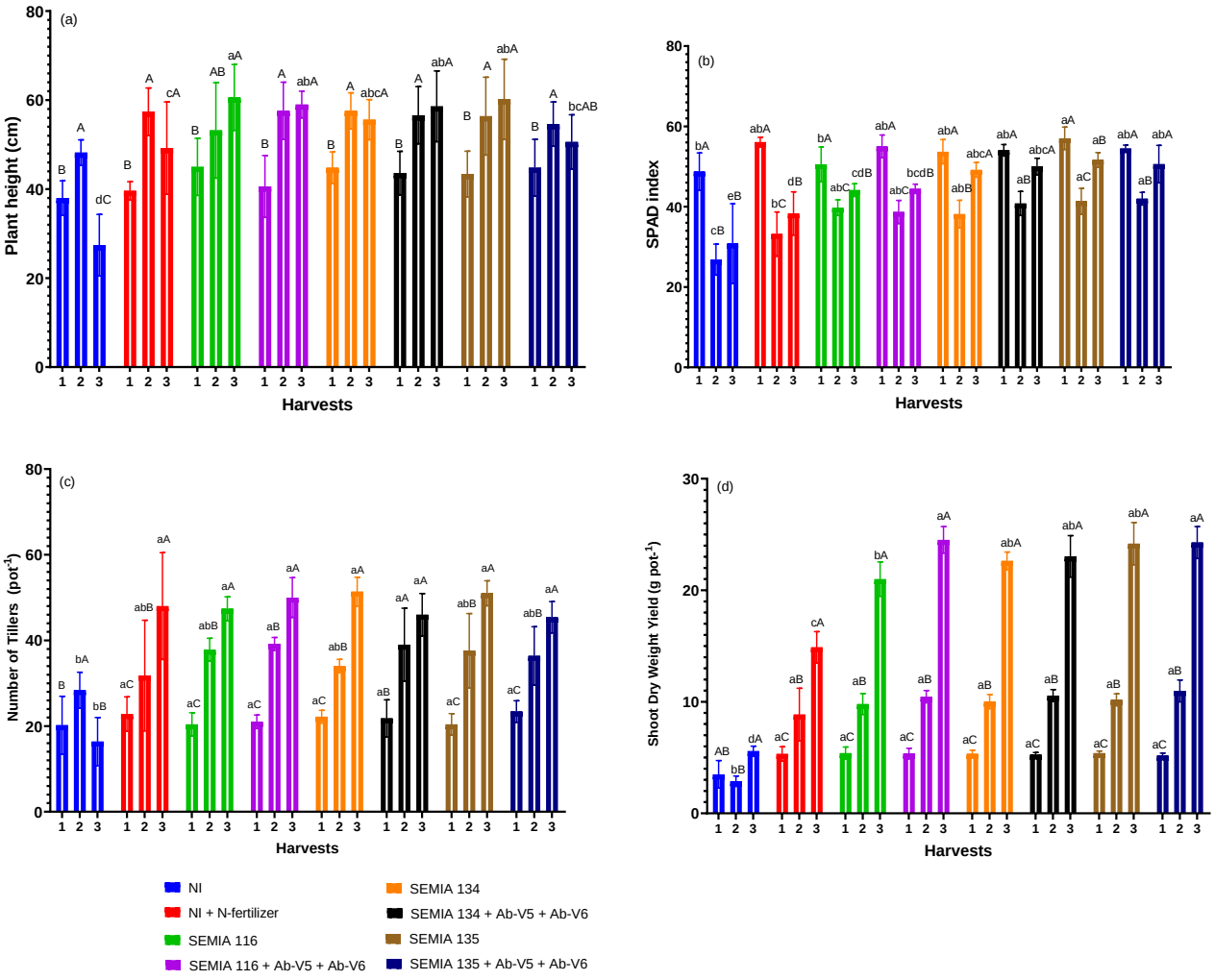

Figure 3. Plant height (cm) (a); relative chlorophyll index (SPAD index) (b); number of tillers (units pot^{-1}) (c) and shoot dry weight yield (g pot^{-1}) (d) in alfalfa inoculated with *S. meliloti* and co-inoculated with *A. brasilense* (Ab-V5 + Ab-V6) of the three harvests. Error bars represent the standard errors of the means. Averages followed by the same lowercase letter for treatments and upper case for harvests do not differ by t test (LSD) (P≤0,05).

There was a significant effect of treatment × cut for the SPAD index (Figure 3b), which varied from 35.5 to 50.0 for the average of the three cuts, and was similar to the range of 31.4 to 44.9 reported by [44] with the same alfalfa cultivar 'Crioula'. There were differences between the cuts, and in the second cut, the plants inoculated with *S. meliloti* + (Ab-V5 + Ab-V6) showed the highest values. It was also observed that the values of the SPAD index were more expressive in the third cut for the treatments involving the inoculation or co-inoculation with strains SEMIA 134 and SEMIA 135 than in the other treatments. The SPAD value in treatment T_8 (co-inoculation with SEMIA 135) was 32.0% higher than that in NI control, again demonstrating the importance of seed inoculation.

Considering that SPAD values above 30 indicate adequate nutrition with regard to N in alfalfa [44], it was observed that only the NI control showed low values in the second cut (26.8). As the SPAD index is used as an indicator of responses of plants that have higher N content, in the present study, except in plants

inoculated with strains of *S. meliloti* + (Ab-V5 + Ab-V6), the SPAD indexes decreased with the successive cuts in the NI and NI + N treatments (Figure 3b), indicating a probable decrease in the photosynthetic mechanism of the plants, possibly owing to the decrease in the contents of structural carbohydrates in the reserve mechanism in plants [45]. The number of tillers (NT) showed significant differences for the interaction treatments × cuts and ranged from 21.7 to 36.7 between cuts, with increase from the first to the third cut (Figure 3c); however, among the treatments, the NT varied significantly in the third cut, and in the third cut was lowest in the NI treatment.

