AGRONOMY SCIENCE AND BIOTECHNOLOGY

RESEARCH ARTICLE

Selection of alfalfa genotypes for dry matter yield and persistence with repeated measures

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ABSTRACT

The biggest challenge in the alfalfa breeding program is to obtain cultivars with high persistence, high productivity, and adaptability. Therefore, studies about selection methods are necessary for the success of alfalfa breeding programs. This study aimed to evaluate dry matter yield and persistence in alfalfa for selecting genotypes, using appropriate statistical models for experiments with repeated measures. The experiment was conducted at Embrapa Southeast Livestock, in São Carlos, state of São Paulo, Brazil in a randomized blocks design, in plots subdivided in time, with three replicates. Eight genotypes were evaluated, and the agronomic trait evaluated was dry matter yield. The experiments in split-plots were used with two and three errors and generalized linear models with the following correlation structures: composite symmetry (CS), heterogeneous composite symmetry (HCS), auto regressive (AR), heterogeneous auto regressive (HAR), and variance components (VC). The best model was selected according to the lowest value of the Akaike Information Criterion (AIC), and three methodologies were used to identify the genotype with greater productivity and persistence: Average test for multiple comparisons, adaptability, and stability by multi-information, and similarity between genotype and ideotype. The interaction between genotypes and cuts was significant, demonstrating the existence of the different behavior of the alfalfa genotypes over the cuts. Different methodologies allowed to measure the average yield of the alfalfa genotype and the persistence over the cuts. PSB 4 genotype demonstrated promissory behavior in terms of productivity and persistence throughout the production cycle of alfalfa.

Keywords: Biometrics, information summary, structures of variance, evaluation of models, stand persistence, *Medicago sativa* L.

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INTRODUCTION

Alfalfa (*Medicago sativa* L.) is one of the oldest forage species and the most globally cultivated perennial forage legume (Annicchiarico, Barrett, Brummer, Julier, & Marshall., 2015), owing to its high productivity, protein content, palatability, digestibility, and nitrogen fixation capacity (Herrmann et al., 2018; Annicchiarico et al., 2015), which allow the culture to be used in the feeding of dairy herds, with excellent results in milk production.

The cultivation of alfalfa is still considered incipient in Brazil, and it is mainly carried out in the South region of the country (Santos et al., 2020; Assis et al., 2010). However, interest in this forage has grown rapidly and the expansion of the cultivation area in the country depends on the development of cultivars adapted to the tropical environment. Alfafa breeding programs are essential for the development of alfalfa cultivars adapted to different regions of the country and the achievement of genotypes with high quality and high productivity in intensive milk production systems. Long-term stand persistence is important for the economical production of alfalfa since it reduces the costs of seeding over a longer period.

Persistence is a complex trait affected by many components, including grazing, mechanical harvesting equipment, the intensity of harvest management, diseases and pests, weather, and inter and intraspecies plant competition. Thus, one of the challenges of the alfalfa breeding programs is to obtain cultivars with high persistence, high productivity, and better adaptability (Tucak et al., 2014), which will provide more cuts over the years and a higher dry matter yield per cut.

Agricultural experiments with alfalfa are most frequently arranged in experimental randomized block designs, in which the genotypes are allocated in the plots, and the cuts are allocated in the subplots. As the cut time cannot be randomly allocated to the subplots, the appropriate analysis for this situation is that of repeated measures.

In view of the above, this study aimed to evaluate dry matter yield and persistence in alfalfa for the selection of genotypes, using appropriate statistical models for experiments with repeated measures.

MATERIAL AND METHODS

Field experiments

The experiment was conducted in the experimental field of Embrapa Pecuária Sudeste, in São Carlos, state of São Paulo (22° 01'10” S, 47° 53'38” W). It was arranged in a randomized complete block design, in a parcel subdivided in time, with three replications. Eight genotypes were evaluated, namely, Mecha, Ruano, Barbára SP Inta, Prointa Patrícia, PSB4, PSB5, P5715, and Crioula.

