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ORIGINAL ARTICLE

Morphoagronomic characterization and reproductive versatility in polymorph clover

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Abstract - *Trifolium polymorphum* Poir. is a legume of natural pastures that combines amphicarpy with vegetative reproduction, but with little morphoagronomic information in populations of southern Brazil. In this study, plant height and diameter, number of primary stolons, dry matter, number of inflorescences, number of flowers per inflorescence, number of flowers with legume with seeds, number of flowers without legume or with legume without seeds, seed production, number and dry weight of storage roots were evaluated in populations of *T. polymorphum*. Little variation was observed for most characteristics, including low dry matter and aerial seed production. There were positive correlations between plant height and dry matter (r = 0.44), plant diameter and dry matter (r = 0.43), number of inflorescences and number of flowers per plant (r = 0.98) and number of inflorescences and seed production (r = 0.84). *T. polymorphum* presented low dry matter yield, however, the reproductive versatility of the species with the amphicarpic condition combined with vegetative propagation are mechanisms that allow the species to increase its frequency and contribution in natural pastures.

Keywords: Forage. Seeds. Amphicarpy. Natural pasture. Leguminosae. *Trifolium polymorphum*.

Caracterização morfoagronômica e versatilidade reprodutiva em trevo polimorfo

Resumo - *Trifolium polymorphum* Poir. é uma leguminosa das pastagens naturais que combina anficarpia com reprodução vegetativa, mas com escassas informações morfoagronômicas em populações do sul do Brasil. Nesse estudo foram avaliados altura e diâmetro das plantas, número de estolões primários, matéria seca, número de inflorescências, número de flores por inflorescência, número de flores com legumes com sementes, número de flores sem legumes ou com legumes sem sementes, produção de sementes, número e peso seco das raízes de reserva em populações de *T. polymorphum*. Pouca variação foi observada para a maioria das características, incluindo baixa produção de matéria seca e de sementes aéreas. Ocorreram correlações positivas entre altura da planta e matéria seca (r = 0,44), diâmetro da planta e matéria seca (r = 0,43), número de inflorescência e número de flores por planta (r = 0,98) e número de inflorescências e produção de sementes (r = 0,84). O *T. polymorphum* apresentou baixa produção de matéria seca, no entanto, a versatilidade reprodutiva da espécie com a condição anficárpica aliada a propagação vegetativa são mecanismos que possibilitam à espécie aumentar a sua frequência e contribuição nas pastagens naturais.

Palavras-chave: Forragem. Sementes. Anficarpia. Pastagem natural. Leguminosae. *Trifolium polymorphum*.

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Introduction

Plants have developed various reproduction strategies that allow them to transmit genetic material, the purpose of the whole organism, even in the face of climate change and environmental stress (SWIFT *et al.*, 2016). *Trifolium polymorphum* Poir. (Fabaceae) is a winter stoloniferous species that grows in the natural grasslands of Rio Grande do Sul, southern Brazil, reaching Uruguay, Argentina, Paraguay, and Chile (BURKART, 1987). This legume combines amphicarpy - the production of aerial and subterranean flowers and seeds in the same individual (CHEPLICK, 1987; LEV-YADUN, 2000), with vegetative reproduction by regrowth of storage roots (REAL *et al.*, 2007; SPERONI *et al.*, 2009, 2014; CONTERATO and SCHIFINO-WITTMANN, 2014; CONTERATO *et al.*, 2019). During winter, it is an essential qualitative component of natural pastures due to good forage quality and excellent palatability with protein values of 22 % (COLL and ZARZA, 1992; SPERONI and IZAGUIRRE, 2003). However, Speroni and Izaguirre (2003) cited herbivory by cattle as one of the causes for the low aerial seed production of *T. polymorphum*. Speroni *et al*. (2014) mentioned that cattle consume most aerial flowers, and the remaining flowers show low fruiting. In Rio Grande do Sul, the species develops in the state's southern half, is appetized by cattle, and does not produce much green mass, but it is of great importance because of its hibernal growth (KAPPEL, 1967).

Cattle production is one of the main economic activities in southern Brazil. It has natural pastures as its forage base (CARVALHO *et al*., 2009); however, few morphological and agronomic evaluations have been conducted on *T. polymorphum*. This information is essential for a better understanding of the contribution of the species to the natural pastures of Rio Grande do Sul and the reproductive strategies adopted for the persistence of the species. In this writing, we evaluated morphological characteristics, dry matter production, aerial and subterranean seed production, and storage roots in different populations of *T. polymorphum* collected at several sites in Rio Grande do Sul state.