For SDW parameter there was interaction between treatments × cuts (Figure 3d); the second lowest yield was obtained with the NI treatment, and in the third cut there an outstanding performance was achieved by the co-inoculated treatments in comparison to both NI and NI + N treatments. An increase in SDW was observed from the first to the third cuts in all treatments, except for the NI control. All *S. meliloti* showed good performance, but it is worth mentioning that co-inoculation of SEMIA 116 and *A. brasilense* produced 16.8% more SDW than with single inoculation with SEMIA 116 (Figure 3d).

There was a significant relation between the RDW and RV (Figure 4b and Figure 4c), which ranged from 3.3 to 17.6 g per pot, with treatments NI and NI + N being inferior to all other treatments (Figure 4b). The treatment co-inoculated with *S. meliloti* SEMIA 134 produced 89.2% RDW when compared to the NI + N treatment. For the RV, emphasis should be given to the co-inoculation with strain SEMIA 116, with, 45.8% higher than the NI + N (Figure 4c).

The increase in the RDW with the diazotrophic bacteria demonstrated that the stimulation of nodulation can occur as a direct response to the increase in the quantity of roots and RV. Similar results were obtained by [46]; [47], wherein inoculation with *A. brasilense* promoted the formation of root hair in common beans (*Phaseolus vulgaris*) and alfalfa, respectively. As rhizobial infection occurs through the formation of infectious threads in the root hair [48], stimulation of a greater number of epidermal cells to differentiate into infectious root hair cells can increase the potential of nodule initiation [49].

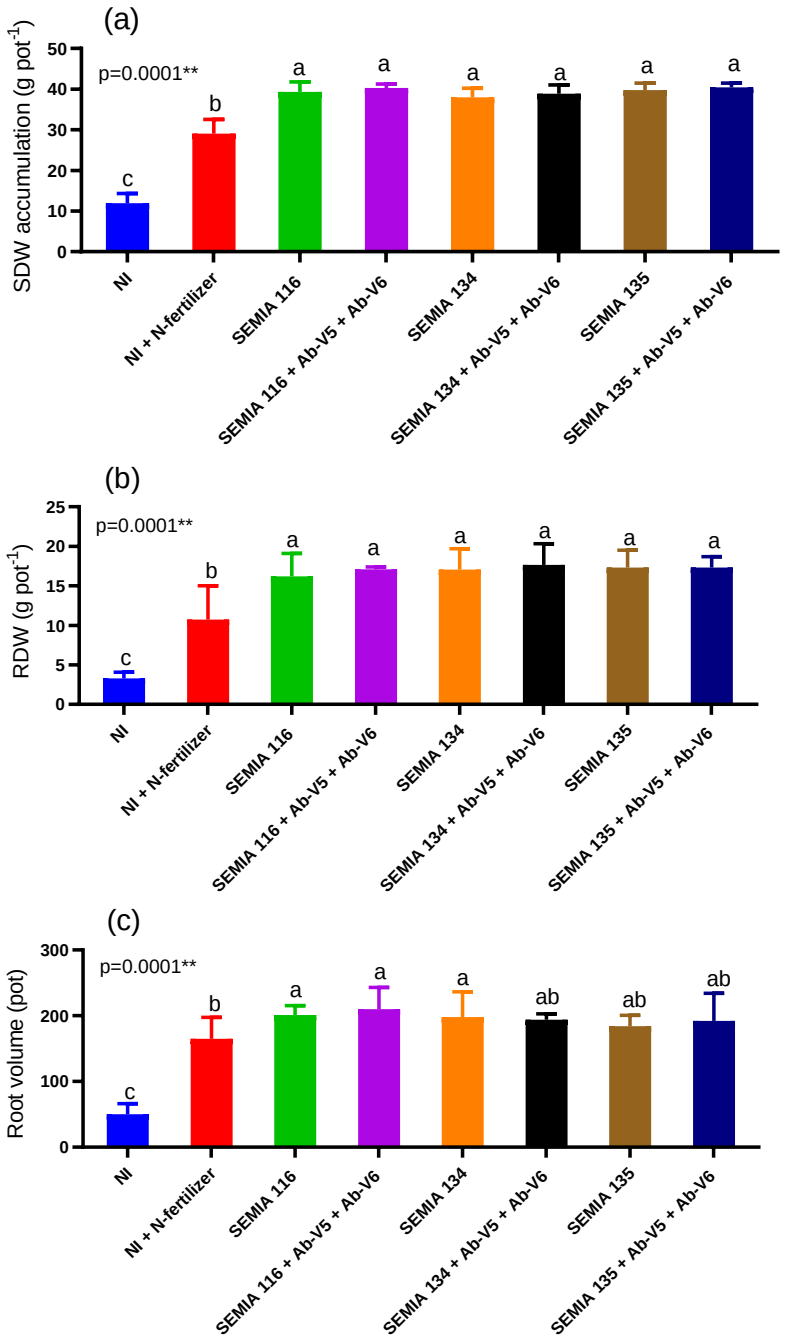

Figure 4. Shoot dry weight yield accumulation (g pot^{-1}) (a); root dry weight (g pot^{-1}) (b) and root volume (pot) (c) in alfalfa inoculated with *S. meliloti* and co-inoculated with *A. brasilense* (Ab-V5 + Ab-V6) of the three harvests. Error bars represent the standard errors of the means. Averages followed by the same lowercase letter for treatments do not differ by t test (LSD) (P≤0,05).

In addition to BNF, PGPBs are also known for their ability to produce phytohormones. [50] verified the effect of co-inoculation with *S. meliloti* and *Herbaspirillum frisingense* on alfalfa and concluded that inoculation with these strains had a beneficial effect on the symbiosis process with regard to the seed growth, the SDW and RDW, as well as increased number of nodules and nitrogenase activity. These results are consistent with those reported in the present experiment, as the PGPB showed beneficial effects,

resulting in an increase in the SDW, RDW, and RV (Figure 3d and Figure 4a and 4b and 4c).

Interaction between treatments × cuts was verified in the total N content (NC) of shoots an (Figure 5a). The NC was lower in the NI and NI + N treatments than in other treatments, except for the first cut, in that NI + N did not differ and ranged from 22.7 to 31.9 g kg^{-1} N, whereas in the third cut, the co-inoculated treatment with *S. meliloti* SEMIA 135 was 58.3% higher than in the NI + N treatments. It should be noted that even without significant differences, the co-inoculation with *S. meliloti* SEMIA 135 was 8.1% higher in NC than the same treatment single inoculated with the same strain. According to [51] studying the efficiency of inoculants in alfalfa also found similar results, with the inoculated treatments being superior to the treatment both non-inoculated controls, demonstrating the efficiency of the inoculants in the cultivation of alfalfa.

