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ORIGINAL ARTICLE

Reproductive strategies in the persistence of polymorph clover, an amphicarpic species

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Abstract - Amphicarpic plants produce aerial and subterranean fruits on the same plant. *Trifolium polymorphum* Poir. is an amphicarpic legume that also reproduces vegetatively by regrowing storage roots. Because of this, resource allocation information for different reproductive strategies is of great interest. In this study, the production of aerial and subterranean seeds and storage roots was evaluated in populations of *T. polymorphum*. The Pinheiro Machado and Eldorado do Sul populations produced on average the highest (165.50) and lowest (61.87) number of inflorescences per plant, respectively. Total aerial seed production did not differ between populations. Aerial flowers produced more seeds than subterranean flowers. There were positive correlations between number of inflorescences and total aerial seeds (r = 0.73), the number of subterranean legumes and total subterranean seeds (0.94) and between number and weight of storage roots (r = 0.83). Amphicarpy associated with vegetative reproduction is an important strategy for the persistence and increase frequency of *T. polymorphum* in the natural pastures of the Rio Grande do Sul, where intense grazing and trampling can destroy periodically the aerial part of the plants.

Keywords: *Trifolium polymorphum.* Aerial seeds. Subterranean seeds. Vegetative reproduction. Natural pastures.

Estratégias reprodutivas na persistência de trevo polimorfo, uma espécie anficárpica

Resumo - As plantas anficárpicas produzem frutos aéreos e subterrâneos na mesma planta. *Trifolium polymorphum* Poir. é uma leguminosa anficárpica que também se reproduz vegetativamente pelo rebrote de raízes de reserva. Por causa disso, informações sobre a alocação de recursos para diferentes estratégias reprodutivas são de grande interesse. Neste estudo, foi avaliada a produção de sementes aéreas e subterrâneas e de raízes de reserva em populações de *T. polymorphum*. As populações Pinheiro Machado e Eldorado do Sul produziram em média o maior (165,50) e o menor (61,87) número de inflorescências por planta, respectivamente. A produção total de sementes aéreas não diferiu entre as populações. As flores aéreas produziram mais sementes do que as flores subterrâneas. Houve correlações positivas entre número de inflorescências e total de sementes aéreas (r = 0,73), número de legumes subterrâneos e total de sementes subterrâneas (0,94) e entre número e peso de raízes de reserva (r = 0,83). A anficarpia associada à reprodução vegetativa são estratégias importantes para a persistência de *T. polymorphum* nas pastagens naturais do Rio Grande do Sul, onde o pastejo intenso e o pisoteio podem destruir periodicamente a parte aérea das plantas.

Palavras-chave: *Trifolium polymorphum.* Sementes aéreas. Sementes subterrâneas. Reprodução vegetativa. Pastagem natural.

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Introduction

Diversity in plant reproductive biology proves the wide adaptive ability among plants (Barret, 2010). In addition, plant species combine different reproductive strategies or reproduction modes to ensure persistence over time and space (Speroni *et al*., 2014). One of these strategies is amphicarpy, a kind of seed heteromorphism by which the same plant produces aerial and subterranean flowers and fruits (Baskin and Baskin, 2014) that differ in size/mass (Conterato *et al*., 2019), dispersal ability (Zhang *et al*., 2015), and dormancy (Kaul *et al*., 2000). Zhang *et al*. (2020) cited 67 amphicarpic species from 39 genera of flowering plants, of which the largest group was the family Fabaceae. The two types of propagules have different ecological roles: the aerial flowers, usually cross-pollinated, produce genetically variable seeds with the possibility of dispersion away from the mother plant, while the subterranean self-pollinated flowers produce seeds that germinate *in situ*, ensure the parental genotype and local persistence (Kaul *et al*., 2000; Cheplick, 1987). Amphicarpy has been hypothesized to be an adaptive response to different pressures of selection, including herbivory, drought, fire, desiccation, predation of seeds (Cheplick, 1987; Kaul *et al*., 2000; Kumar *et al*., 2012; Speroni *et al*., 2014).