Materials and Methods

In May 2021, aerial seeds of *T. polymorphum* accessions/populations collected in different locations in Rio Grande do Sul (Table 1) were scarified with sandpaper n°180 and placed to germinate in tubes (one seed per tube) filled with a commercial substrate composed of pine bark, vermiculite, acidity corrective and macronutrients. In early August, ten plants of each access (except the access Quaraí with eight plants) were transferred to the field area of the Centro Estadual de Diagnóstico e Pesquisa Florestal/CEFLOR/Santa Maria/RS (29°39'54,2"S and 53°54'38,9"W), at 0.80 m spacing between plants of the same population within the line and 1 m between lines of different populations in a completely randomized arrangement. Each plant was considered an experimental unit. The field area was fertilized according to the technical recommendations of the Comissão de Química e Fertilidade do Solo - CQFS-RS/SC (2016). Weeding and manual cleaning of weeds were performed. As a result of the intense heat, the plants were watered daily from October 2021 to January 2022.

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Table 1. Collection site and physiographic region of evaluated *T. polymorphum* accessions.

Access/ Collection site	Physiographic Region
23. Santa Margarida do Sul (30°30'27"S and 54°19'01"W)	Campanha
26. Santa Margarida do Sul (30°21'37,579"S and 54°10'47,704"W)	Campanha
30. Caçapava do Sul (31°01'483"S and 53°46'840"W)	Serra do Sudeste
31. Hulha Negra (31°08'976S and 53°48'628"W)	Campanha
33. Bagé (31°15'080"S and 54°13'009"W)	Campanha
35. Santana do Livramento (30°53'27"S and 55°31'58"W)	Campanha
39. Quaraí (30°01'793S and 56°25'667"W)	Campanha
40. Uruguaiana (29°43'16S and 56°55'852"W)	Campanha

The following characteristics were evaluated: plant height (PLH), plant diameter (PLD), number of primary stolons (ST1), dry matter production (DM), number of inflorescences (INF), number of flowers per inflorescence/plant (F/INF), number of flowers with seed legumes (FSL), number of flowers without legumes or with seedless legumes (FwL/LwS), number of probably viable seeds (VS) (full, developed and yellow seeds), number of probably non-viable seeds (empty and brown seeds) (NVS), total number of seeds (TS), number (SR) and dry weight (DW) of storage roots per plant. Plant height and diameter were measured with a ruler graduated in centimeters. The evaluation of dry matter yield was performed in a single cut in October 2021 when most accessions were in flowering, since at the end of flowering, approximately in December, their aerial part disappears during the summer (SPERONI and IZAGUIRRE, 2003). All plants were individually cut close to the ground using scissors, placed in paper bags and dried at 55 °C - 60 °C in a forced ventilation oven until constant weight and weighed to determine dry matter. In December 2021, the aerial inflorescences of each plant were collected and placed in paper bags for seed counting. In January 2022, all plants were removed from the experimental area to count the subterranean legumes and seeds, and the storage roots. After counting, the storage roots were dried at 55 °C - 60 °C in a forced ventilation oven until constant weight. In April 2023, a sample of 120 aerial seeds visually classified as probably viable were scarified with sandpaper n°180 and put to germinate in four Petri plates with filter paper moistened with distilled water in order to assess seed germination. The Petri dishes were kept in a Mangelsdorf germinator with a water slide to maintain humidity for 39 days, with 8.5 hours of daily artificial light and a temperature of 21 °C. The germination was evaluated every two days, and the germinated seeds were removed from the plates to avoid contaminating the other seeds. Seeds with radicle emission of approximately 1.5 mm were standardized as germinated.

The data were submitted to analysis of variance (ANOVA). The Shapiro-Wilk test has tested the assumption of the data's normality ($P > 0.05$). Variables that did not present a normal distribution were transformed. When differences were observed, the Tukey HSD test compared the means at a 5 % significance level. A simple linear correlation analysis (Pearson) was performed between the variables. Statistical analyses were performed using R software (version 4.1.2.).

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Results and Discussion

There was no significant difference between populations for most of the characteristics evaluated in *T. polymorphum* (Table 2). However, plants from the Uruguaiana and Quaraí populations had significantly greater heights (1.925 cm and 1.860 cm, respectively) compared to those from the Santa Margarida do Sul population (0.95 cm), not significantly different from the other populations. The largest plant diameters observed in the Uruguaiana (61.50 cm) and Bagé (58.60 cm) populations differed from those of the Quaraí population (22.62 cm), the other populations being statistically similar. The variation in the number of primary stolons was not significant.