The experimental unit was four meters long, with 0.2 m spacing between rows. The useful area of the plot to obtain the data was the two central rows, and 0.5 m from each end of the row was eliminated. Planting fertilization was carried out by scattering, and triple superphosphate, potassium chloride and FTE BR-12 were applied as sources of P, K, and micronutrients, respectively, following the chemical analysis of the soil.

Soil tillage consisted of plowing and two passes with a disk harrow. Lime and fertilizers were broadcast on the planting area, according to soil chemical analysis. Triple superphosphate was applied as a P source, potassium chloride as a K source, and FTE BR-12 as a micronutrient source. Seeds were inoculated with strains of

Rhizobium *melilotti* - SEMIA 116. After each harvest, the plants received cover fertilization according to soil analysis. The alfalfa was irrigated, except for harvests 8, 9, 10, 19, 20, and 21, for which irrigation was suspended. Irrigation was performed by a central pivot, with management based on the difference between evaporation and rainfall. Weeds were controlled by a single application of Pivot, at a dosage of 1 L ha^{-1}, and Fusilade, at a dosage of 1.5 L ha^{-1} (Brighenti & Castro 2008).

The agronomic trait dry matter yield (DMY, kg ha^{-1}), obtained by the manual cutting of the plants at eight to10 cm above the ground, when each cultivar reached the flowering stage, was evaluated over 30 cuts, for long-term stand persistence.

Statistical analysis

Split-plot with two errors (Error A and Error B)

Experiments in split-plots are used when it is desired to study the effect of two factors simultaneously. In these experiments, the plots are divided into equal parts, called subplots, and can be distributed following a statistical design, such as randomized block designs.

According to the structure of the subplots, two types of experiments can be distinguished in subdivided plots: Split-plot into space (there is a subdivision in each plot, called a subplot), and split-plot over time (plots are not subdivided into subareas, but data are obtained over time, forming subplots). Therefore, the analysis of variance was carried out considering the experiment in the split-plot over time. The plots were represented by the genotypes, and the subplots, by the cuts. The following statistical model was used:

$$Y_{ijk} = \mu + g_i + b_j + \delta_{ij} + t_k + \varepsilon_{ijk}$$

where: Y_{ijk} is the observation of DMY for the ith genotype, on the jth block and kth cut; μ is the general mean effect; $g_i$is the ith genotype effect; b_j is the jth block effect; δ_{ij} is the experimental error associated with plot ij; t_k is the experimental error associated with the kth cut; it is the effect of ith genotype and kth cut interaction; ε_{ijk} is the experimental error for the ith genotype, on jth block and kth cut.

Split-plot with three errors (Error A, Error B and Error C)

Steel & Torrie (1982) presented an alternative model for an experiment in split-plot, considering three errors:

$$Y_{ijk} = \mu + g_i + b_j + \delta_{ij} + \tau_k + \theta_{jk} + (g\tau)_{ik} + \varepsilon_{ijk}$$

where: Y_{ijk} is the observation of DMY for the ith genotype, on the jth block and kth cut; μ is the general mean effect; $g_i$is the ith genotype effect; b_j is the jth block effect; δ_{ij} is the experimental error associated with plot ij; t_k is the experimental error associated with kth cut; $\theta_{jk}$is the experimental error associated with the ith genotype and kth cut; $(g\tau)_{ik}$ it is the effect of ith genotype and kth cut interaction; ε_{ijk} is the experimental error for the ith genotype, on jth block and kth cut.

Models with different structures of variance and covariance

The schemes in split-plot in time, it is assumed that the residual variances are homogeneous in the different times and null covariance between any two times, that is, ε_{ijk} errors are independent, identically distributed and, with approximate normal distribution. Thus, V(e) = R = σ^2I, where σ^2 is the residual variance estimate and I is the n-order identity matrix. If these assumptions are met, ANOVA can be performed according to the split-plot model with two errors.