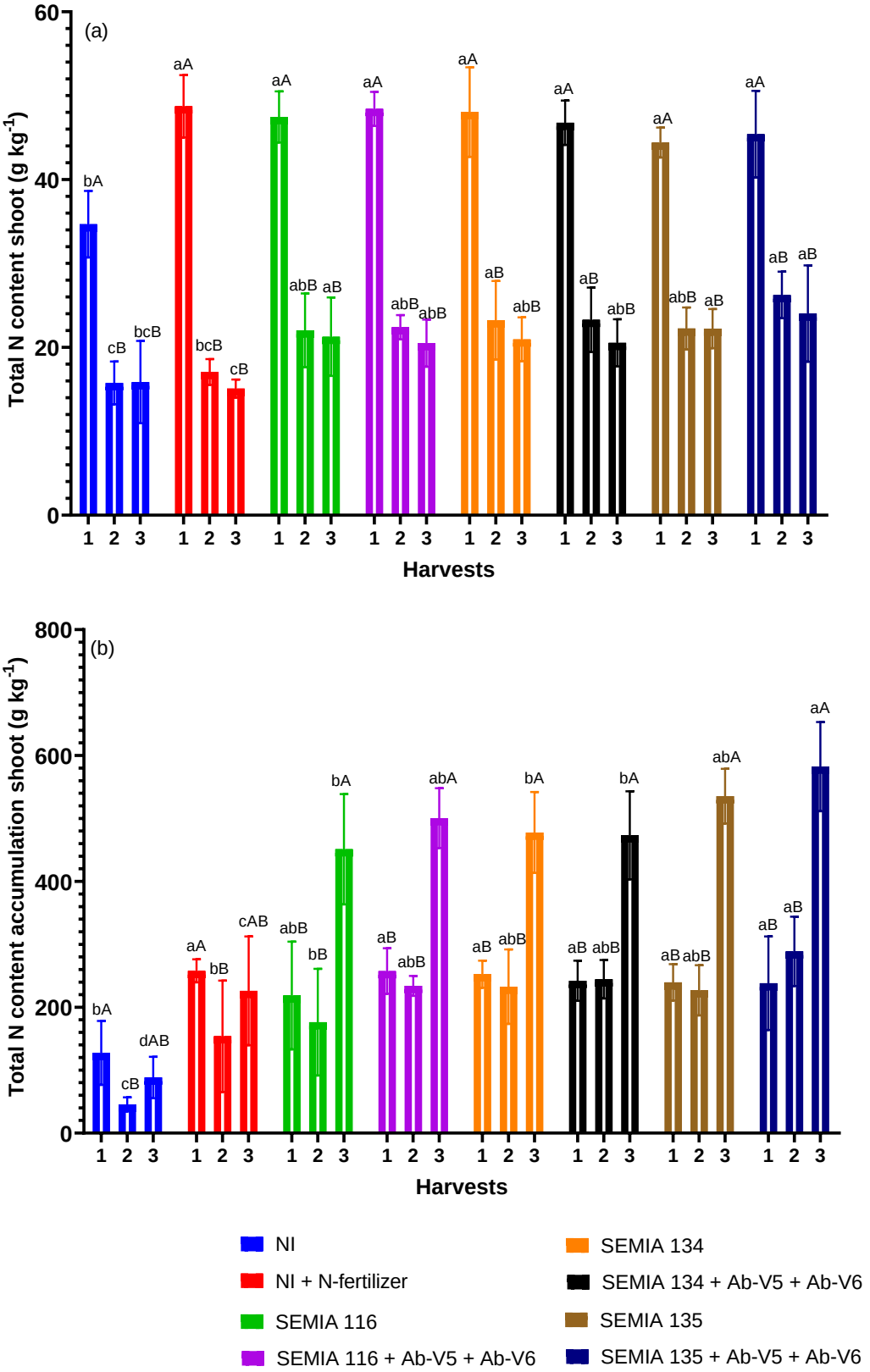

Figure 5. Total nitrogen content (g kg^{-1}) (a) and total N accumulation (g kg^{-1}) (b) of shoots in alfalfa inoculated with *S. meliloti* and co-inoculated with *A. brasilense* (Ab-V5 + Ab-V6) of the three harvests. Error bars represent the standard errors of the means. Averages followed by the same lowercase letter for treatments and upper case for harvests do not differ by t test (LSD) (P≤0,05).

In the second and third cuts, the NC in the NI + N treatment, that received 50 kg ha^{-1} N indicate that this dose was not sufficient to guarantee a satisfactory growth of alfalfa, which was reflected by lower N levels, while the inoculation with the rhizobia strains allowed good performance. According to [52]; [4], [2], the ideal N content in SDW for alfalfa grown in the tropics should be in the range of 24.0- to 35.0 g kg^{-1}. However, differences in the NC depend on the time of cut and the stage of development. According to [53] found that when harvested at the first cut, the NC was 43 g kg^{-1} N (growing phase) and 22 g kg^{-1} N (end of flowering). For the total N accumulation, there was an interaction between treatments × cuts (Figure 5b). In the first cut, the lowest N accumulation was verified in the NI control, whereas in the second cut, the highest values were achieved in the co-inoculated and in the NI + N treatments co-inoculated treatments. In the third cut, the co-inoculation treatment with strain SEMIA 135 resulted in the accumulation of 157.5% more N than that the NI + N; although not significant difference, it produced 8.8% more N than *S. meliloti* SEMIA 135. This shows the possible activity of PGPB in total N accumulation in plants. In the average of the three cuts, the co-inoculation treatment with *S. meliloti* SEMIA 135 resulted in the accumulation of 370 g kg^{-1} N per pot when compared to the NI +N treatment. Similar results were verified by [54], who found increases in total N accumulation in plants from the first to the third year, from 382 to 649 kg ha^{-1}; in our experiment, from the increases were from 229 to 417 g kg^{-1} per pot (Figure 5b).