The genus *Trifolium* is one of the most important in the Fabaceae family with approximately 250 species (Lewis *et al*., 2005), with a wide distribution throughout the temperate and subtropical regions of the world (Zohary and Heller, 1984). Many species of clover are widely cultivated as animal fodder, soil and pasture improvement, honey plants, hay and silage, and in horticulture (Lewis *et al*., 2005). *Trifolium polymorphum* Poir. is a perennial, stoloniferous winter species that occurs in Uruguay, Argentina, Paraguay, Chile, and southern Brazil (Burkart, 1987; Dalla Rizza *et al*., 2007). During winter, it is an important qualitative component in natural pastures for its good quality and palatability (Coll and Zarza, 1992; Speroni and Izaguirre, 2003), with protein values of 18.01 % (Moraes *et al*., 1989). In the southern region of Brazil, *T. polymorphum* occurs in the states of Rio Grande do Sul and Paraná (Iganci *et al*., 2020) and it has an interesting reproductive attribute because it combines amphicarpy with vegetative propagation by regrowth from storage roots (Speroni and Izaguirre, 2003; Conterato and Schifino-Wittmann, 2014) (Fig. 1). As in other amphicarpic species, its subterranean seeds are heavier and fewer than the aerial ones (Conterato *et al*., 2019). While the production of subterranean seeds is not affected by the removal of aerial biomass, Speroni *et al*. (2014) cited that most aerial flowers are consumed by livestock, and the remaining flowers show low fruit set. However, few studies have focused on resource allocation for different reproductive strategies to ensure species regeneration and persistence. In this study we evaluated the production of aerial seeds, subterranean seeds and storage roots in *T. polymorphum* populations in the absence of cutting.

Materials and Methods

In March 2015, aerial seeds of natural *T. polymorphum* populations/accessions previously collected in the distribution area of the species (in the southern half of the state of Rio Grande do Sul , Brazil: accession: 06- Rio Pardo (29º59’S, 52º22’W), 11- Aceguá (31º45’S, 54º03’W), 17- Eldorado do Sul (30º05’S, 51º36’W), 20- Pinheiro Machado (31º34’S, 53º22’W), 23- Santa Margarida do Sul (30°20'S, 54°4'W), 25- Vila Nova do Sul (30°20', 53°52’W)) were scarified with sandpaper number 180 and germinated in Petri dishes on moistened filter paper. Ten germinated seedlings from each population were transferred to

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plastic boxes (34 cm × 14 cm × 11.5 cm) filled with commercial substrate composed of pine bark, vermiculite, acidity corrector and macronutrients (one plant per box) and kept in an open area of the Secretaria da Agricultura, Pecuária, Produção Sustentável e Irrigação (30°20′27″S; 54°19′01″W), São Gabriel, Rio Grande do Sul/Brazil. In December 2015, after the senescence of the aerial part, the plastic boxes were kept in this area and watered daily to preserve the storage roots. In March 2016, a storage root of each plant from 2015 was transplanted to plastic boxes (one plant per box) in an open area, aiming at the collection of aerial and subterranean seeds and storage roots of *T. polymorphum*. Each box was considered a replicate. The plants were watered daily, and invasive plants were removed manually. No chemical fertilization was applied to the plants during the course of the experiment.

Figure 1. *Trifolium polymorphum* Poir.: a) aerial inflorescences and young storage roots; b) storage roots and mature subterranean legumes.

In November 2016, the aerial inflorescences of each plant were collected separately in paper bags and kept in the refrigerator. In the first half of December 2016, the plastic boxes were removed from the experimental area due to high temperatures and transferred to a covered area, where they no longer received water to facilitate the removal and counting of the subterranean legumes. Afterward, the substrate of the flower pots was removed, the storage roots were counted, and the subterranean legumes of each plant were collected in paper bags and kept in the refrigerator at a temperature of 4 ºC to 6 ºC . Later, in 2018, aerial and subterranean legumes were manually opened and seeds counted. A visual assessment according to appearance was estimated for putatively viable seeds (full, developed, and yellow seeds and putatively non-viable seeds (empty and/or dark brown seeds) according to Conterato *et al*. (2019) and Conterato *et al*. (2020). The evaluation of the populations in this study was carried out in 2018, as the collection of aerial inflorescences and underground legumes took place at the end of 2016. As there was a significant production of inflorescences per plant in the populations, counting the aerial seeds was a very time-consuming task. In addition, underground legumes and seeds were also evaluated.