For the use of the F test to be correct, providing an exact type I error for testing all hypotheses, the ε_{ijk} errors must meet the assumptions. Otherwise, the chance of type I error increases, as the F values to test these effects are underestimated and there is a tendency not to reject the null hypothesis and consequently conclude that the effects are insignificant (Freitas et al., 2011).

In experiments with repeated measures, observations in the same experimental unit are usually correlated. Ignoring the covariance structure can affect tests and estimates of fixed effects (Littell et al., 2000; Hoffmann, 2016). The package *nlme* (Pinheiro et al., 2020) of the R software (R CORE TEAM, 2019) has the function *gls,* which adjusts a linear model using generalized least squares. The errors can be correlated and/or have uneven variances, so the function allows incorporating different correlation structures. Thus, generalized linear models were evaluated with the following correlation structures: composite symmetry (CS), heterogeneous composite symmetry (HCS), auto-regressive (AR), heterogeneous auto-regressive (HAR) and, variance components (VC).

Evaluation of models

The best model was selected according to the lowest value of the Akaike Information Criterion (AIC) (Akaike, 1974), which was used to verify the effects of the interaction between the factors. Then, three methodologies were used to identify the genotype with greater productivity and persistence: Average test for multiple comparisons, adaptability and stability by multi-information and, similarity between genotype and ideotype.

Average test for multiple comparisons

The procedure of comparing averages was performed using the Tukey test ($P < 0.05$), with the specific residual variance value for each cut. Analyzes of variance (ANOVA's) were performed individually and after verifying that the ratio between the largest and smallest mean square of the residue of the cuts did not exceed the ratio 7:1 (Pimentel Gomes, 2009).

The genotypes were ranked over the 30 cuts, and the number of times it was classified in the high productivity group was indicated by the letter A. This classification aims to quantify the persistence of each genotype to assist in the selection.

Adaptability and stability study by multi-information (Silva Junior et al., 2022)

The estimates of the following parameters were considered for adaptability and stability analysis by multi-information:

Average potential: Expresses the productive capacity of the genotype and particularizes the cuts (environmental variations) in general, as favorable, or unfavorable.

Plasticity: Refers to the ability of the genotype to alter its physiology or morphology according to the exposed cuts.

Measurement of relative contribution to the interaction: It is a measure that quantifies the contribution of genotype x cut interaction.

Recommendation index associated with the i^{th} genotype: This index has used the concept of the genotype of the greatest productive potential, in terms of average, and less environmental variability (cuts). The recommendation index estimate is based on the methodology of Annicchiarico (1992). The procedures for the calculations were initially performed with the transformation of the averages of each cultivar in each cut since the percentage of the cut average is the standard deviation and the average of the percentages of each cultivar is estimated later.

Adaptability or responsiveness of genotype i: It is a measure of the genotypic ability to respond to improvements in the environment. The adaptability estimate is obtained by regression coefficients (β_{1i}), which are the linear response of genotype i to environmental variation, obtained from the following model proposed by Finlay and Wilkinson (1963) and Eberhart and Russell (1966).

Stability or predictability: It is a measure of the predictability of genotype i behavior in response to environmental variations, considering a linear regression model, as described by Eberhart and Russell (1966). The stability parameter (σ^2_{di}) is estimated by the analysis of variance method from the mean square of the regression deviation of each genotype (QMD_i) and the mean square of the residue. Alternatively, predictability can be measured through the model determination coefficient that measures the proportion of total variation explained by the linear behavior of the genotype.

Champion pattern: The best genotype is superior to all others in all cuts. Most of the time, this genotype does not exist or is not present in the experiment, but it is possible to quantify the distance of the evaluated genotypes to this hypothetical one, called the champion pattern.