The total N content of the roots (NCR) was significant for treatments (Figure 6a). The NCR ranged from 13.1 to 17.8 g kg^{-1}, and were lower in the NI and NI + N treatments. The values were also higher than the average of 11.3 g kg^{-1} obtained by [2]. Although not statistically different, co-inoculation with *S. meliloti* SEMIA 135 resulted in NCR 9.2% higher than that in single inoculation with SEMIA 135. The total N accumulation in roots showed significant differences for the treatments and varied from 43.5 to 312.3 g kg^{-1} per pot in treatments NI and co-inoculation with *S. meliloti* SEMIA 135, respectively (Figure 6b). The co-inoculation with *S. meliloti* SEMIA 135 accumulated 120 % more N than that in plants of NI + N treatment.

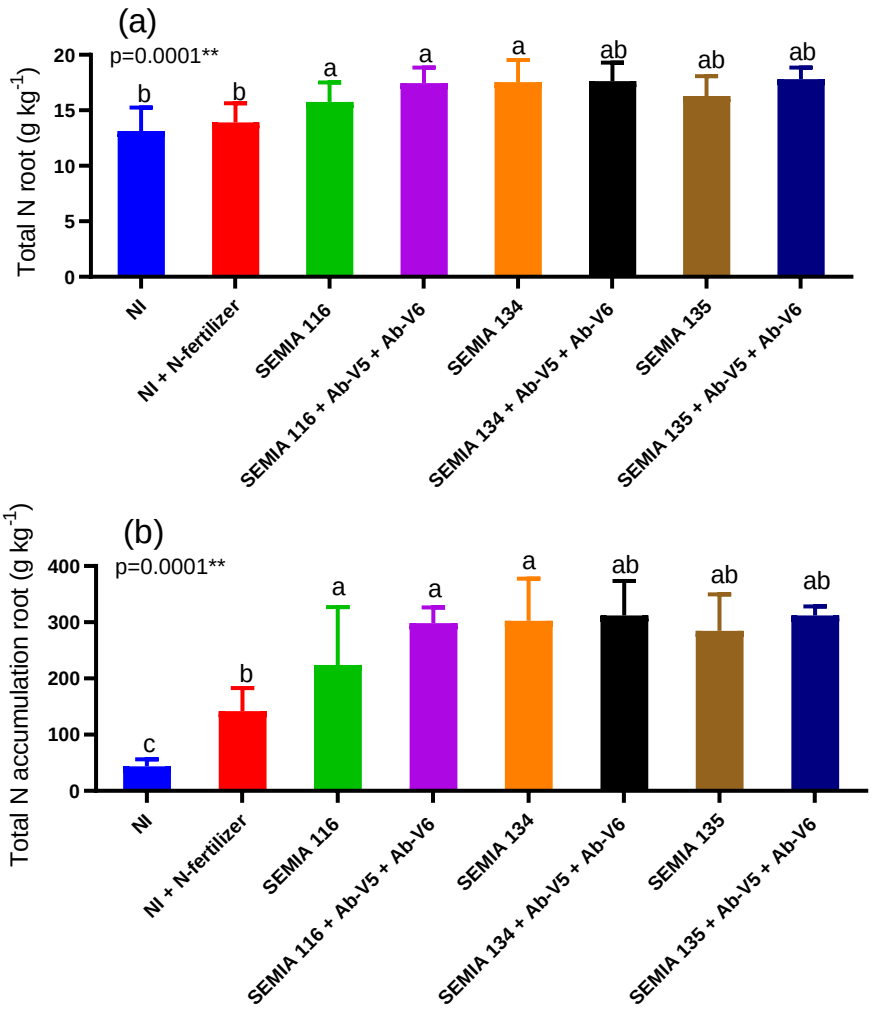

Figure 6. Total nitrogen content (g kg^{-1}) (a) and total N accumulation (g kg^{-1}) (b) in roots in alfalfa inoculated with *S. meliloti* and co-inoculated with *A. brasilense* (Ab-V5 + Ab-V6) of the three harvests. Error bars represent the standard errors of the means. Averages followed by the same lowercase letter for treatments do not differ by t test (LSD) (P≤0,05).

It is worth mentioning that, although without statistical difference, co-inoculation with *S. meliloti* SEMIA 135 and *A. brasilense* resulted in higher values than the single inoculation with SEMIA 135 for the following parameters: 8.1% in total N content of shoots; 9.8% in total N accumulation and 9.2% in NCR. According to [55] demonstrated an increase in the rhizobium population when grown with PGPB. According to [56], when describing the relationship between the plant growth-promoting *Burkholderia* sp. and *S. meliloti* PP3, found that in combination, plant growth was promoted, due to increased synthesis of indole-3-acetic acid and phosphate solubilization.

PGPB stimulate plant growth by facilitating the absorption of nutrients by the plant. It is suggested that the increase in mineral uptake by plants is owing to a general increase in root volume [57].

Nutritive value

Alfalfa nutritional properties varied with the rhizobial strains and the co-inoculation with *A. brasilense*

(Ab-V5 + Ab-V6) and the strains of *S. meliloti* used. The crude protein (CP) content showed significant interaction between treatments × cuts (Figure 7a). Except for the first cut in the NI control, this treatment, as well as the NI +N had the lowest levels of CP. The CP content was satisfactory and varied on average from 14.2- to 20.1%, and the co-inoculation treatment with *S. meliloti* SEMIA 135 resulted in 19.3% more CP than that in the NI + N treatment. According to [58] obtained similar results in the evaluation of nine alfalfa cultivars. As in several other parameters, co-inoculation resulted in 8.6% higher CP content then single inoculation with SEMIA 135, although not statistically different. According to [59] reported average levels of 18% to 25%, and these levels are close to that of the present study, which varied from 14% to 20%.