Table 2. Mean values for plant height, plant diameter, number of primary stolons, and dry matter production in *T. polymorphum*.

Access	Plant height (cm)	Plant diameter (cm)	Number of primary stolons	Dry matter (g)
39. Quaraí	1.925 a*	22.62 c	4.75 a	1.160 b
40. Uruguaiana	1.860 a	61.50 a	5.50 a	2.905 a
33. Bagé	1.570 ab	58.70 a	5.00 a	1.859 ab
30. Caçapava do Sul	1.520 ab	44.40 ab	5.60 a	2.572 ab
26. Santa Margarida do Sul	1.330 ab	48.00 ab	5.30 a	1.982 ab
35. Santana do Livramento	1.120 ab	43.90 ab	5.10 a	0.876 b
31. Hulha Negra	1.110 ab	42.60 ab	4.60 a	2.630 ab
23. Santa Margarida do Sul	0.950 b	39.10 bc	4.70 a	2.025 ab

*Means followed by the same letter in each column do not differ significantly by Tukey's test at 5 %.

The reduced height growth of *T. polymorphum* plants differs from the species' 5-15 cm height quoted by Speroni and Izaguirre (2003). However, *T. polymorphum* is a species of prostrate stoloniferous habit (BURKART, 1987; ZOHARY and HELLER, 1984), and the cultivation of isolated plants may have favored the growth in diameter due to the absence of competition, as mentioned for *T. riograndense* (CONTERATO *et al*., 2010). Also, in Uruguayan populations of *T. polymorphum*, plant yield scores were quite variable (1-9, with 1 being the smallest and 9 being the largest plants). The number of stolons was also highly variable, with an average of 1.3 and a maximum of 23 stolons per plant (REAL *et al*., 2007). Unlike the results presented here, Conterato *et al*. (2013) found average values of 20.93 cm diameter and 8.50 cm height in plants of aerial origin in *T. argentinense*. In *T. riograndense*, a significant variation was observed in the first cut related to height (3.0 to 6.8 cm), diameter (4.5 to 28.2 cm), and the number of primary stolons (1.9 to 4.9) in the first cut, as well as in the second cut there was variation for these characteristics (CONTERATO *et al*., 2010).

T. polymorphum allowed only one cut with low dry matter production and slight variation among accessions (Table 2). Only the difference between the largest (Uruguaiana with 2.905 g) and the smallest (Quaraí and Santana do Livramento with 1.16 g and 0.876 g, respectively) yields was significant. Differently, the other native species *T. riograndense* allowed two cuts with total dry matter production greater than 7 g per plant (7.22 g to 8.73 g) in some accessions (CONTERATO *et al*., 2010). The low production of dry matter in

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T. polymorphum corroborates with the low growth rate in diameter, height and stolon growth (SOUZA *et al.*, 1988), and the low production of dry matter in one cut per year compared to the cultivars BR1 Bagé, Regal, Jacui and Guaiba of white clover (*T.repens*) (MORAES *et al.*, 1989). Despite the low dry matter production, the authors observed that *T. polymorphum* exhibited good forage quality (18.01 % crude protein). Besides, it is appetizing to cattle and of great importance because it appears in winter when native pasture are paralyzed in their growth (KAPPEL, 1967). In Uruguay, *T. polymorphum* is an essential component of natural pastures for its excellent adaptation, quality, and palatability (SPERONI and IZAGUIRRE, 2003).

The number of inflorescences per plant, number of flowers per inflorescence, number of flowers with vegetables with seeds, number of flowers without vegetables or with vegetables without seeds, of probably viable seeds and probably nonviable seeds did not differ among populations (Table 3), as did the variation in total seed production per plant (11 to 182.20 seeds) (P = 0.0563) (detailed data not shown). Although without significant variation, plants from the Santa Margarida do Sul population (population 23) numerically produced the highest values for these variables (Table 3), and only one plant from this same population produced six subterranean (probably nonviable) seeds from five subterranean fruits. The non-differentiation in the production of probably viable and probably nonviable aerial seeds among the populations may be related to the small sample size and the significant variation in inflorescences and flowers produced by the plants. Although there was no significant aerial seed production of *T. polymorphum* after cutting, these data are essential because there is a paucity of information on seed production in native forage species. Under the conditions tested, 55 % of the seeds germinated (66 seeds), and 45 % did not germinate (54 seeds, 47 of which were hard seeds and seven dead seeds), highlighting the importance of tests to evaluate the germination potential of seeds.