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

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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.).

Results and Discussion

The results and discussion will be analyzed at the population level, however in some *T. polymorphum* plants no mature underground legumes were observed. For many of the characteristics evaluated, there were no differences between populations. However, the number of inflorescences (165.50) and putatively viable seeds (1106.33) in the Pinheiro Machado population differed significantly from the Eldorado do Sul population (61.87, 44.38, respectively), with no difference between the other populations (Table 1). This difference may be related to the number of inflorescences per plant, the number of flowers per inflorescence and number of flowers per plant bearing legumes with seeds, indicating that the greater the number of inflorescences per plant, the more seeds could be produced, possibly due to the greater number of flowers (Conterato *et al*., 2023). Differences in the number of putatively non-viable seeds and the total number of seeds were not significant. The large production of inflorescences, of putatively viable and non-viable seeds in the absence of cutting in *T. polymorphum* differs from Conterato *et al*. (2023), who reported reduced number of inflorescences and seeds after a cut when most accessions were in flowering and from Speroni *et al*. (2014) who mentioned that most of the aerial flowers are consumed by cattle and the rest of the flowers show low fruit set. These results support herbivory or cutting as one of the possible causes of low aerial seed production in *T. polymorphum*.

An expressive production of aerial seeds makes possible the colonization of new places; however the number of putatively non-viable seeds was also high (Table 1). In contrast, more than 90 % of the seeds were putatively viable in two populations of *T. polymorphum* grown in 2016 in the same area (Conterato *et al*., 2019). Given that in this study the visual classification into putatively viable and non-viable seeds occurred in 2018 (and not at the end of 2016), it is not possible to know whether the seeds were already putatively non-viable when the inflorescences were collected or if, over time, many seeds became putatively non-viable because of inadequate storage conditions, e.g., temperature and humidity. However, even when stored under optimal conditions, all seeds will eventually lose their viability as they age and die, a process called seed deterioration or ageing (McDonald, 1999; Ebone *et al*., 2019). These questions are important because seeds can also be used to provide for the long-term preservation of genetic diversity and serve as the focal point of efforts aimed at environmental restoration (Bewley *et al*., 2013; Baskin and Baskin, 2020) and the evolution of natural populations (Akash *et al*., 2022). A significant production of aerial seeds in *T. polymorphum* can be used to increase their frequency in the natural pastures in Rio Grande do Sul. The Pinheiro Machado (160.83) and Rio Pardo (133.00) populations produced more subterranean legumes than Eldorado do Sul population (1.00). There was significant variation in the production of putatively viable subterranean seeds among Pinheiro Machado (26.50), Santa Margarida do Sul (65.75) and Eldorado do Sul (0.75) populations. For putatively non-viable seeds, the variation was significant between populations Pinheiro Machado (200.17), Rio Pardo (125.71) compared to Aceguá (20.50) and Eldorado do Sul (0.62). In *T. polymorphum*, the production of subterranean seeds was lower than that of aerial seeds

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Table 1. Average values in *Trifolium polymorphum* Poir. for number of inflorescence (IN), number of putatively viable aerial seeds (VAS), number of putatively non-viable aerial seeds (NVAS), total aerial seeds (TAS), number of subterranean legumes (SL), number of putatively viable subterranean seeds (VSS), number of putatively non-viable subterranean seeds (NVSS), total subterranean seeds (TSS), number of storage roots (SR) and green weight of storage roots (WSR).