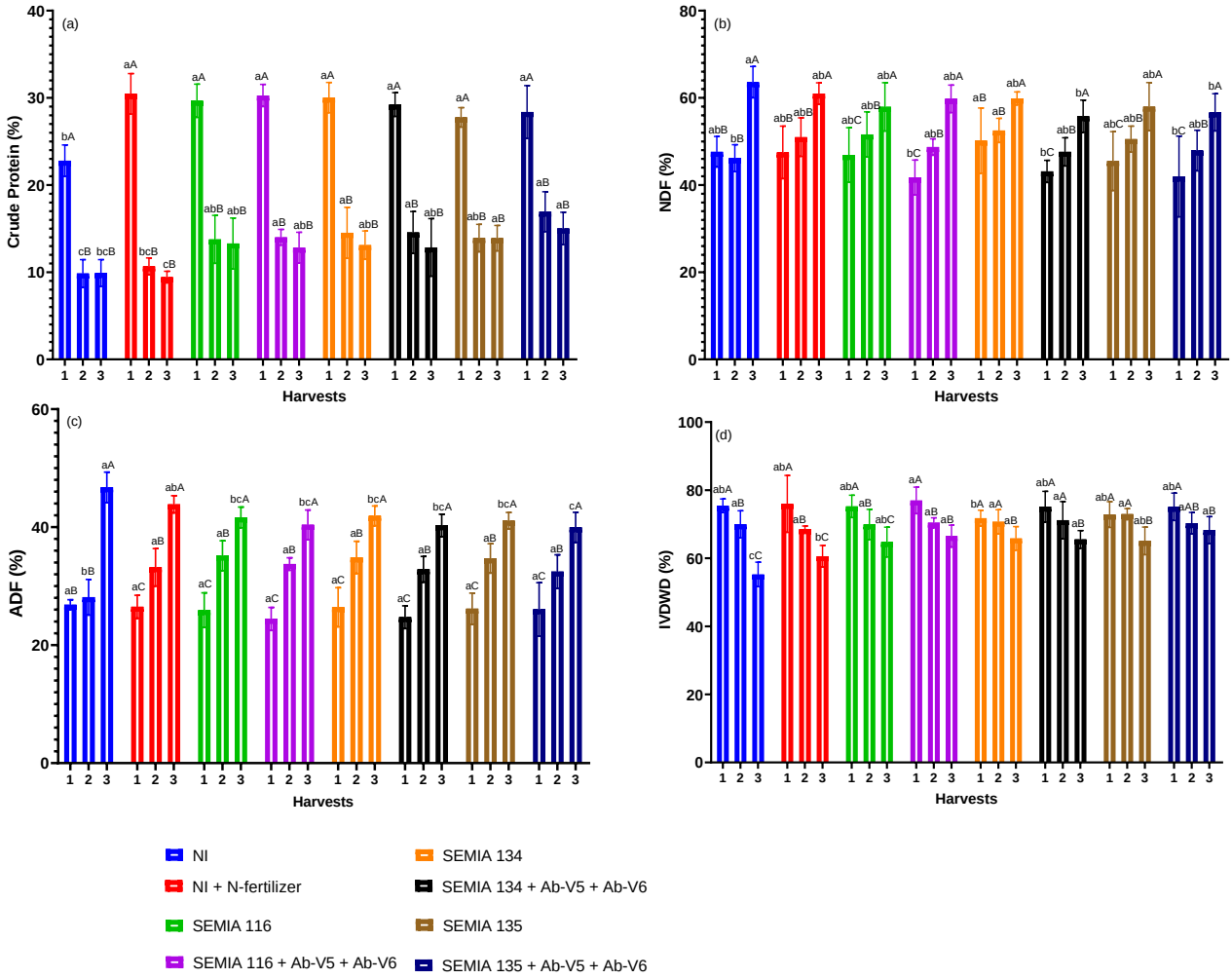

Figure 7. The crude protein (CP) (%) (a); neutral detergent fiber (NDF) (%) (b); acid detergent fiber (ADF) (%) (c) and in vitro digestibility dry weight (IVDWD) (%) (d) in shoots in alfalfa inoculated with *S. meliloti* and co-inoculated with *A. brasilense* (Ab-V5 + Ab-V6) of the three harvests. Error bars represent the standard errors of the means. Averages followed by the same lowercase letter for treatments and upper case for harvests do not differ by t test (LSD) (P≤0,05).

According to [60] evaluating the effect of different strains of *S. meliloti* found CP levels higher than those obtained in the present study, and only in the first cut the levels were higher than those reported by these authors. The effective application of strains of *S. meliloti* proved to be essential for increasing the CP

content by 19 to 42% when compared to the NI and NI + N treatments, respectively. The NDF showed significant differences for the treatments × cuts interaction (Figure 7b). In the mean of the three cuts, all co-inoculated treatments presented the lower NDF content, except in the third cut, with a good performance with the co-inoculation with SEMIA 116. The NDF content indicates the dry weight intake rate of alfalfa; the higher the value, the lower is the forage quality, thereby compromising the performance of animals [59]. The percentage of NDF makes up the cell wall structure, comprising mainly structural carbohydrates, which are of low availability in the digestion process [61]. Therefore, the reduction in NDF content implies an increase in the constituents of the cellular content, soluble carbohydrates, proteins, and lipids, which have greater availability.

The ADF showed significant differences for the treatment × cut interaction (Figure 7c). The ADF of the plants did not vary significantly between treatments in the first cut, whereas in the second cut, the value in the NI treatment was higher than that in the other treatments. In the third cut, the inoculated plants showed significantly lower ADF content than that in both non-inoculated controls. High ADF values indicate low energy production, i.e., the quality of forage is reduced [59]. The levels of ADF in the first two cuts were well below that of 24 to 35%, indicated as the maximum tolerated level, and in the third cut was higher (Figure 7c). According to [62] working with 'Crioula' reported values below 24% and [63] found average values of 30% ADF, which are similar to those obtained in the first cut and higher than those obtained in the other cuts. The ADF fraction represents the fibrous fraction of the food or the composition of the cell wall and is closely linked to digestibility as lignin is the main chemical component of the forage cell wall [61].

The IVDWD was significant for the treatments × cuts interaction (Figure 7d). In the first and second cuts, there were no differences between the treatments. In the third cut, the inoculated treatments were superior to the negative and positive controls, and the co-inoculation with SEMIA 135 showed 12.7% greater digestibility than that in the NI + N treatment. On average, the digestibility varied from 67 to 71%, staying within the value considered adequate by [64]. According to [62] obtained values from 68 to 71%, which are similar to those found in the present study. IVDWD depends on the cellulose and lignin content. As lignin is virtually indigestible, intense cell wall lignification in advanced stages of alfalfa growth tends to reduce the IVDWD coefficient. In the present experiment, treatments NI and NI + N in the third cut presented lower IVDWD than in other treatments, and high levels of NDF and ADF.

