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Agronomic performance of interspecific *Paspalum* hybrids under nitrogen fertilization or mixed with legumes

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Abstract

Nitrogen supply and mixtures with legumes affect agronomic performance of pastures, and both practices can guide breeding decisions in *Paspalum* hybrids. The goals of this study were: (a) quantify herbage accumulation (HA), leaf accumulation (LA), cold tolerance, and N use efficiency (NUE) in *P. plicatulum* × *P. guenoarum* hybrids subjected to N fertilization or grown in a mixture with legumes; (b) compare the grass–legume system to a grass–N fertilizer system; and (c) select the best hybrid for future cultivar releases. A randomized complete block design with three replications and a split-plot treatment arrangement was used for 2 yr, with five N rates (0, 60, 120, 240, and 480 kg N ha^{-1}) and a grass–legume mixture [grass + white clover (*Trifolium repens* L.) + birdsfoot trefoil (*Lotus corniculatus* L.)] as whole plots, and six genotypes as subplots (hybrids: 1020133, 102069, 103084, 103061; and controls: *P. guenoarum* 'Azulão' and *Megathyrsus maximus* 'Aruana'). Higher N rates increased HA, LA, and cold tolerance. Higher NUE was obtained between 60 and 120 kg N ha^{-1}. In the grass–legume mixture HA was similar to the rates of 60 and 120 kg N ha^{-1}. Hybrid 1020133 had HA similar to the controls, LA greater than Aruana, and greater cold tolerance and NUE at 60 kg N ha^{-1} than Azulão and Aruana. Hybrid 1020133 should be selected for further animal performance studies. The agronomic performance of perennial pastures can be improved through N management, and NUE should be a selection criterion in forage breeding.

1 | INTRODUCTION

Livestock production depends largely on grasslands, which are the main feed source in tropical and subtropical regions (Pontes et al., 2016). Soil fertility is an important factor influencing production and longevity of perennial pastures. Thus, if nutrient deficiency is a limiting production factor in soils, the application of synthetic N fertilizer or the association between grasses and legumes may increase productivity and persistence in perennial grass pastures. Selecting improved grass hybrids with enhanced N use efficiency (NUE) would

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Abbreviations: DM, dry matter; HA, herbage accumulation; LA, leaf accumulation; NUE, nitrogen use efficiency.

warrant a long-term solution to limiting N conditions in tropical soils.

Nitrogen is an important nutrient for plant growth, being a constituent of proteins that actively participate in the synthesis of organic compounds, which make up the structure of the plant (Costa et al., 2013; Lavres et al., 2004). Several studies reported the positive effect of N fertilizer on forage yield of different perennial warm-season grasses (Machado et al., 2019; Pontes et al., 2016; Seepaul, Macoon, Reddy, & Evans, 2016). However, prices for synthetic N sources have increased over the past several years and these higher prices may reduce profitability in forage/livestock systems (Interrante, Biermacher, Kering, & Butler, 2012). Industrial production of N fertilizer uses non-renewable reserves of fossil fuels. Moreover, N losses can have negative impacts on the environment, as it is associated with global warming effects and contamination of groundwater and surface water (Schröder, 2014).

Considering the high N costs, an alternative approach for increasing production without additional N inputs and attendant environmental impacts is desirable. Grass–legume mixtures can be considered a viable alternative to synthetic N fertilizer application to grass monocultures (Adjesiwor, Islam, Zheljazkov, Ritten, & Garcia, 2017; Cox et al., 2017). The N input in a grass–legume mixture comes from the symbiotic legume–bacteria biological N fixation (Carlsson & Huss-Danell, 2003). Therefore, forage legumes have the potential to transfer N fixed to grasses and contribute to increased productivity and nutritive value in pastures (Cox et al., 2017; Lüscher, Mueller-Harvey, Soussana, Rees, & Peyraud, 2014). The use of N-fixing legumes to replace synthetic N has been reported in several temperate legumes, including hairy vetch (*Vicia villosa* Roth), field pea (*Pisum sativum* L.) and arrowleaf clover (*Trifolium vesiculosum* L.) (Interrante et al., 2012), and in white clover (*Trifolium repens* L.), hairy vetch, and alfalfa (*Medicago sativa* L.) (Biermacher et al., 2012).

The grass genus *Paspalum* is an important forage constituent in natural grasslands in South America (Chase, 1929), and its center of diversity covers a wide range of ecological zones in Argentina, Uruguay, and southern Brazil (Zuloaga & Morrone, 2005). *Paspalum* species have been genetically improved in the United States, Argentina, and Brazil with the aim of generating forage cultivars for subtropical regions (Acuña, Blount, Quesenberry, Kenworthy, & Hanna, 2009; Aguilera, Sartor, Galdeano, Espinoza, & Quarin, 2011; Weiler et al., 2018). Plicatula group is a taxonomic informal category within the genus *Paspalum* (Chase, 1929), and its species comprise different ploidy levels ranging from sexual diploid to apomictic polyploid cytotypes (Novo et al., 2016). *Paspalum plicatulum* and *P. guenoarum* species were introduced to cultivation and sown in other areas of the world; however, these species are primarily tetraploid and apomictic, and the current commercial cultivars had been selected from natural populations (Aguilera et al., 2011).

Core Ideas

- Herbage accumulation and persistence of *Paspalum* genotypes increased with N supply.
- Yield for grass–legume mixture was similar to the N rates at 60 and 120 kg N ha^{-1}.
- Hybrid 1020133 had higher herbage accumulation, greater NUE, and improved cold tolerance.

The chromosome duplication of a sexual diploid *P. plicatulum* ecotype resulted in the generation of a sexual tetraploid plant that could be crossed to apomictic ecotypes to generate segregating progenies (Sartor, Quarin, & Espinoza, 2009). Hybridization is one of the most prominent methods applied in forage grass breeding (Fernandes et al., 2014), and it has been the primary tool used for improvement in the Plicatula group (Aguilera et al., 2011; Novo et al., 2016, 2017). Previous studies reported that hybrids exhibited genetic potential for improved forage traits, such as herbage accumulation (HA), cold tolerance, and cattle preference (Motta et al., 2016, 2017; Novo et al., 2017).

The development of improved cultivars with higher HA and adapted to subtropical environmental conditions can contribute to improved animal performance in these regions. In addition, evaluating the response of novel interspecific hybrids under different N rates or in mixtures with legumes can provide information about agronomic management decisions and can aid in cultivar selection. However, quantitative information required for optimizing HA in interspecific *Paspalum* hybrids subjected to differing N fertilization rates or in mixtures with legumes is unknown. The goals of this study were to: (a) determine HA, leaf accumulation (LA), cold tolerance, plant persistence and NUE in four hybrids of *P. plicatulum* × *P. guenoarum* subjected to varying N rates for 2 yr; (b) compare agronomic performance of the grass–legume system to a grass–N fertilizer system in subtropical conditions; and (c) select a superior hybrid for further evaluation within the breeding program, for traits such as seed yield and animal performance.