Accession	Aerial legumes					
	IN	VAS	NVAS	TAS		
20 - Pinheiro Machado	165.50 a*	1106.33 a	478.66 a	1585.00 a		
23 - Santa Margarida do Sul	134.75 ab	917.12 ab	352.25 a	1269.37 a		
25 - Vila Nova do Sul	130.88 ab	642.00 ab	717.88 a	1359.88 a		
06 - Rio Pardo	111.00 ab	372.57 ab	629.57 a	1002.14 a		
11 - Aceguá	99.00 ab	727.62 ab	283.00 a	1010.62 a		
17 - Eldorado do Sul	61.87 b	44.38 b	334.00 a	378.37 a		
Accession	**Subterranean legumes**				**Storage roots**	
	SL	**VSS**	**NVSS**	**TSS**	**SR**	**WSR(g)**
20 - Pinheiro Machado	160.83 a	26.50 a	200.17 a	226.67 a	50.16 ab	5.20 b
23 - Santa Margarida do Sul	92.88 ab	65.75 a	40.12 ab	105.88 abc	94.87 a	30.40 a
25 - Vila Nova do Sul	52.67 ab	14.56 ab	40.11 ab	54.67 abc	44.66 b	10.97 ab
06 - Rio Pardo	137.00 a	14.43 ab	125.71 a	140.14 ab	36.14 b	9.77 b
11 - Aceguá	35.00 ab	9.38 ab	20.50 b	29.88 bc	44.12 b	11.38 ab
17 - Eldorado do Sul	1.00 b	0.75 b	0.62 b	1.38 d	52.37 ab	20.75 ab

*Values followed by the same letter in each column are not significantly different from each other (F-test, at P = 0.05). Accession 17: Depressão Central physiographic region; accession 06, 11, 20, 23, 25: Campanha physiographic region.

Table 2. Correlations among the analyzed characteristics in *Trifolium polymorphum* Poir.: number of inflorescence (IN), number of putatively viable aerial seeds (VAS), number of putatively non-viable aerial seeds (NVAS), total aerial seeds (TAS), number of subterranean legumes (SL), number of putatively viable subterranean seeds (VSS), number of putatively non-viable subterranean seeds (NVSS), total subterranean seeds (TSS), number of storage roots (SR) and green weight of storage roots (WSR).

	IN	VAS	NVAS	TAS	SL	VSS	NVSS	TSS	SR	WSR
IN	-	0.66*	0.48*	0.73*	0.47*	0.36*	0.43*	0.48*	-0.22	-0.36*
VAS	-	-	-0.27	0.92*	0.42*	0.48*	0.43*	0.51*	-0.06	-0.19
NVAS	-	-	-	0.62*	0.27	0.08	0.23	0.22	0.24	-0.29
TAS	-	-	-	-	-0.45*	0.43*	0.44*	0.50*	-0.15	-0.27
SL	-	-	-	-	-	0.55*	0.90*	0.94*	-0.11	-0.26
VSS	-	-	-	-	-	-	0.33*	0.56*	0.12	-0.01
NVSS	-	-	-	-	-	-	-	0.97*	-0.15	-0.30*
TSS	-	-	-	-	-	-	-	-	-0.10	-0.27
SR	-	-	-	-	-	-	-	-	-	0.83*
WSR	-	-	-	-	-	-	-	-	-	-

* Significant at 5 % probability, t-test ($p < 0.05$).

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as occurs in most amphicarpic species such as *Gymnarrhena micranta* Desf. (Kaul *et al*., 2000), *Trifolium argentinense* (Conterato *et al*., 2013), *Persicaria thunbergii* (Nam *et al*., 2017), *Emex australis* (Borger and Wilkins, 2022) e *Scrophularia arguta* (Rodríguez-Riãno *et al*., 2022). These results agree with Speroni *et al*. (2009) who observed four ovules in most aerial flowers and two ovules in each subterranean flower and Conterato *et al*. (2019) where mostly subterranean legumes formed one or two seeds, while the aerial legumes formed up to four seeds. Although few in number, subterranean seeds ensure the persistence of the population, maintain the parental genotype and according guarantee the maintenance of the population *in situ* (Cheplick, 1994), and could decrease competition between seedlings.