Recommendation Index: This is a measure that makes it possible to characterize each genotype by its proximity to the genotypes considered to be patterned.

After obtaining each of the parameters described above, they were organized in a table containing the most varied information resulting from different adaptability and stability study proposals, which, together, reveal important characteristics of each cultivar for its recommendation. This allows the simultaneous analysis of the indices characterizing the multi-information analysis.

Similarity between genotype and ideotype

Information on the performance of the genotypes in each block and cut was used to define the ideotype, and the highest DMY value was attributed to the ideotype. For the study on the similarity between the genotypes and the ideotype, the information from the 30 sections was summarized in two components obtained through dimensionality reduction via main components. The Euclidean distance between the ideotype and the genotypes was used as a measure of similarity, and the smallest Euclidean distance is indicative of greater similarity. The GENES software (Cruz, 2016) was used to perform the analyses.

RESULTS AND DISCUSSION

The dry matter yield (DMY) of the alfalfa exhibited high and low production peaks throughout the productive period and cuts (Figure 1). This oscillation is a characteristic frequently observed in perennial pasture species (Faveri, 2015;

Edwards & Chapman, 2011; Freitas et al., 2011). The oscillation pattern occurs due to alfalfa biological aspect, added to its plasticity under different environmental conditions.

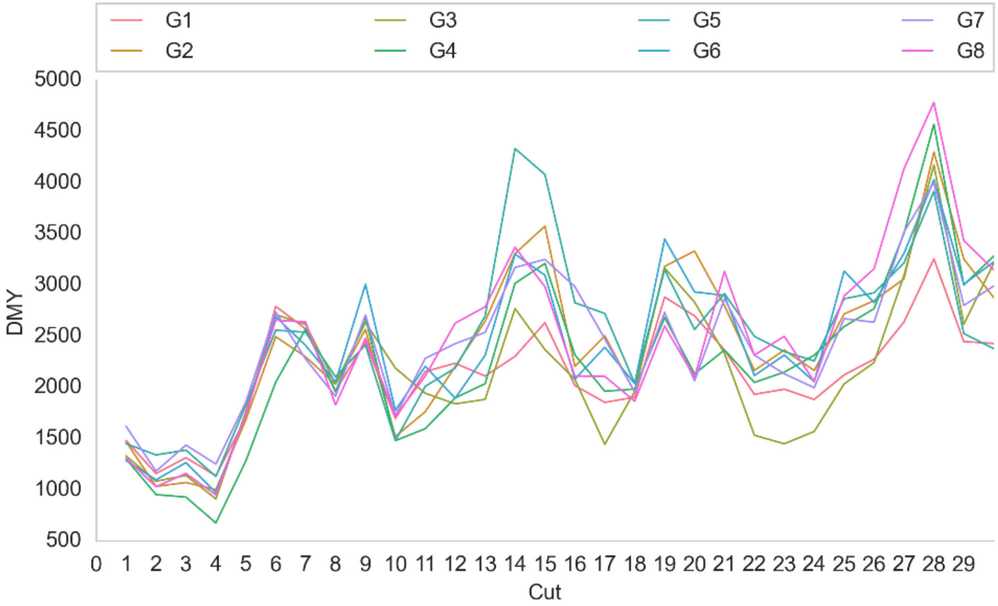

Figure 1. Dry matter yield (DMY) in eight alfalfa genotypes G1 (Mecha), G2 (Ruano), G3 (Barbára SP), G4 (Prointa Patrícia), G5 (PSB4), G6 (PSB5), G7 (Crioula) and, G8 (P5715) throughout the productive period with 30 cuts.

The analysis of variance for the split-plot model, considering two and three errors (error A and error B) and three errors (error A, error B and error C), is shown in Tables 1 and 2, respectively. It was possible to observe that the model with three error structures (A, B and C) is more adequate, due to the AIC estimate being smaller in relation to the structure of two errors. Estimates of genetic parameters in perennial species differ from annual ones. This is because the influence of production cycles is removed using models through appropriate statistical modeling (Cecon et al., 2008).