2 | MATERIAL AND METHODS

2.1 | Experimental design

The study was conducted at the Agronomic Experimental Station of the Federal University of Rio Grande do Sul (UFRGS), Rio Grande do Sul, Brazil (30°05′ S, 51°39′ W, 34 m asl). The climate in the region is classified as subtropical humid (Cfa), according to the Köppen classification (Moreno, 1961). The average minimum and maximum monthly temperature during the last 40 yr were 8.5 ˚C (July) and 30.2 ˚C (January), respectively, and the average annual precipitation was approximately 1,450 mm (Bergamaschi et al., 2013). The average monthly minimum and maximum temperature and rainfall during the conduct of the study are listed in Table 1.

The soil at the experimental site was classified as Dystrophic Red Argisol. Soil analyses were assessed 6 months before the starting experiment. Soil samples were collected at 0–15 cm depth across treatments, then bulked and submitted to the Soil Analysis Lab–UFRGS. The soil pH (H_2O) was 5.5; P was 8.9 mg dm^{-3}; K was 105 mg dm^{-3}; organic matter was 15 g kg^{-1}; Ca was 2.4 $cmol_c$ dm^{-3}; Mg was 1.0 $cmol_c$ dm^{-3}, and Al was not present at a toxic concentration. The experimental area was fertilized following the local recommendations (CQFS, 2004): 1.5 Mg ha^{-1} of Filler-type limestone (Relative Total Neutralizing Power = 100%, fine particle size), 80 kg P ha^{-1} as triple superphosphate, and 60 kg K ha^{-1} as potassium chloride.

The experiment consisted of 108 experimental units arranged in a split-plot under a randomized complete block design with three replications. Whole plot treatments were five N fertilization rates: 0, 60, 120, 240, and 480 kg N ha^{-1}; and one grass–legume mixture. For the grass–legume mixture, two species were sown over grass genotypes swards: white clover ‘BRSURS Entrevero’ and birdsfoot trefoil (*Lotus corniculatus* L.) ‘URSBRS Posteiro’ at a rate of 4 and 10 kg seed ha^{-1}, respectively. Subplot treatments included four *Paspalum* interspecific hybrids (1020133, 102069, 103084, and 103061) and two controls: *Paspalum guenoarum* ‘Azulão’ and *Megathyrsus maximus* Aruana. Hybrids 1020133 and 102069 resulted from a cross between *P. plicatulum* ecotype 4PT and Azulão, whereas hybrids 103084 and 103061 resulted from a cross between 4PT and *P. guenoarum* ecotype Baio. The four hybrids (1020133, 102069, 103084, and 103061) exhibited good forage performance in different edaphoclimatic conditions (Saraiva, 2015). Ecotype Azulão is native to the subtropical and temperate regions of southern Brazil, Argentina, and Paraguay (Steiner et al., 2017), and showed good forage performance in subtropical conditions (Pereira et al., 2012). Aruana is a perennial grass used in intensive beef production systems in Brazil (Fernandes et al., 2014). The hybrids and controls were vegetatively propagated and transplanted to the experiment in December 2014 in pure stands in six rows (10 tillers row^{-1}) with 20 cm between rows (2.4-m^2 plot size). Weeds were manually removed from the plots (once) to reduce competition.

2.2 | Crop management and data collection

The N rates were supplied during the spring and summer divided into four applications per year, and N source was ammonium sulfate (21% N and 23% S). In January 2015, the

TABLE 1 Average monthly minimum–maximum temperature (˚C) and rainfall (mm) during the experimental period (September 2015–May 2017) compared with the 40-yr average (1970–2009)

	Temperature (minimum–maximum)				Rainfall			
Month	**2015**	**2016**	**2017**	**40-yr avg.**	**2015**	**2016**	**2017**	**40-yr avg.**
	˚C				mm			
Jan.	21.7–30.9	21.3–32.0	21.3–31.3	19.3–30.2	160	109	197	106
Feb.	21.2–30.2	21.8–31.8	22.4–32.2	19.0–29.4	95	139	51	106
Mar.	20.1–29.2	19.5–27.8	19.4–29.1	18.0–28.2	53	302	166	102
April	17.1–26.8	19.3–27.1	17.0–25.9	14.5–25.2	73	216	115	110
May	14.8–23.6	12.3–19.6	15.6–22.8	11.2–21.8	136	73	180	108
June	11.6–21.2	8.3–16.4		8.7–18.6	170	7		154
July	12.7–20.2	10.4–20.1		8.5–18.7	309	151		144
Aug.	16.2–26.2	12.1–22.0		9.7–20.2	109	99		134
Sept.	13.8–22.1	12.4–22.0		11.2–21.4	184	91		142
Oct.	15.4–23.6	15.5–24.8		13.8–24.0	307	192		129
Nov.	17.5–25.7	15.9–27.5		15.4–26.6	124	104		109
Dec.	20.2–29.0	20.0–30.9		17.5–29.0	100	128		111

experimental site received 60 kg N ha^{-1} to stimulate seedling establishment. Plots were cut at a 10-cm stubble height, and the experimental units receiving the grass–legume mixture were sown with the legumes using coated seeds on 17 Apr. 2015 and 30 Mar. 2016. It was necessary to sow the legume species again due to the drought that occurred in the summer of 2016, which affected the plant stands.

Data collection occurred from September 2015 to May 2017: the first year began in September 2015 and ended in April 2016; the second began in September 2016 and ended May 2017. Stubble height management varied during the experiment: in Year 1, plots were harvested at 10-cm stubble height when the higher N rate treatments (240 and 480 kg N ha^{-1}) reached 30–35 cm; in Year 2, plots were harvested at 15-cm stubble height when genotypes reached 30 cm in each whole-plot treatment. The harvest criterion was modified in Year 2 because 10-cm stubble height impaired regrowth in low N rate treatments.

Herbage accumulation was evaluated from two 0.25-m^2 samples per subplot in both years. Forage samples were manually separated into leaf blade, stem plus leaf sheath, inflorescence, and dead fractions. The grass–legume treatment was separated by the same components previously mentioned and by each legume species (birdsfoot trefoil and white clover). Samples were dried at 60 ˚C in a forced-air oven for 72 h and then weighed. Herbage accumulation, LA, and legume dry matter yield were calculated as the sum of all harvests per plot in each year of the experiment.