The high number of putatively non-viable subterranean seeds in the boxes may be related to the formation of subterranean seeds that are closer to the soil surface and less protected from high temperatures, or they may have occurred seed deterioration as suggested for aerial seeds. Not all *T. polyporphum* plants produced subterranean seeds, reinforcing field observations from Uruguayan populations that underground flowering can occur simultaneously with aerial flowering during the winter and springer months, or not occur throughout the year (Speroni *et al*., 2010). This occasional underground seed formation in *T. polymorphum* has been cited as an alternative security system in terms of population persistence (Conterato *et al*., 2019), while in *Centrosema rotundifolium*, the production of subterranean seeds is an effective mechanism for the species to maintain a reserve of them in the soil, and therefore contribute to the persistence of plants in grazing (Schultze-Kraft *et al*., 1994). As subterranean *T. polymorphum* seeds are away from the harvest horizon of herbivores (Speroni *et al*., 2014), they may be able to germinate close to the mother plant as they are already in the soil and recolonize the site, provided that they maintain viability. For most amphicarpic species the subterranean cleistogamous seeds are dispersed to a favorable and predictable microsite (i.e., the parental microsite) (Trapp, 1988).

The Santa Margarida do Sul population had the highest number and weight of storage roots (average of 94.87 and 30.40g, respectively). In the other populations, there was little significant variation for these two variables (Table 1). Given that the storage roots are below the grazing level of herbivores (such as subterranean seeds), they are an important mechanism for the regeneration and persistence of *T. polymorphum* since each storage root, when regrowth, can originate a new plant after disturbance such as herbivory (Speroni *et al*., 2014) or after natural senescence of the aerial part of the plant in summer (Speroni and Izaguirre, 2003). Besides, this form of vegetative reproduction maintains the parental genotype and favors persistence in unfavorable habitats for reproduction sexual, allowing plants to persist vegetatively year after year without the need for seed regeneration (Conterato *et al*., 2019). According to Yang and Kim (2016), vegetative reproduction has high competitive ability. However, long periods of asexual reproduction may reduce the dispersal capacity of the species over large distances and affect genetic diversity within population (McLellan *et al*., 1997). In *T. polymorphum* Real *et al*. (2007), by using SSR explained the high polymorphism between populations by cross-pollination aerial seed dispersal and the low intra-patch variability by vegetative propagation and underground seeds.

The analysis of the correlation coefficient showed a positive correlation between the number of inflorescences and the number of putatively viable aerial seeds (r = 0.66) and total aerial seeds (r = 0.73). Positive correlations also occurred between the number of putatively viable seeds and total aerial seeds (r = 0.92) and between the total number of putatively non-viable aerial seeds and total aerial seeds (r = 0.62) evidencing the importance of these variables for seed production (Table 2). These data indicate that the greater the number of inflorescences per plant, more aerial seeds will be formed, most likely due to the greater number of flowers, as observed in populations of *T. polymorphum* maintained in the field (Conterato *et al*., 2023). The number of subterranean pods was positively correlated with the number of putatively viable subterranean seeds (r = 0.55), the number of putatively non-viable subterranean seeds (r = 0.90) and the total number of subterranean seeds (r = 0.94). The total number of subterranean seeds was also positively correlated with the number of putatively viable subterranean seeds (r = 0.56) and the number of putatively non-viable subterranean seeds (r = 0.97) (Table 2). These correlations were expected since subterranean legume carry seeds. A high correlation (r = 0.83) occurred between number of storage roots and storage root weight, indicating that more storage roots lead to an increase in root weigh.

The reproductive system of *T. polymorphum*, allows the species to grow in disturbed habitats such as the natural pastures of Rio Grande do Sul, a place of intense grazing and trampling in which *T. polymorphum* overcomes by combining the amphicarpy (aerial and subterranean seeds) and vegetative reproduction (regrowth of storage roots). In this context, an efficient production of seeds and storage roots can be used to increase the persistence and frequency of *T. polymorphum* in natural pastures of Rio Grande do Sul, improving forage production and nutritional quality.

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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.

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