Table 1. Analysis of variance for dry matter yield (DMY), considering the split-plot model with errors A and B.

Variation Source	DF	Mean Square	F-test	p-value
Genotype	7	1707809	1.52^{ns}	0.24
Block	2	1906123		
Error A	14	1127274		
Cut	29	11633304	53.16**	0
Genotype x cut	203	253243	1.15^{ns}	0.11
Error B	464	218823		

** and ns: significant at 1% and not significant by the test of F, respectively; Model AIC: 11094.06; DF: Degree of freedom.

Table 2. Analysis of variance for dry matter yield (DMY), considering the split-plot model with errors A, B and C.

Variation Source	DF	Mean Square	F- test	p-value
Genotype	7	1707809	1.51 ^{ns}	0.24
Block	2	1906123		
Error A	14	1127274		
Cut	29	11633304	23.58**	0
Error B	58	493325		
Genotype x cut	203	253243	1.41**	0.002
Error C	406	179608		

** and ns: significant at 1% and not significant by the test of F, respectively; Model AIC: 10971.73; DF: Degree of freedom.

In experiments with repeated measures, it is appropriate to evaluate the behavior of individuals using models that take dependence over time into account. These measurements are performed in the same experimental unit and on more than one occasion (Xavier & Dias, 2001). When two models assume that the residuals are independent, experiments organized in repeated measures schemes tend to generate dependency. The experimental units used are the same for the factor levels evaluated over time. In this scenario, it is possible to choose models that capture the autocorrelation and that allow the addition of different correlation structures (Keselman et al., 1998; Hoffmann, 2016). Therefore, our result shows that the AIC estimates were better when compared with different error structures (Table 3). When evaluating different error structures, the lowest AIC estimate was the heterogeneous autoregressive AR1H, which indicates that this structure is the one that best fits data from repeated measures over time, which in our case for the alfalfa crop.

Table 3. Comparative values of AIC obtained from models with different error structures considering CS: composite symmetry; CSH: heterogeneous composite symmetry; AR1: auto regressive; ARH1: heterogeneous auto regressive; CV: components of variance.

VC	CS	CSH	AR1	AR1H
8048.38	8018.48	7856.03	7880.72	7737.36

Table 4 shows the result of the model with the AR1H error structure for dry matter production of eight alfalfa genotypes evaluated in 30 cuts. In this Table, it was possible to observe dependence between the cuts. When carrying out traditional analysis of variance with two errors, it can lead to wrong conclusions about the estimates, especially when we consider the genotype x cut interaction. This is since it presents dependence between the cuts, which impacts on the non-homogeneity of the errors. The model that does not consider an adequate covariance structure can provide false estimates. Therefore, the choice of the most promising materials will be demonstrated below using different approaches that helped in the choice of genotypes with high persistence and productivity. These approaches consist of the frequency of high-performance classes, multi-information analysis and similarity between genotype and ideotype.

Table 4. Analysis considering the AR1H error structure for dry matter yield (DMY) of eight alfalfa genotypes evaluated over the 30 cuts.

FV	DF	F-value	p-value
Block	2	0.231	0.7936
Genotype	7	1.344	0.2275
Cut	29	60.577	< 0001
Genotype *x* Cut	203	1.268	0.0201

DF: Degree of freedom.

High performance class frequency

The PSB 4 genotype stood out for having superior average performance during 28 cuts according to the quantification of the number class A (NCA) by the average test (Table 5).

Table 5. Dry matter yield (DMY, Kg ha^{-1}) of the genotypes and number of classes A (NCA) by the Tukey (with $\alpha = 0.05$) test during 30 cuts.