The NUE (kg dry matter [DM] kg^{-1} N applied) was calculated according to the following equation: NUE = (yield fertilized plot – yield unfertilized plot)/(applied N rate). Cold tolerance was visually estimated on 14 June and 22 July 2016 after frost events, with temperatures of 2 ˚C, using a 1–5 scale, where 1 is the least cold tolerance, and 5 is the greatest cold tolerance. A scoring system from 1 (few plants present in the plot) to 5 (plot fully covered with plants) was used to assess persistence after the second harvesting season.

2.3 | Data analysis

Data were analyzed using linear models on R statistical software (R Core Team, 2019). Analysis of variance (ANOVA) was performed fitting a linear model with a split-plot treatment arrangement for all variables to test the statistical significance of the main factors and their interactions: year, block, N rate, and genotype. The graphs were created using the package *ggplot2* in R (Vu, 2011). A Tukey-HSD test was used to separate treatment means at $P < .05$ using the *lsmeans* package (Lenth, 2016) in R.

3 | RESULTS

3.1 | Herbage accumulation and leaf accumulation

Significant year × genotype interaction ($P < .001$) effect was observed for HA. Therefore, results are presented by year for all variables where this interaction was significant. In Years 1 and 2, hybrid 1020133 had the greatest HA among the hybrids; however, it was not different from Azulão and Aruana (Figure 1a). There was also a year × genotype interaction ($P < .001$) effect on LA. In Year 1, hybrid 1020133 had the highest LA among all genotypes. In Year 2, hybrid 1020133 and Azulão had similar LA, which were higher than those of other genotypes (Figure 1b).

There was a N rate × genotype interaction ($P < .001$) effect on HA. Significant differences were found for HA among genotypes depending on N rate or grass–legume mixture, but larger differences were found at higher N rates. Hybrid 1020133, Azulão, and Aruana showed the greatest increase in HA at greater N application rates, especially at 240 and 480 kg N ha^{-1} (Figure 2a).

Herbage accumulation for hybrid 1020133, Azulão, and Aruana was approximately double at 480 kg N ha^{-1} compared with 0 kg N ha^{-1}. Among N rates and grass–legume mixture, hybrid 1020133 had higher or equal HA to the controls, except at 480 kg N ha^{-1} (Figure 2a).

A genotype × N rate interaction ($P < .01$) was also observed for LA. For this variable, significant differences among genotypes were observed under different N application rates and in the grass–legume mixture, except when N was not applied. Hybrid 1020133 and Azulão exhibited a greater LA response when greater N rates were applied (Figure 2b). Leaf accumulation increased 2.2 times for 1020133 and 2.1 times for Azulão at 480 kg N ha^{-1} compared with 0 kg N ha^{-1}. In all treatments, hybrid 1020133 showed higher or equal LA than the controls (Figure 2b).

There was a year × N rate interaction ($P < .001$) for HA. In Years 1 and 2, HA increased with N application rates. In Year 1, HA ranged from 11,368 kg ha^{-1} at 0 kg N ha^{-1} to 19,033 kg ha^{-1} when fertilized with 480 kg N ha^{-1} (Figure 3a). The HA of the grass–legume mixture (13,221 kg ha^{-1}) was greater than that of the 0 kg N ha^{-1} treatment and was equal to HA of the grass fertilized with 60 kg N ha^{-1} (12,996 kg ha^{-1}). In Year 2, HA ranged from 7,386 kg ha^{-1} (0 kg N ha^{-1}) to 16,036 kg ha^{-1} when fertilized with 480 kg N ha^{-1}. Greater HA was observed for the grass–legume mixture (10,495 kg ha^{-1}) than for the 0 and 60 kg N ha^{-1} grass fertilization treatments, which was equal to the 120 kg N ha^{-1} fertilized grass treatment (11,128 kg ha^{-1}).

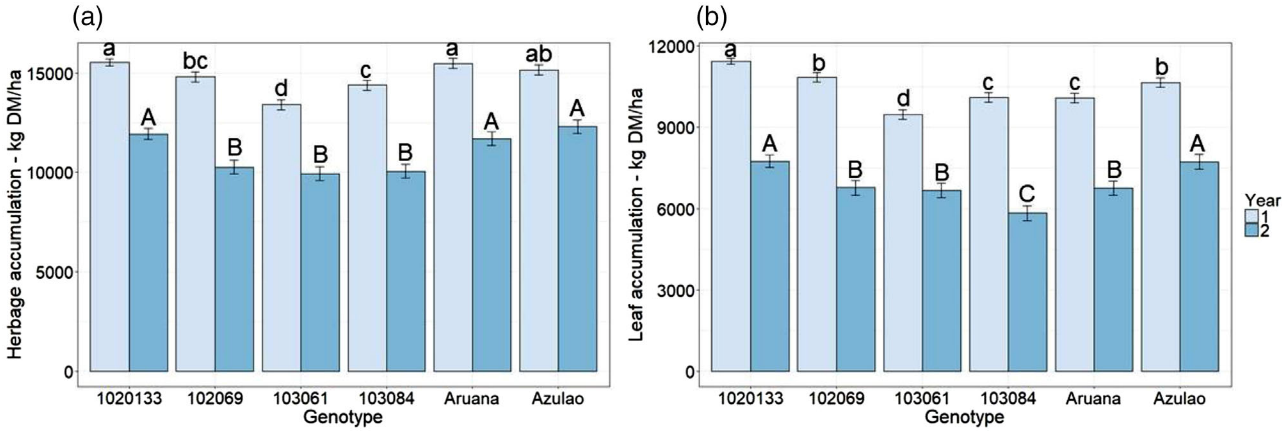


FIGURE 1 (a) Year × genotype interaction effects on herbage accumulation; and (b) leaf accumulation of four interspecific *Paspalum* hybrids, *P. guenoarum* 'Azulão' and *M. maximus* 'Aruana' in southern Brazil. Distinct letters (lowercase letters = Year 1 and uppercase letters = Year 2) indicate significant differences according to Tukey's test ($P < .05$). Bars represent mean values, and the error bars represent standard errors for each mean

Similarly, there was a year × N rate interaction ($P < .001$) effect on LA. The higher N rates provided greater LA. In Years 1 and 2, LA increased with N application. In Year 1, LA ranged from 8,107 kg ha^{-1} when no N applied to 14,116 kg ha^{-1} with application of 480 kg N ha^{-1} (Figure 3b). The grass–legume mixture (7,242 kg ha^{-1}) showed the lowest performance for LA. In Year 2, LA ranged from 4,479 kg ha^{-1} at 0 kg N ha^{-1} to 11,412 kg ha^{-1} when fertilized with 480 kg N ha^{-1}. The grass–legume mixture (4,134 kg ha^{-1}) showed LA equal to 0 kg N ha^{-1}.