Table 3. Number of inflorescences (INF), number of flowers per inflorescence/plant (F/INF), number of flowers with seed legumes (FSL), number of flowers without legumes or with seedless legumes (FwL/LwS), number of seeds probably viable (VS), number of seeds probably not viable (NVS), number (SR) and dry weight (DW) of reserve roots per plant in *T. polymorphum* accessions.

Access	INF	F/INF.	FSL	FwL/LwS	VS	NVS	SR	DW (g)
39. Quaraí	-	-	-	-	-	-	21.25 a	3.19 a
40. Uruguaiana	7.88 a*	124.00 a	19.25 a	106.88 a	14.33 a	15.00 a	16.50 a	3.00 a
33. Bagé	1.00 a	24.00 a	6.50 a	17.50 a	-	11.00 a	13.10 a	1.64 a
30. Caçapava do sul	-	-	-	-	-	-	32.55 a	2.37 a
26. Santa Margarida do Sul	9.25 a	185.00 a	66.00 a	135.50 a	60.66 a	48.33 a	31.55 a	2.88 a
35. Santana do Livramento	-	-	-	-	-	-	20.87 a	2.08 a
31. Hulha Negra	2.80 a	80.60 a	36.80 a	43.80 a	41.40 a	25.50 a	21.80 a	1.69 a
23. Santa Margarida do Sul	17.40 a	321.60 a	114.60 a	207.00 a	127.00 a	55.20 a	22.40 a	2.42 a

*Means followed by the same letter in each column do not differ significantly by Tukey's test at 5 %.

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Generally, amphicarpic plants produce more aerial seeds than subterranean seeds, validating these data with those of Conterato *et al*. (2019), Weiss (1984); Cheplick and Quinn (1983); Choo *et al*. (2014) and Zhang *et al*. (2017) who evaluated other amphicarpic species. The production of probably viable and probably non-viable aerial seeds after cutting in *T. polymorphum* was lower than that obtained by Conterato *et al*. (2019), where the plants evaluated did not undergo any cutting or grazing of the inflorescences. However, in that study, during cutting for dry matter production evaluation, many inflorescences were cut along with the vegetative part, probably reducing aerial seed production to some extent, in addition to the numerous aerial flowers that contained mature seedless legumes or aerial flowers that did not even form legumes. According to Speroni *et al*. (2014), *T. polymorphum* makes a significant energy investment in aerial and underground flowering; however, most aerial flowers are consumed by livestock and the remaining flowers show low fruit set, but they are essential for incorporating genetic variability and long-distance dispersal in a species that combines autogamous subterranean flowers with vegetative propagation.

The importance of subterranean seeds in amphicarpic plants may differ between plant species and the environment (CHOO *et al*., 2014). In *T. polymorphum*, subterranean seeds can be referred to as an alternative security system in terms of population persistence and *in situ* reproduction (CONTERATO *et al*., 2019), confirming our data of no subterranean seed formation in all plants with data obtained by other authors (SPERONI *et al*., 2014; CONTERATO and SCHIFINO-WITTAMANN, 2014). Similarly, in *Amphicarpaea bracteata* and *A. edgeworthii*, subterranean seeds form a transient seed bank (ZHANG *et al*., 2015), while in *Centrosema rotundifolium* (Fabaceae), subterranean seed production is an effective mechanism of the species to maintain a reserve of them in the soil and therefore to contribute to the persistence of the plants under grazing (SCHULTZE-KRAFT *et al*., 1994).

The mean variation in the number and dry weight of storage roots (P = 0.5509) was not significant among populations, and the values of the number of storage roots were similar to those obtained in populations of *T. polymorphum* (CONTERATO and SCHIFINO-WITTMANN, 2014) and in *T. argentinense* (CONTERATO *et al*., 2013). The production of storage roots in *T. polymorphum* evidences the allocation of resources for an important reproductive strategy in addition to amphicarpy to ensure the persistence of the species and maintain the parental genotype (CONTERATO and SCHIFINO-WITTAMAN, 2014), as occurs in *C. rotundifolium* (SCHULTZE *et al*., 1997) and *T. argentinense* (CONTERATO *et al*., 2013). Such a strategy enables plants to persist vegetatively year after year in unfavorable environments without the need for regeneration through seeds.