4. CONCLUSIONS

Application of N-fertilization increases the production cost, making alfalfa cultivation unviable. Inoculation with three strains of *Sinorhizobium meliloti* highly promoted alfalfa growth, considering several parameters, including plant height, relative chlorophyll index, number of tillers, shoot and root dry weight, nutritive value, and with an emphasis on root volume, and total N content and total N accumulated in shoots and roots. No further increases were observed with the co-inoculation with the PGPB *A. brasilense*. Studies in field and greenhouse conditions are necessary to verify the benefits of the use of PGPB in the cultivation of alfalfa.

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6. REFERENCES

[1] Borreani G., Tabacco E. The effect of a baler chopping system of fermentation and losses of wrapped big bales of alfalfa. *Agron. J*., **98**, 1-7. (2006).

[2] Moreira, A., Fageria, N. K. Liming influence on soil chemical properties, nutritional status and yield of alfalfa grown in acid soil. *Revista Brasileira de Ciência do Solo,* **34**:1231–1239. 2010.

[3] Rassini, J.B., Ferreira, R.P., Camargo, A.C. Alfalfa cultivation and establishment [Cultivo e estabelecimento da alfafa]. In *Alfalfa Cultivation and Use in the Tropics* [Cultivo e Utilização da Alfafa nos Trópicos], eds. R. P. Ferreira, J. B. Rassini, A. A. Rodrigues, A. R. Freitas, A. C. Camargo, and F. C. Mendonça. 39–51. São Carlos, Brazil: Embrapa Pecuária Sudeste. 2008.

[4] Moreira, A., *et al. Soil Fertility and Nutritional Status of Alfalfa Grown in the Tropics*. São Carlos, Brazil: Embrapa Pecuária Sudeste. 2007.

[5] Ormeño-Orrillo, E., Hungria, M., Martínez-Romero, E. Dinitrogen-fixing prokaryotes. In *The Prokayotes: Prokaryotic Physiology and Biochemistry*, eds. E. Rosemberg, E. F. De Long, S. Lory, E. Stackebrandt, and F. Thompson. 427–451. Berlin Heidelberg: Springer-Verlag. 2013.

[6] Moreira, A, Bernardi, A.C.C., Rassini, J.B. Soil correction, nutritional status and alfalfa fertilization [Correção do solo, estado nutricional e adubação da alfafa]. In *Alfalfa Cultivation and Use in the Tropics* [Cultivo e Utilização da Alfafa nos Trópicos], eds. R. P. Ferreira, J. B. Rassini, A. A. Rodrigues, A. R. Freitas, A. C. Camargo, and F. C. Mendonça. 97–137. São Carlos, Brazil: Embrapa Pecuária Sudeste. 2008.

[7] Hungria, M., Campo, R.J., Souza, E.M. & Pedrosa, F.O. Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. *Plant and Soil.* **331**(1/2): 413-425, (2010). https://doi.org/10.1007/s11104-009-0262-0

[8] Hungria, M., Nogueira, M.A. & Araujo, R.S. Co-inoculation of soybeans and common beans with rhizobia and *azospirilla*: strategies to improve sustainability. *Biology and Fertility of Soils* **49**(7):791-801, 2013.

[9] Hungria, M., Nogueira, M.A. & Araújo R.S. Inoculation of *Brachiaria* spp. with the plant grow-promoting bacterium *Azospirillum brasilense*: an environment-friendly component in the reclamation of

degraded pastures in the tropics. *Agriculture, Ecosystems and Environment,* **221**:125–131. 2016.

[10] Bashan, Y., De-Bashan, L.E. How the plant growth-promoting bacterium *Azospirillum* promotes plant growth–A critical assessment. *Advances in Agronomy,* **78**:77–136. 2010.

[11] Pereg, L., Luz, E. & Bashan, Y. Assessment of affinity and specificity of *Azospirillum* for plants. *Plant and Soil*, **399**:389-414. 2016.

[12] Fukami, J., Cerezini, P. & Hungria, M. *Azospirillum*: benefits that go far beyond biological nitrogen fixation. *AMB Express,* **8**(1):73. 2018.

[13] Dominguez-Nuñez, J.A., Muñoz, D., Planelles, R., Grau, J.M., Artero, F., Anriquez, A. & Albanesi, A. Inoculation with *Azospirillum brasilense* enhances the quality of mesquite *Prosopis juliflora* seedlings. *Forest System,* **21**:364–372. 2012.

[14] Vogel, G.F., Martinkoski, L. & Ruzicki, M. Efeitos da utilização de *Azospirillum brasilense* em poáceas forrageiras: importâncias e resultados. *ACSA Agropecuária Científica no Semi-Árido,* **10**:1–6. 2014.

[15] Leite, R.C., Santos, J.G.D., Silva, E.L., Alves, C.R.C.R., Hungria, M. & Santos, A.C. Productivity increase, reduction of nitrogen fertiliser use and drought-stress mitigation by inoculation of Marandu grass (*Urochloa brizantha*) with *Azospirillum brasilense. Crop and Pasture Science,* **70**(1):61-67. 2018.

[16] Leite, R.C., Santos, A.C., Santos, J.G., Leite, R.C., Oliveira, L.B.T., & Hungria, M. Mitigation of Mombasa Grass (*Megathyrsus maximus*) dependence on nitrogen fertilization as function of inoculation with *Azospirillum brasilense*. *Revista Brasileira de Ciência do Solo,* **43**:p.e0180234. 2019.

[17] Duarte, C.F.D., Cecato, U., Hungria, M., Fernandes, H.J., Biserra, T.T., Galbeiro, S., Toniato, A.K.B., & Silva, D.R. Morphogenetic and structural characteristics of *Urochloa* species under inoculation with plant-growth-promoting bacteria and nitrogen fertilisation. *Crop and Pasture Science,* **71**(1):82-89. 2020.