Genotypes	DMY	NCA
Mecha (G1)	2162.82	22
Ruano (G2)	2419.28	23
Barbára SP Inta (G3)	2175.51	15
Prointa Patrícia (G4)	2242.69	17
PSB 4 (G5)	2543.69	**28**
PSB 5 (G6)	2382.47	24
Crioula T (G7)	2399.17	27
P 5715 (G8)	2433.03	25

Table 6 shows the performance of genotypes between cuts. It demonstrates the environmental index and the classification of the cuts estimated as favorable and unfavorable during the multi-cut. According to this Table, the environmental index was negative for the first five cuts. This justifies a limitation in expressing the productive potential in the face of environmental adversities. On the other hand, the last six cuts showed a positive environmental index. Which presents better performance of genotypes with high productivity. Regarding the intermediate cuts, there was oscillation with alternation of positive and negative environmental indices (Table 6).

Table 6. Average dry matter yield (DMY, kg ha^{-1}), residual variance (RV), environmental index (Ei) for classification regarding favorable or unfavorable environment and coefficient of variation experimental (CV%).

Cuts	Average	RV	Ei	CV(%)	Cuts	Average	RV	Ei	CV(%)
1	1406.46	14328.09	-938.373	8.51	16	2328.67	71716.84	-161.644	11.50
2	1110.42	11427.61	-1234.42	9.63	17	2183.54	239316.64	-161.292	22.40
3	1213.42	7191.04	-1131.42	6.99	18	1963.33	6544.81	-381.498	4.12
4	1004.54	15550.09	-1340.29	12.41	19	2981.25	130032.36	6.364.168	12.10
5	1698.79	30276.00	-646.041	10.24	20	2583.33	87734.44	2.385.006	11.47
6	2587.42	29756.25	2.425.856	6.67	21	2693.75	115532.01	3.489.181	12.62
7	2491.17	21933.61	1.463.356	5.95	22	2116.67	99666.49	-228.164	14.92
8	1986.50	28257.61	-358.332	8.46	23	2156.25	102336.01	-188.582	14.84
9	2618.96	82656.25	2.741.256	10.98	24	2037.42	32869.69	-307.416	8.90
10	1698.79	38455.21	-646.039	11.54	25	2630.42	198203.04	2.855.843	16.93
11	2009.46	30765.16	-335.372	8.73	26	2711.46	81967.69	3.666.256	10.56
12	2165.21	79073.44	-179.624	12.99	27	3310.42	111622.81	9.655.856	10.09
13	2377.54	77061.76	327.093	11.68	28	4129.58	112896.00	1.784.752	8.14
14	3197.83	157291.56	8.530.018	12.40	29	2886.96	95542.81	5.421.256	10.71
15	3150.42	265843.36	8.055.856	16.37	30	2915.00	147917.16	5.701.681	13.19

Cut 28 was considered the best performance, since it had the highest environmental index and the highest average DMI. This fact can also be observed in Figure 1. In this cut, it obtained a high productivity for all genotypes. The results of the classification of favorable and unfavorable environments are also shown in Figure 2, where the axes are represented by two main components. A pattern was observed between cycles of favorable and unfavorable environments, in which there is a greater cycle of unfavorable environments in the first years of cutting and a greater cycle of favorable conditions after the 25th cut. Kallenbach et al. (2002) identified that in the initial cuts, all genotypes showed low persistence. This can be explained by the characteristics of the alfalfa culture.

Alfalfa productivity and persistence are directly related to management. The regrowth of the plant occurs due to carbohydrate reserves in the roots and the canopy of the plant. This reserve is accumulated during the forage growth period (Rodriguez & Eroles, 2008). The canopy is a storage structure for reserve substances and where new plant shoots will emerge. Over time, the canopy increases, providing greater accumulation of carbohydrate reserves in the roots and in the canopy, consequently resulting in greater productivity and persistence.