3.2 | Legume proportion on herbage accumulation

Significant genotype ($P < .05$) effects were observed for legume proportion in HA in the grass–legume mixture. In Year 1, hybrid 103061 (34%) had the greatest legume as a proportion of HA, and hybrid 102069 (25%) had similar legume proportion than Azulão (23%) and hybrid 103084 (18%) and greater than hybrid 1020133 (16%) and Aruana (11%) (Figure 4a). In Year 2, hybrid 103084 (52%) had greater legume as a proportion of HA than hybrid 1020133 (42%) and Azulão (36%) but was not different from hybrids 103061 (49%) and 102069 (45%), and Aruana (44%).

There was also a significant legume species ($P < .001$) effect for legume dry matter yield. In Year 1, white clover (1,874 kg DM ha^{-1}) had greater dry matter yield compared with birdsfoot trefoil (576 kg DM ha^{-1}) (Figure 4b). In Year 2, there was an increase in dry matter yield of white clover (3,318 kg DM ha^{-1}), whereas birdsfoot trefoil (608 kg DM ha^{-1}) showed similar yield to Year 1.

3.3 | Nitrogen use efficiency

The N rate ($P < .001$) had a significant effect on NUE in Year 1. The highest NUE was observed at 60 kg N ha^{-1} (32 kg kg^{-1} N applied) across all genotypes (Figure 5a). There was no difference in NUE between the 120 and 240 kg N ha^{-1} rates, and both rates had greater NUE than 480 kg N ha^{-1} (5 kg kg^{-1} N applied).

In Year 2, there was a N rate × genotype interaction ($P < .05$) for NUE. At the rate of 60 kg N ha^{-1}, hybrid 1020133 showed greatest NUE among genotypes (Figure 5b). At the rate of 120 kg N ha^{-1}, hybrid 1020133 and Azulão had greater NUE than hybrids 103061 and 103084, and Aruana. There was no difference for NUE of genotypes in N rates of 240 and 480 kg N ha^{-1}. At the rate of 240 kg N ha^{-1}, NUE ranged from 16 kg^{-1} N (hybrid 103061) to 26 kg kg^{-1} N (hybrid 1020133). At the rate of 480 kg N ha^{-1}, NUE ranged from 12 kg^{-1} N (hybrid 103061) to 22 kg kg^{-1} N for Aruana. In general, NUE increased from a N rate of 60 kg N ha^{-1} to 120 kg N ha^{-1}, and then decreased under higher rates (240 and 480 kg N ha^{-1}) N rates, except for Aruana (Figure 4b).

3.4 | Cold tolerance

The only a significant genotype ($P < .001$) effect was on cold tolerance. Hybrid 1020133 had greater cold tolerance among all genotypes, except for hybrid 102069. Azulão ecotype and hybrids 103084 and 103061 showed greater cold tolerance than Aruana (Figure 6a). Greater differences in the visual score for cold tolerance were observed between hybrid 1020133 and Aruana (Figure 6b).

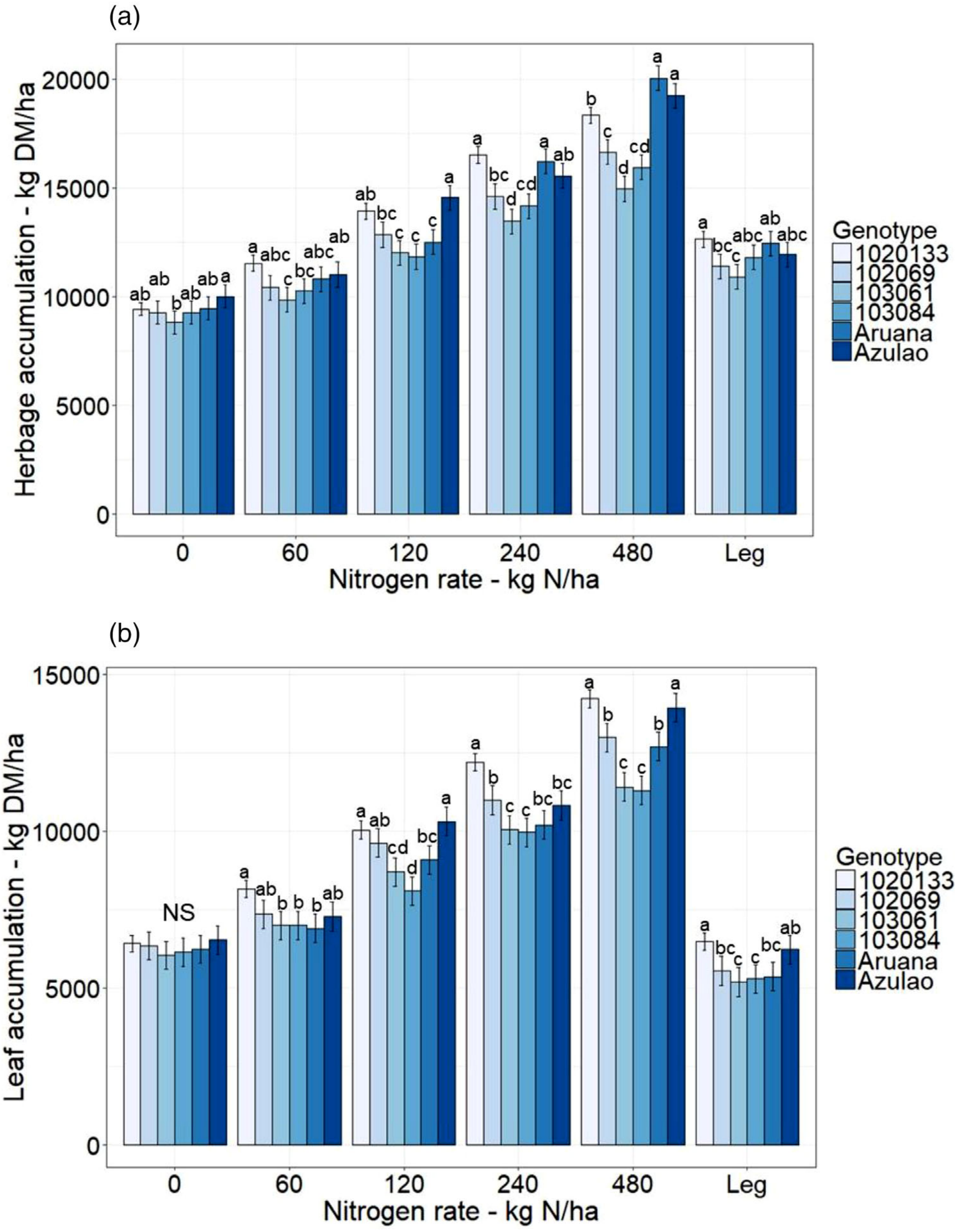


FIGURE 2 (a) Nitrogen rate × genotype interaction effects on total herbage accumulation; and (b) leaf accumulation of four interspecific *Paspalum* hybrids, *P. guenoarum* 'Azulão' and *M. maximus* 'Aruana' in southern Brazil. Leg, grass–legume mixture. NS, not significant. Bars represent mean values, and the error bars represent standard errors for each mean