[18] MAPA (Ministério da Agricultura, Pecuária e Abastecimento). *Instrução Normativa nº. 13, de 24 de março de 2011*. Brasília, Brazil: MAPA. 2011.

[19] Hungria, M., Araujo, R.S. Relato da VI Reunião de laboratórios para recomendação de estirpes de *Rhizobium* e *Bradyrhizobium*. In: *Microbiologia do Solo: Desafios para o Século XXI*, eds. M. Hungria, E. L. Balota, A. Colozzi-Filho, and D. S. Andrade, 476-489. Londrina, Brazil: IAPAR/EMBRAPA-CNPSo. 1995.

[20] Ribeiro, R.A., Ormeño-Orrillo, E., Dall'agnol, R.F., Graham, P.H., Martínez-Romero, E., & Hungria, M. Novel *Rhizobium* lineages isolated from root nodules of common bean (*Phaseolus vulgaris* L.) in

Andean and Mesoamerican areas. *Research in Microbiology,* **164**:740-748. 2013.

[21] Klepa, M.S., Urquiaga, M.C.O., Somasegaran, P., Delamuta, J.R.M., Ribeiro, R.A., & Hungria, M. *Bradyrhizobium niftali* sp. nov., an effective nitrogen-fixing symbiont of partridge pea [*Chamaecrista fasciculata* (Michx.) Greene], a native caesalpinioid legume broadly distributed in USA. *International Journal of Systematic and Evolutionary Microbiology,* **69**:3448-3459. 2019.

[22] Delamuta, J.R.M., Menna, P., Ribeiro, R.A., & Hungria, M. Phylogenies of symbiotic genes of *Bradyrhizobium* symbionts of legumes of economic and environmental importance in Brazil support the definition of the new symbiovars pachyrhizi and sojae. *Systematic and Applied Microbiology,* **40**(5):254–265. 2017.

[23] Edgar, R.C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research,* **32**(5):1792–1797. 2004.

[24] Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution,* **30**(12):2725–2729. 2013.

[25] Felsenstein, J. Evolutionary trees from DNA sequences: a Maximum Likelihood approach. *Journal of Molecular Evolution,* **17**:368–376. 1981.

[26] Gouy, M., Guindon, S., & Gascuel, O. Sea view version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution,* **27**(2):221–224. 2010.

[27] Chibeba, A.M., Kyei-Boahen, S., Guimarães, M.F., Nogueira, M.A., & Hungria, M. Isolation, characterization and selection of indigenous *Bradyrhizobium* strains with outstanding symbiotic performance to increase soybean yields in Mozambique. *Agriculture, Ecosystems and Environment,* **246**:291-305. 2017.

[28] Sneath, P., Sokal, R. *Numerical Taxonomy: The Principles and Practice of Numerical Classification.* San Francisco, USA: W. H. Freeman & Co. 573 p. 1973.

[29] Jaccard, P. The distribution of flora in the alpine zone. *New Phytologist,* **11**(2):37–50. 1912.

[30] Hungria, M., O'Hara, G.W., Zilli, J.E., Araujo, R.S., Deaker, R., & Howieson, J. G. Isolation and growth of rhizobia. In: *Working with Rhizobia,* eds. J. G. Howieson, and M. J. Dilworth, 39-60. Canberra, Australia: Australian Center for International Agricultural Research (ACIAR). 2016.

[31] Fukami, J., Abrante, J.L.F., Del Cerro, P., Nogueira, M.A., Megías, M., Ollero, F.J., & Hungria, M.

Revealing different strategies of quorum sensing in *Azospirillum brasilense* strains Ab-V5 and Ab-V6. *Archives of Microbiology,* **200**(1):47–56. 2018.b

[32] Raij B. van, Andrade, J.C., Cantarella, H., & Quaggio, J.A. *Chemical Analysis for Fertility Evaluation of Soil Tropical* [Análise Química para Avaliação da Fertilidade de Solos Tropicais]. Campinas, Brazil: Instituto Agronômico de Campinas. 2001.

[33] Werner, J.C., Paulino, V.T., Cantarella, H., Andrade, N.O., & Quaggio, J.A. Forajes [Forrageiras] In *Fertilization and Liming Recommendation for the São Paulo State* [Recomendação de Adubação e Calagem para o Estado de São Paulo], eds. B. Raij, H Cantarella, J. A Quaggio, and A. M. Furlani. 245–258. Campinas, Brazil: Instituto Agronômico de Campinas. 1997.

[34] Allen, S.E., Terman, G.L., Clements, L.B., & Mikkelsen, R. *Greenhouse Techniques For Soil-Plant Fertilizer Research*. Muscle Shoals. USA: Tennessee Valley Authority, 1976.

[35] Hungria, M., Araujo, R.S. *Manual of Methods Employed in Agricultural Microbiology Studies* [Manual de Métodos Empregados em Estudos de Microbiologia Agrícola]. Brasília, Brazil: EMBRAPA-SPI. 1994.

[36] Goering, H.K.; Van Soest, P.J. Forage fiber analyses: apparatus, reagents, procedures, and some applications. Agricultural Research Service, US Department of Agriculture, (1970).

[37] Holden, L.A. Comparison of methods of in vitro dry matter digestibility for ten feeds. *Journal of dairy science*, **82**(8):1791-1794, (1999).

[38] Pimentel-Gomes, F., Garcia, C.H. Estatística aplicada a experimentos agronômicos e florestais: exposição com exemplos e orientações para uso de aplicativos. Piracicaba: FEALQ. (2002).

[39] Ribeiro, R.A., Barcellos, F.G., Thompson, F.L., & Hungria, M. Multilocus sequence analysis of Brazilian *Rhizobium* strains microsymbionts of common beans (*Phaseolus vulgaris*) reveals unexpected taxonomic diversity. *Research in Microbiology*, **160**(4)297-306. 2009.

[40] Menna, P., Pereira, A.A., Bangel, E.V., & Hungria, M. Rep-PCR of tropical rhizobia for strain fingerprinting, biodiversity appraisal and as a taxonomic and phylogenetic tool. *Symbiosis*, **48**(1-3):120-130. 2009.