Adaptability and stability study by multi-information (Silva Junior et al., 2022)

The simultaneous use of some methodologies, using multi-information criteria for cultivar recommendation, can extract information that cannot be observed using each methodology separately. However, it is important to note that this information is appropriate for annual crops. In this study, we used an analogous strategy to measure persistence. This analysis strategy of the multi-information technique that contains the information resulting from different proposals for studying the potential of the genotype and its persistence is shown in Table 7.

For each parameter, we have a reference estimate that corresponds to the maximum and minimum value for the genotype, making it possible to obtain ranking and greater credibility of both genotypes, according to each studied parameter. According to the description of the average potential for favorable, unfavorable, and general environments, the PSB 4 genotype was the best classified.

As for the recommendation index for favorable and unfavorable environment, it ranked second (Table 7). Thus, this genotype is associated with high productivity and persistence and performs well in both favorable and unfavorable environments (cycles). For the standard champion parameter, PSB4 ranked first for favorable and general environment and second for unfavorable environment. For this methodology, the genotypes with the best performance were Ruano and PSB 4, according to analyzes by Lin & Binns (1988). In terms of plasticity and interaction contribution, the PSB 4 genotype ranked sixth and fifth, respectively (Table 7). Silva Junior et al. (2022) demonstrated that multi-information estimation is an efficient tool for adaptability and stability studies. These authors argue that the challenge for breeders is to know which methodologies for assessing adaptability and stability should be used to recommend a particular cultivar for a specific or broad region.

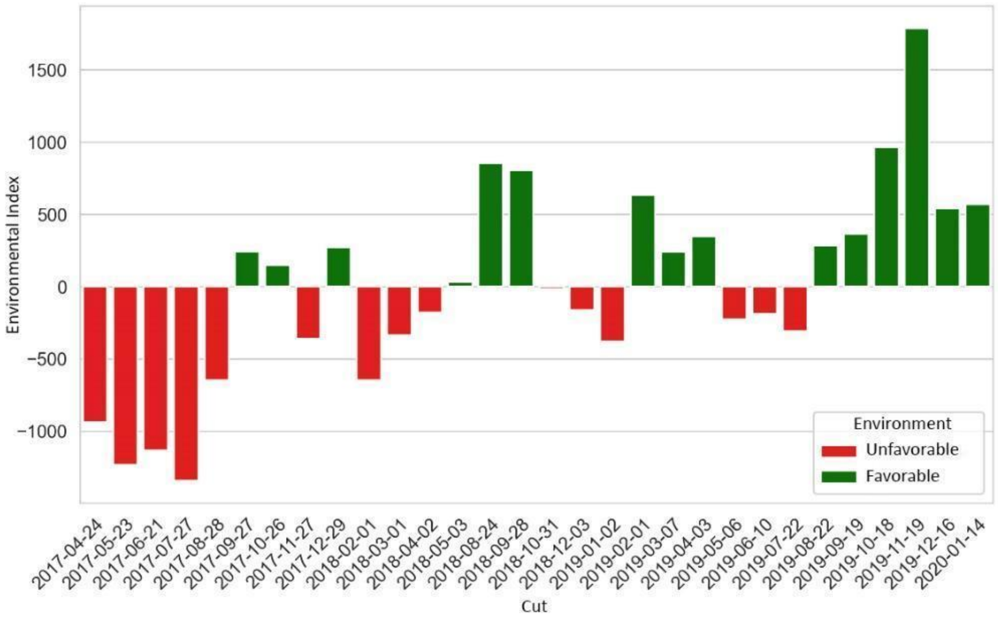

Figure 2. Average dry matter yield (DMY) and classification of environments as favorable and unfavorable according to the environmental index of the cuts

The similarity between the genotype and ideotype methods can provide an increase in genetic gain, characterizing alfalfa genotypes with desirable productive, morphological and chemical characteristics (Vasconcelos et al., 2010). Furthermore, it allows the indication of a single genotype, as well as the use of multiple ideotypes, according to the researcher's interest, as shown by Nascimento et al. (2015). The present study presented three different concordant proposals for the same result. The results were satisfactory, and the methodologies can help alfalfa breeders to identify superior genotypes for productivity and persistence throughout alfalfa.