3.5 | Persistence

Nitrogen rate had a significant ($P < .001$) effect on stand persistence. Estimated visual scores ranged from 3.4 at 0 kg N ha^{-1} to 4.9 when fertilized at 480 kg N ha^{-1} (Figure 7). Greater persistence scores were observed for N rates of 240 and 480 kg N ha^{-1} when compared with the 0, 60 kg N ha^{-1} and grass–legume mixture but did not differ from 120 kg N ha^{-1}. Grass–legume mixture had persistence similar to the 60 and 120 kg N ha^{-1} treatments. In general, all genotypes exhibited acceptable persistence (data not shown), with hybrids 1020133 and 102069 showing greater persistence than hybrids 103084 and 103061; however, they were not statistically different from Azulão and Aruana.

4 | DISCUSSION

Interspecific hybridization has been the primary tool used for genetic improvement in the Plicatula group in the genus *Paspalum* (Aguilera et al., 2011; Novo et al., 2017). Crossing artificially induced sexual tetraploid germplasm with locally

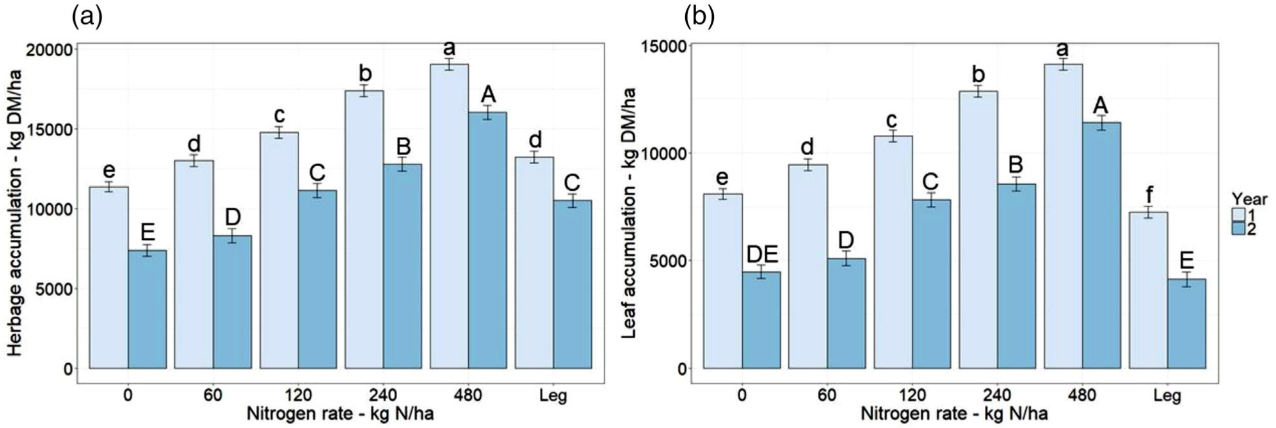


FIGURE 3 (a) Year × N rate interaction effects on herbage accumulation; and (b) leaf accumulation of four interspecific *Paspalum* hybrids, *P. guenoarum* 'Azulão' and *M. maximus* 'Aruana' in southern Brazil. Leg, grass–legume mixture. Distinct letters (lowercase letters = Year 1 and uppercase letters = Year 2) indicate significant differences according to Tukey's test ($P < .05$). Bars represent mean values, and the error bars represent standard errors for each mean

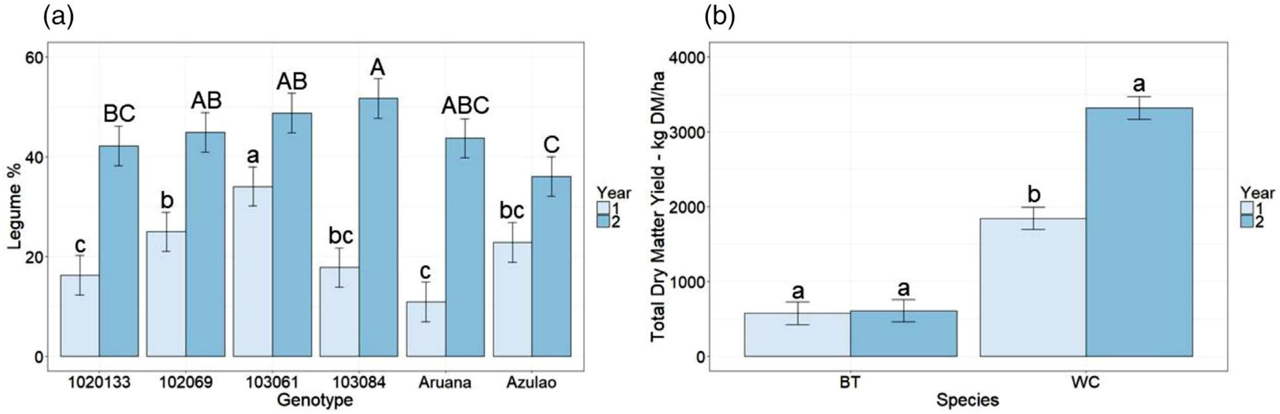


FIGURE 4 (a) Legume proportion on herbage accumulation; and (b) Dry matter yield of birdsfoot trefoil–BT and white clover–WC in Years 1 and 2. Distinct letters (lowercase letters = Year 1 and uppercase letters = Year 2) indicate significant differences according to Tukey's test ($P < .05$). Bars represent mean values, and the error bars represent standard errors for each mean