[41] MAPA (Ministério da Agricultura, Pecuária e Abastecimento). *Instrução Normativa nº. 30, , de 12 de novembro de 2010*. Brasília, Brazil: MAPA. 2010.

[42] Biondi, E., Pilli, E., Giuntini, E., Roumiantseva, M.L., Andronov, E.E., Onichtchouk, O.P., Kurchak,

O.N., Simarov, B.V., Dzyubenko, N.I., Mengoni, A., & Bazzicalupo, M. Genetic relationship of *Sinorhizobium meliloti* and *Sinorhizobium medicae* strains isolated from Caucasian region. *FEMS Microbiology Letters,* **220**:207–213. 2003.

[43] Elboutahiri N., Thami-Alami, I., & Udupa, S.M. Phenotypic and genetic diversity in *Sinorhizobium meliloti* and *S. medicae* from drought and salt affected regions of Morocco. *BMC Microbiology*, **10**:15. 2010.

[44] Moreira, A., Moraes, L.A.C., & Fageria, N.K. Zinc and amino-acids on the yield and nutritional state of alfalfa grown in the tropical soil. *Journal of Plant Nutrition,* **38**:780–794. 2015.

[45] Nuernberg, N.J., Milan, P.A., & Silveira, C.A.M. *Alfalfa Production Manual* [Manual de Produção de Alfafa]. Florianópolis, Brazil: EMPASC. 1990.

[46] Itzigsohn, R., Kapulnik, Y., Okon, Y., & Dovrat, A. Physiological and morphological aspects of interactions between *Rhizobium meliloti* and alfalfa (*Medicago sativa*) in association with *Azospirillum brasilense*. *Canadian Journal of Microbiology,* **39**:610–615. 1993.

[47] Burdman, S., Volpin, H., Kigel, J., Kapulnik, Y., & Okon, Y. Promotion of nod gene inducers and nodulation in common bean (*Phaseolus vulgaris*) roots inoculated with *Azospirillum brasilense* Cd. *Applied and Environmental Microbiology,* **62**:3030–3033. 1996.

[48] Long, S.R. *Rhizobium*-legume nodulation: life together in the underground. *Cell,* **56**:203–214. 1989.

[49] Yahalom, E., Okon, Y., & Dovrat, A. *Azospirillum* effects on susceptibility to Rhizobium nodulation and on nitrogen fixation of several forge legumes. *Canadian Journal of Microbiology,* **33**:510–514. 1987.

[50] Niewiadomska, A., Swerdrzynska, D. Effect of the co-inoculation of lucerne (*Medicago sativa* L.) with *Sinorhyzobium meliloti* and *Herbaspirillum frisigense* in relation to the interations between bacterial strains. *Archives of Environmental Protection,* **37**(4):37–48. 2011.

[51] Xavier, D.F., Gomes, F.T., Lédo, F.J.S., & Pereira, A.V. Efficiency of rhizobia inoculants on nodulation of alfalfa in a "Cerrado" soil. *Revista Brasileira de Zootecnia,* **34**(3):781–785. 2005.

[52] Moreira, A., Malavolta, E., Moraes, L.A.C., & Heinrichs, R. Sources and Rates of Phosphorus on Nitrogen and Micronutrients Levels in Alfalfa and Centrosema. *Boletim de Indústria Animal,* **59**(2):157–165. 2002.

[53] Pietrzak, S. Estimation of nitrogen fixed symbiotically by legume plants. *Woda Środowisko Obszary Wiejskie,* **11**(3):197–207. 2011.

[54] Symanowicz, B., Skorupka, W. Effect of mineral fertilization on nitrogenase activity, yield, nitrogen content and uptake with alfalfa (*Medicago sativa* L.) yield. *Journal of Elementology,* **24**(1):181–191. 2019.

[55] Prasad, H., Chandra, R. Growth pattern of urdbean *Rhizobium* sp. with PSB and PGPR in consortia. *Journal of the Indian Society of Soil Science,* **51**:76–78. 2003.

[56] Pandey, P., Maheswari, D.K. Two-species microbial consortium for growth promotion of *Cajanus cajan*. *Current Science,* **92**:1137–1142. 2007.

[57] Biswas, J.C., Ladha, J.K., & Dazzo, F.B. Rhizobia inoculation improves nutrient uptake and growth of lowland rice. *Soil Science Society of America Journal,* **64**:1644–1650. 2000.

[58] Moreira, A., Evangelista, A.R., & Rodrigues, G.H.S. The alfalfa cultivars evaluation in the region of Lavras, Minas Gerais, Brazil. *Pesquisa Agropecuária Brasileira,* **31**(6):407–411. 1996.

[59] Gashaw, M. Review on biomass yield dynamics and nutritional quality of alfalfa (*Medicago sativa*). *Journal of Harmonized Research in Applied Science,* **3**(4):241–251. 2015.

[60] Delic, D., Stajkivic, O., Milieie, B., Kuzmanovic, D., Rasulue, N., Radovic, J., & Tomic, Z. Effects of diferente strains of *Sinorhizobium meliloti* on alfalfa (*Medicago sativa* L.) biomass yield. *Biotechnology in Animal Husbandry*, **23**(5-6):601-607, 2007.

[61] Silva, D.J., Queiroz, A.C. *Análise de alimentos: métodos químicos e biológicos*. 3.ed. Viçosa, MG: Universidade Federal de Viçosa, 235p. 2002.

[62] Bernardi, A.C.C., Cardoso, R.D., Mota, E.P., & Ferreira, R.P. Yield, nutritional status and quality of alfalfa under grazing and weed occurrence in response to liming, gypsum and potassium fertilization. *Boletim de Indústria Animal,* **70**(1):67–74. 2013.

[63] Monteiro, A.L.G., Costa, C., & Silveira, A.C. Dry matter production and seasonal distribution and chemical composition of alfalfa cultivates (*Medicago sativa* L.). *Revista Brasileira de Zootecnia,* **27**:868–874. 1998.

[64] Conrad, H.R., Pratt, A.D., & Hibbs, J.W. Regulation of feed intake in dairy cows. I. Change in importance of physical and physiological factors with increasing digestibility. *Journal of Dairy Science,* **47**:54–62. 1964.