The analysis of correlation coefficients (Table 4) revealed a positive correlation between plant height and total dry matter (r = 0.44) and between plant diameter and total dry matter (0.43), indicating that taller and larger plants in diameter lead to an increase in dry matter production. In *T. riograndense*, Conterato *et al*. (2010) reported a positive correlation between total dry matter production and plant diameter with plant height, and Bortolini *et al*. (2006) found a positive correlation between stature and leaf area and between total dry matter production and leaf area in white clover. Such results confirm those Rosso and Pagano (2001) obtained, which show that large-leaved populations showed higher dry matter yields.

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Table 4. Simple linear correlation between the analyzed traits in T. polymorphum: PLH (plant height), PLD (plant diameter), ST1 (number of primary stolons), DM (total dry matter), INF (number of inflorescence per plant), F/INF (number of flowers per inflorescence/plant), FLS (number of flowers per plant with seed legumes), FwL/LwS (number of flowers per plant without legumes or flowers with legumes without seeds), TS (total seeds per plant), VS (viable seeds per plant), NVS (non-viable seeds per plant), SR (number of storage roots per plant), DW (dry weight storage roots per plant).

	PLH	PLD	ST1	DM	INF	F/ INF	FSL	FwL/ LwS	TS	VS	NVS	SR	DW
PLH		0.17	-0.05	0.44*	-0.16	-0.21	-0.30	-0.13	-0.34	-0.26	-0.28	0.07	0.14
PLD			0.35*	0.43*	0.00	-0.04	-0.15	0.02	-0.18	-0.11	-0.17	0.12	0.15
ST1				0.02	-0.17	-0.25	-0.37	-0.20	-0.37	-0.34	-0.37	0.02	0.03
DM					0.16	0.14	0.18	0.15	0.20	0.13	-0.24	0.21	0.20
INF						0.98*	0.84*	0.98*	0.84*	0.77*	-0.79*	-0.20	-0.22
F/INF							0.91*	0.98*	0.91*	0.84*	-0.85*	-0.15	-0.20
FSL								0.79*	0.99*	0.98*	0.81*	0.14	0.02
FwL/ LwS									0.79*	0.69*	0.80*	-0.22	-0.25
TS										0.98*	0.85*	0.16	0.02
VS											0.69*	0.20	0.19
NVS												0.03	-0.10
SR													0.89*

*Significant at 5 % probability by t-test (P < 0.05).

Plant diameter was weakly correlated (r = 0.35) with the number of primary stolons, indicating that a greater number of primary stolons does not increase plant diameter. Stolon growth is possibly an essential variable for plant diameter, as cited for *T. riograndense* (MORAES *et al*., 1989; CONTERATO *et al*., 2010). This could also be applied to *T. polymorphum* since the species occurs in natural pastures associated with other species, such as grasses, and its persistence is related to the production of stolons where the storage roots and seeds are formed. The number of inflorescences per plant was positively correlated with the number of flowers per plant (r = 0.98), number of flowers per plant bearing legumes with seeds (r = 0.84), number of flowers per plant without legumes or with legumes without seeds (r = 0.98) and total seeds per plant (r = 0.84), evidencing the importance of these variables as a reproductive strategy of the plants. These results indicated that the higher the number of inflorescences per plant, the more seeds could be produced, possibly due to the higher number of flowers. The high positive correlation between the number of storage roots and dry weight of storage roots (r = 0.89) evidences that more storage roots lead to an increase in root weight. Because *T. polymorphum* is an amphicarpic species that also reproduces vegetatively, both reproductive strategies contribute to the species' success.

Data on morphological and agronomic characteristics such as dry matter production and seed production in native forage species are scarce but essential since much of the livestock activity in Rio Grande do Sul is developed in native pasture. The results showed low dry matter production in *T. polymorphum*. However, the amphicarpic condition with the production of aerial and subterranean seeds by the same plant,

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added to the vegetative propagation by regrowth of storage roots, are mechanisms that allow the species to increase its frequency and contribution in the natural pastures of Rio Grande do Sul.

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Conflict of Interests

The authors declare that the research was conducted in the absence of any potential conflicts of interest.

Ethical Statements

The authors confirm that the ethical guidelines adopted by the journal were followed by this work, and all authors agree with the submission, content and transfer of the publication rights of the article to the journal. They also declare that the work has not been previously published nor is it being considered for publication in another journal.

The authors assume full responsibility for the originality of the article and may incur on them any charges arising from claims, by third parties, in relation to the authorship of the article.

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