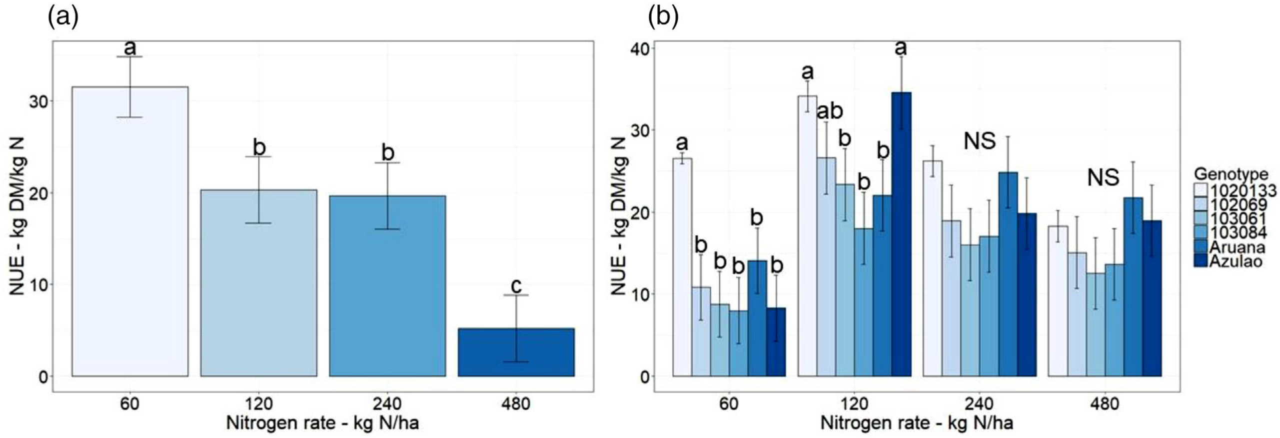


FIGURE 5 (a) Nitrogen rate effect on NUE in Year 1; and (b) Genotype × N rate interaction effects on NUE in Year 2. Distinct letters indicate significant differences according to Tukey's test ($P < .05$). NS, not significant. Bars represent mean values, and the error bars represent standard errors for each mean

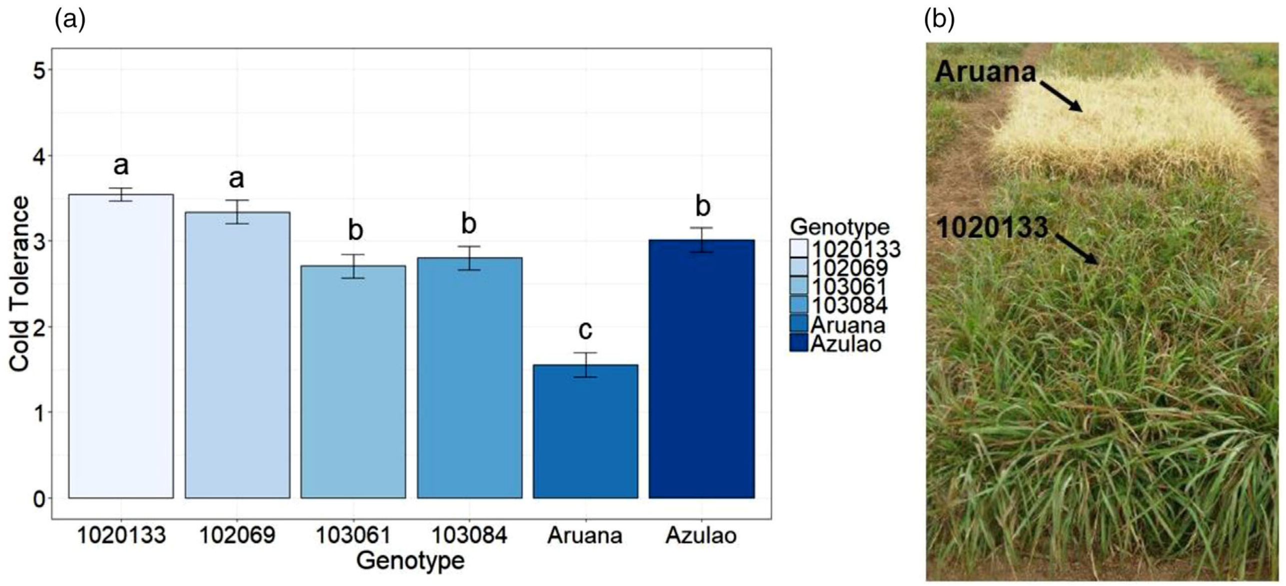


FIGURE 6 (a) Cold tolerance of four interspecific *Paspalum* hybrids, *P. guenoarum* 'Azulão' and *M. maximus* 'Aruana' in response to N rates or in mixture with legumes; and (b) visual evaluation of the damage after frost occurrence in Aruana and hybrid 1020133 in July 2016. Cold tolerance score, where: value = 1 means the least cold tolerance (canopy with many dead leaves) and value = 5 means the greatest cold tolerance (canopy with few dead leaves). Distinct letters indicate significant differences according to Tukey's test ($P < .05$). Bars represent mean values, and the error bars represent standard errors for each mean

adapted tetraploid apomicts can generate highly productive F_1 hybrids that exploit heterosis. Besides increasing pasture yield, cultivar development should also be focused on tolerance, persistence, and return on investment under edaphic stress (Parsons et al., 2011). In this study, HA, cold tolerance, persistence, and NUE were determined for four interspecific *Paspalum* hybrids, Azulão and Aruana as controls, grown under five N rates and in a grass–legume mixture with birdsfoot trefoil and white clover for 2 yr.

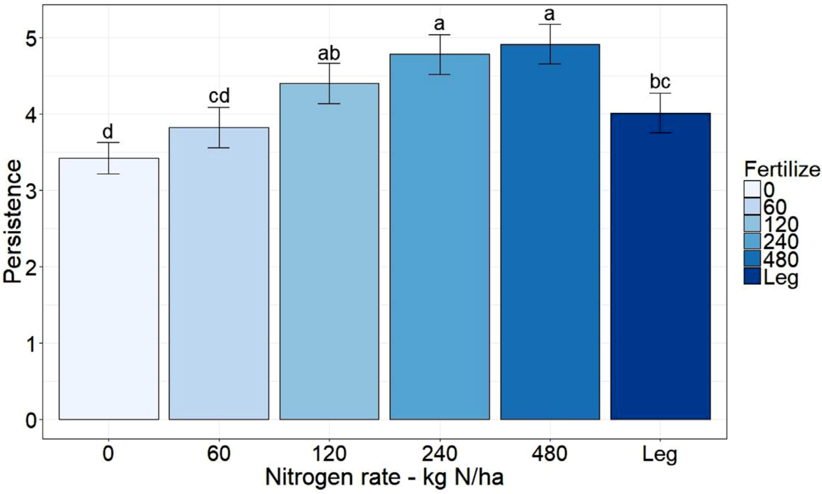
4.1 | Herbage accumulation and leaf accumulation

Large phenotypic variation was observed among genotypes for HA and LA; however, genotypic performance was dependent upon year and N rate. In Years 1 and 2, hybrid 1020133 produced more HA than the other hybrids, but it was not different from Azulão and Aruana. Hybrid 1020133 resulted from hybridization between a 4PT sexual tetraploid and the apomictic ecotype Azulão. Azulão is a locally adapted genotype that produces high HA (Pereira et al., 2012; Steiner et al., 2017), possibly explaining the similar HA and the response to N fertilization between these two related genotypes. Aruana was used as a control in this experiment due to its known forage potential in southern Brazil (Motta, Dall'Agnol, Pereira, Machado, & Simioni, 2017), and its high regrowth capacity after each grazing/cutting cycle (Lavres et al., 2004). A study focused on evaluating several genotypes of *M. maximus* reported HA of 12,900 kg ha^{-1} for Aruana at 250 kg N ha^{-1} (Fernandes et al., 2014), confirming its ability to respond to high N rates. Therefore, our results suggest that interspecific hybridization techniques used in *Paspalum* species may provide native germplasm with excellent HA, which can be used to recover degraded native pasture areas or be cultivated in livestock production systems.