Table 7. Genotypic recommendation form based on multi-information analysis for the PSB 4 genotype.

		Genotype PSB 4		
Description		**Value**	**Rank**	**Reference**
Average potential	General Environment	2543.7	1	(2162.8; 2543.7) $\mu = 2344.8$
	Favorable Environment	3112.3	1	(2563.6; 3112.3) μ =2344.8
	Unfavorable Environment	1975.1	1	(1618.7; 1975.1) μ =1805.3
Plasticity (QMG/A)		1932268	6	(806637.6; 2220994.0)
Measurement of relative contribution to the interaction	S^2GxA (%)	13.69	5	(9.8; 15.7)
	SQGxA(%)	14.49	5	(7.9; 17.8)
Recommendation index	General Environment (%)	94.62	1	(74.8; 94.6)
	Favorable Environment (%)	96.81	2	(70.4; 97.3)
	Unfavorable Environment (%)	92.18	2	(75.4; 93.0)
Adaptability or responsiveness of genotype (%)		1.08*	-	(0.7; 1.2)
Stability (%)		86.89**	-	(80.3; 93.1)
Champion pattern	General Environment	62408	1	(62408.9; 316302.8)
	Favorable Environment	95966	1	(95966.1; 532088.6)
	Unfavorable Environment	28851	2	(21868.8; 205224.2)
Recommendation Index	1 Centroid		I	
	7 Centroid		VII	

Similarity between genotype and ideotype

The P5715 (8) and PSB4 (5) genotypes were the ones that presented the greatest similarities with the ideotypes (Figure 3).

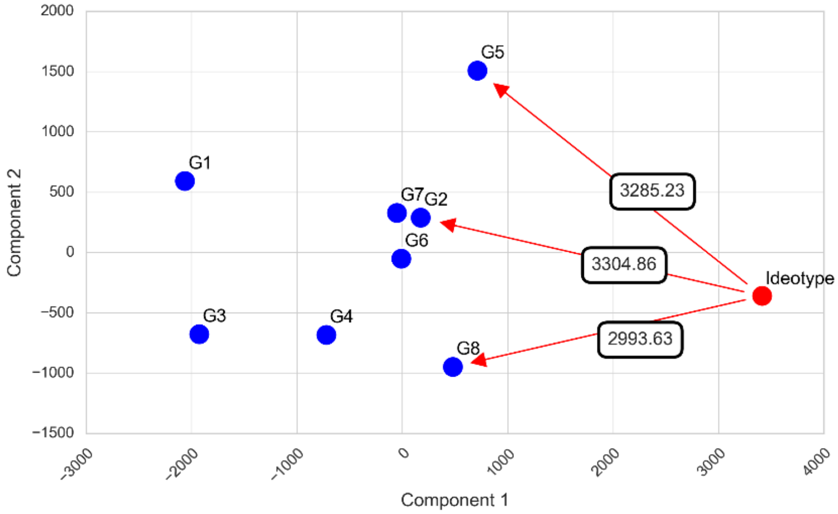

Figure 3. Similarity between the ideotype and the evaluated genotypes G1 (Mecha), G2 (Ruano),G3 (Barbára SP), G4 (Prointa Patrícia), G5 (PSB4), G6 (PSB5), G7 (Crioula) and G8 (P5715).

CONCLUSIONS

The interaction between genotypes and cuts was significant, demonstrating the existence of the different behavior of the alfalfa genotypes over the cuts.

Different methodologies allowed to measure the average yield of the alfalfa genotype and the persistence over the cuts.

The PSB 4 genotype demonstrated promissory behavior in terms of productivity and persistence throughout the production cycle of alfalfa

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