Our findings revealed that N fertilization provided significant increases in HA. Averaged across genotypes, HA increased by 67% (Year 1) and 117% (Year 2) at 480 kg N ha^{-1} compared with 0 kg N ha^{-1}. These results are consistent with other studies that also showed an increase in HA of *P. guenoarum* (Towsend, 2008), *Brachiaria brizantha* (Costa, Faquin, & Oliveira, 2010), and *P. notatum* (Machado et al., 2019) as N fertilization rates increased. Nitrogen has an effect on plant growth, development, and physiological processes and determines crop productivity (Seepaul et al., 2016). Herbage accumulation of hybrid 1020133, Azulão, and Aruana increased dramatically with higher N rates. Large genotypic differences in HA were observed at higher N rates, whereas lower HA and less variability among genotypes were observed at 0 and 60 kg N ha^{-1}. Generally, when plants are under stress, such as N deficiency, phenotypic variability among genotypes decreases because they cannot express their yield potential (Bartl et al., 2009).

Currently, high N fertilizer prices and increasing concerns/regulations regarding the use of inorganic N fertilizers guided research toward alternative approaches for increasing HA without additional inorganic N inputs. In this study, grass

FIGURE 7 Persistence of four interspecific *Paspalum* hybrids, *P. guenoarum* 'Azulão' and *M. maximus* 'Aruana' in response to N rates or in mixture with legumes. Persistence score, where: value = 1 means the least persistence (few plants present in the plot) and value = 5 means the greatest persistence (many plants present in the plot). Leg, grass–legume mixture. Distinct letters indicate significant differences according to Tukey's test ($P < .05$). Bars represent mean values, and the error bars represent standard errors for each mean



monoculture fertilized with 240 and 480 kg N ha^{-1} rates produced higher HA compared with grass–legume mixtures. According to Lüscher et al. (2014), mixed white clover–grass pastures are usually managed with very low N fertilization inputs, and yield per hectare might be less than from highly fertilized grass pastures. In this study, N fertilizer was not applied to the grass–legume mixture, which possibly explains the lower HA compared with the higher N rates. On the other hand, HA for the grass–legume mixture was 16% greater than the 0 kg N ha^{-1} treatment for the first year. In Year 2, HA for the grass–legume mixture increased by 42 and 26% when compared with the 0 kg N ha^{-1} and 60 kg N ha^{-1} treatments, respectively, and were similar to the 120 kg N ha^{-1} treatment. In *Brachiaria decumbens* 'Basilisk' fertilized with different N rates (0, 50, and 100 kg N ha^{-1}) or grown in mixtures with *Stylosanthes guianensis* 'Mineirão', HA was similar among treatments, and yield was greater compared with 0 kg N ha^{-1} (Martuscello et al., 2011). Adjesiwor et al. (2017) reported that meadow bromegrass (*Bromus biebersteinii* Roemer & Schultes) mixed with alfalfa and birdsfoot trefoil showed similar HA than meadow bromegrass fertilized at 56 and 112 kg N ha^{-1}. Cox et al. (2017) observed that tall fescue (*Festuca arundinacea* Schreber), meadow bromegrass, orchardgrass (*Dactylis glomerata* L.), and perennial ryegrass (*Lolium perenne* L.) growing in mixtures with alfalfa, birdsfoot trefoil, and cicer milkvetch (*Astragalus cicer* L.) showed greater HA than any grass monoculture fertilized with 67 kg N ha^{-1} and equal to or higher production than grass fertilized with 134 kg N ha^{-1}.

Our findings also revealed that the hybrid 1020133 and the genotypes Azulão and Aruana in mixtures with legumes showed similar HA to Aruana when fertilized at 120 kg N ha^{-1}. Therefore, results obtained suggest that mixtures with legumes can replace N fertilizer application up to the rate of 120 kg N ha^{-1}. Legumes offer important opportunities for sustainable grassland-based animal production because they can contribute to increasing forage yield, substituting inorganic N-fertilizer inputs with symbiotic N_2 fixation, increasing the nutritive value of herbage and raising the efficiency of conversion of herbage to animal protein (Cox et al., 2017; Lüscher et al., 2014). However, the additional management effort that is required to incorporate legumes into the forage system is generally greater than the labor required to apply N fertilizer in pastures. Legumes typically require greater limestone, and P and K fertilization (Butler & Muir, 2012).

In both years, hybrid 1020133 had greater LA except for Azulão in Year 2. Among treatments, hybrid 1020133 responded strongly to N fertilizer, and was difference from Azulão when fertilized at 240 kg N ha^{-1}. In general, across years and N rates the genetic similarity between hybrid 1020133 and Azulão may explain why there was no difference in LA or HA response to N application rates between these two related genotypes. Previous studies demonstrated LA greater than 10,000 kg ha^{-1} for Azulão (Motta et al., 2017; Steiner et al., 2017) showed the genetic potential of Azulão for LA. The LA of hybrid 1020133 was also greater than that of Aruana in Years 1 and 2, and for all treatments other than 0 kg N ha^{-1}. Fernandes et al. (2014) observed that the LA of Aruana was approximately half the HA. The authors attributed this due to its continuous flowering, favoring stem production. The percentage of leaves impacts ingestive behavior of grazing cattle (Fernandes et al., 2014).When animals grazing the upper grazing strata (higher proportion of leaves), they access a better quality diet, whereas when animals graze the lower strata, their diet quality declines due to a greater proportion of stems (Benvenutti, Pavetti, Poppi, Gordon, & Cangiano, 2015). Therefore, productive species with a higher proportion of leaves provides a better-quality diet that is beneficial for animal performance in pasture-based production systems.

The results also reveal that the N application rates provided significant LA benefit, as was the case for HA. Leaf proportion of HA is greater for *Paspalum* interspecific hybrids of *Paspalum* (Motta et al., 2016, 2017), which may explain a similar response to that observed on HA. Averaged across genotypes, LA increased by 74% (Year 1) and 155% (Year 2) at 480 kg N ha^{-1} compared with 0 kg N ha^{-1}. In grass–legume mixtures, LA decreased by 11% compared with the 0 kg N ha^{-1} in Year 1 and this treatment was similar in Year 2. Competition between species by interception of light may have impaired the development of leaves of the grass. Martuscello et al. (2011) found a similar LA between *B. decumbens* Basilisk fertilized at 50 and 100 kg N ha^{-1} and in a mixture with *S. guianensis* Mineirão; however, they reported a similar LA between the mixture with *Calopogonium mucunoides* and N rate of 0 kg ha^{-1}, and less than other treatments.

4.2 | Legume proportion on herbage accumulation

Considerable variability was observed among grass genotypes for legume proportion of HA. In Year 1, legume proportion ranged from 11% (Aruana) to 34% for the hybrid 103061. In Year 2, legume proportion for all genotypes increased to values between 30 and 52%. According to Lüscher et al. (2014) the advantages presented by the grass–legumes mixture such as increased forage production, greater nutritive value, "energy neutral" N input into grasslands via symbiotic N_2 fixation, and support of non-N_2-fixing plants in the grassland through transfer of symbiotically fixed N are most pronounced in mixed swards with 30–50% legumes.

In Years 1 and 2, white clover produced more dry matter yield (3.3 and 5.5 times, respectively) than birdsfoot trefoil. White clover increased in dry matter yield by 78% in Year 2 compared with Year 1. This value suggests a higher capacity for competition and dry matter yield contribution of white clover in the mixture, especially when compared with birdsfoot trefoil. Legume proportion was less in Year 1, possibly due to increased competitiveness of grass resulting from well-established grass stands, since grasses were planted before the legume species. According to Schwinning and Parsons (1996), grass exploits legumes by taking advantage of the additional N that the legumes bring into the system, whereas suppressing legume growth through competition for light. The increase observed in the legume proportion in Year 2, especially for white clover, can be explained by the trade-off between grass and white clover (i.e., when soil mineral N is low, white clover has a greater relative growth rate than grass, since it can supplement mineral N uptake with N fixation) (Schwinning & Parsons, 1996).

4.3 | Nitrogen use efficiency

Nitrogen use efficiency has been used to describe the capacity for plants to acquire and utilize N for biomass production, and it is expressed as the biomass yield produced per unit N applied (Seepaul et al., 2016). In general, in Year 1, the greatest NUE was observed at 60 kg N ha^{-1} and NUE at 480 kg N ha^{-1} was less. The results observed in Year 1 support previous findings in *Axonopus aureus* (Costa et al., 2010) and *Panicum virgatum* (Obour, Harmoney, & Holman, 2017) that showed a decrease in NUE with increased N application rates. In Year 2, NUE increased with N application rate up to 120 kg N ha^{-1}. Most genotypes showed similar NUE at the higher N application rates of 240 and 480 kg ha^{-1}. In a study with *P. guenoarum*, *P. notatum*, and *P. lividum* subjected to N rates of 0, 60, 180, and 360 kg ha^{-1} Towsend (2008) reported that NUE ranged from 47 to 11 kg DM kg^{-1} N for *P. notatum*, fertilized with 60 kg of N ha^{-1} and *P. guenoarum* at 360 kg N ha^{-1}, respectively, and that maximum NUE was observed with N rates between 60 and 180 kg N ha^{-1}. The results from this study showed that maximum NUE was obtained with N rates between 60 (Year 1) and 120 kg N ha^{-1} (Year 2). Our results also revealed that hybrid 1020133 showed efficient conversion of N to HA and had greater or similar NUE than Azulão and Aruana in Year 2.

4.4 | Cold tolerance

In this study, cold tolerance evaluated by a visual assessment of the damage after frost occurrence, i.e., genotypes with less damage (canopy with green leaves) were classified as cold tolerant, whereas genotypes with greater damage (canopy with dead leaves) were classified as cold susceptible. There is a degree and duration of frost that is lethal to most organisms, due to dehydration of the intracellular environment and physical damage by ice crystals (Jacobsen et al., 2005). Hybrid 1020133 showed greater cold tolerance compared with Azulão and Aruana as shown in Figure 6b, which suggests good adaptation to climatic condition in southern Brazil. Azulão it is known for cold tolerance in subtropical regions (Motta et al., 2016), whereas Aruana is one of the cultivars of *Megathyrsus maximus* species with greater cold tolerance (Corrêa, 2002).

4.5 | Persistence

Although 2 yr of data collection is a short period to assess the persistence of a perennial plant, our results were that N rates greater than 120 kg ha^{-1} provided a benefit for persistence compared with the control and 60 kg N ha^{-1}. Possibly, the cumulative effect of elevated N rates for 2 yr provided favorable growth conditions, reducing the competition between plants for nutrient and increasing their capacity for growing, through the development of tillers. McKenzie, Jacobs, and Keamey (2002) observed that application of 75, 150, and 225 kg N ha^{-1} resulted in increased tiller densities and forage yield of perennial ryegrass compared with 0 kg N ha^{-1}. However, the authors reported that N fertilizer alone would not enhance the persistence of pasture, and that persistence is likely to be influenced by a combination of factors including grazing management and climatic effects, rather than a N fertilizer effect alone.

After 2 yr of harvest, hybrids 1020133 and 102069 had greater persistence than hybrids 103084 and 103061 and were similar to the controls (data not shown). According to Nie, Chapman, Tharmaraj, and Clements (2004) one of the key attributes of plants is their ability to capture and use resources (light, water, and nutrients) to successfully occupy and colonize space. Identification of improved hybrid of *Paspalum* with greater HA as well as persistence may have a significant positive impact on pasture-based production systems.

5 | CONCLUSIONS

Nitrogen application increased HA, LA, and cold tolerance and improved persistence of *Paspalum* interspecific hybrids. Highest NUE is obtained with application of N rates between 60 and 120 kg N ha^{-1}. The grass–legume mixture can be an alternative practice of agronomic management to replace N rates between 60 and 120 kg N ha^{-1}, depending on the year. Hybrid 1020133 responded significantly to N supply and had HA similar to Azulão and Aruana, and LA greater than Aruana; and exhibited greater NUE at 60 kg N ha^{-1} than the other genotypes, and cold tolerance greater than Azulão and Aruana. Hybrid 1020133 is indicated for additional follow up studies, such as seed production and animal performance.

We suggest that replication of the study at different sites and additional years would provide more information, since it is expected that in the long term, the results may vary under different soil types and environmental conditions. In addition, further research would be needed for testing the impact of animal grazing on the response of the most promising genotypes when subjected to N fertilizer or mixed with legumes.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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