

**Reproductive strategies and dimorphic seeds germination in *Trifolium argentinense* Speg., an amphicarpic species**Ionara Fátima Conterato<sup>1\*</sup>, Maria Teresa Schifino-Wittmann<sup>2</sup>, Diego Bitencourt de David<sup>1</sup>, Jorge Dubal Martins<sup>1</sup>

**Abstract** - Native from Rio Grande do Sul natural pastures, *Trifolium argentinense* Speg. has sexual reproduction through aerial and subterranean seeds (amphicarpic) and vegetative reproduction by regrowth from storage roots. In this study the seeds produced at the soil-surface flowers, the depth effect and scarification on the aerial and subterranean seeds germination and the storage roots produced by plants derived from the two types of seeds were evaluated. The aerial (0.10 cm) and soil-surface (0.11 cm) seeds were similar in size, but smaller than the subterranean seeds (0.14 cm), with no significant variation in the production of the three types of seeds. Aerial and subterranean seeds scarified at and sowed 2.5 cm deep germinated better than the scarified and non-scarified seeds sowed on the soil-surface and at 7.0 cm, evincing a depth effect on seed germination. Although amphicarpic, *T. argentinense* first invests in the production of the storage roots making sure the cloning of specific genotypes and allowing the plants to persist vegetatively year after year without the need for regeneration by seeds in unfavorable environments for sexual reproduction, such as in Rio Grande do Sul natural pastures, where hard grazing and trampling can destroy the aerial part of the plants.

**Keywords:** Amphicarpic. Reproductive biology. Dimorphic seeds. Natural pasture. Seeds germination. Storage roots.

**Estratégias reprodutivas e germinação de sementes dimórficas em *Trifolium argentinense* Speg., uma espécie anficárpica**

**Resumo** – Nativa das pastagens naturais do Rio Grande do Sul, *Trifolium argentinense* Speg. tem reprodução sexuada através de sementes aéreas e subterrâneas (anfícarpia) e reprodução vegetativa pelo rebrote das raízes de reserva. Neste estudo foram avaliadas as sementes produzidas nas flores da superfície do solo, o efeito de profundidade e escarificação na germinação de sementes aéreas e subterrâneas e as raízes de reserva produzidas por plantas derivadas dos dois tipos de sementes. As sementes aéreas (0,10 cm) e da superfície do solo (0,11 cm) foram semelhantes em tamanho, mas menores que as sementes subterrâneas (0,14 cm), com nenhuma variação significativa na produção dos três tipos de sementes. Sementes aéreas e subterrâneas escarificadas e semeadas a 2,5 cm de profundidade germinaram melhor que as sementes escarificadas e não escarificadas semeadas na superfície do solo e a 7,0 cm, evidenciando um efeito de profundidade na germinação de sementes. Embora anficárpica, *T. argentinense* investe primeiramente na produção de raízes de reserva assegurando a clonagem de genótipos específicos e permitindo que as plantas persistam vegetativamente ano após ano sem a necessidade de regeneração por sementes, em ambientes desfavoráveis à reprodução sexual, como nas pastagens naturais do Rio Grande do Sul, onde o pastejo intenso e o pisoteio podem destruir a parte aérea das plantas.

**Palavras-chave:** Anfícarpia. Biologia reprodutiva. Sementes dimórficas. Pastagem natural. Germinação de sementes. Raízes de reserva.

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## Introdução

Plants with flowers exhibit diversity in reproductive systems (BARRETT et al., 2008) and different strategies to deal with environmental variables and assure persistence in time and space (SPERONI et al., 2014). One of these strategies hypothesized in answer to different pressures of selection, including herbivory, drought, fire, desiccation, predation of seeds (CHEPLICK, 1987; KAUL et al., 2000, KUMAR et al., 2012) is amphicarp, a type of seeds heteromorphism (BASKIN ; BASKIN, 2014), with production on the same plant of both aerial and subterranean fruits and seeds (CHEPLICK, 1987; LEV-YADUN, 2000). In *Fabaceae* the production of two or more types of different seeds by the same individual is associated with amphicarp (IMBERT, 2002). As chasmogamous and cleistogamous flowers in amphicarpic species are produced under different environmental conditions and/or positions in mother plant, differences in seeds or fruit mass (CHEPLICK; QUINN, 1982; CONTERATO et al., 2010; SPERONI et al., 2014; CHOO et al., 2014; ZHANG et al., 2017), dormancy, germination, vigor and survival (WEISS, 1980; CHEPLICK; QUINN, 1987; BASKIN; BASKIN, 2014) were observed.

In Southern Brazil, there are three native species of *Trifolium*: *Trifolium riograndense* Burkart, *T. argentinense* Speg. and *T. polymorphum* Poir., the two latter being the only amphicarpic species of the genus. These native *Trifolium* species contribute to the high quality production of beef, dairy products, leather and wool with low levels of inputs to the system (DALLA RIZZA et al., 2007). *T. argentinense* is a plant that occur in a low frequency in the natural pastures of the southern half of Rio Grande do Sul, Uruguay and Argentina (BURKART, 1952) and combines two types of reproductive strategies: asexual propagation by regrowth of storage roots and sexual reproduction by two types of flowers: aerial and subterranean with distinct ecological functions. A third type of flower, produced at soil- surface has been observed, but the exact nature of this type of flower is unknown (CONTERATO et al., 2013). Vegetative propagation contributes mainly to the local population growth and preservation of the parental genotype. In contrast, the aerial flowers allow dispersion of seeds over long distances, reduction of intra-specific competition and assure genetic diversity due to the preferential allogamy, while the cleistogamous subterranean flowers contribute to the population local growth (CONTERATO et al., 2013). The subterranean flowers of *T. argentinense* produce larger seeds, heavier and fewer in number than those of aerial flowers (CONTERATO et al., 2010), while those present more anthers, produce more pollen grains and more seeds than the subterranean ones. Soil-surface flowers, possibly cleistogamous had fewer anthers and pollen than did aerial flowers (CONTERATO et al., 2013).

The studies by Conterato et al., (2010) and Conterato et al., (2013) contributed to a better knowledge about *T. argentinense*. However, more information about resource allocation for each different reproductive strategies and about requirements for germination of aerial and subterranean seeds are important and necessary



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for a better understanding of the survival mechanisms and regeneration ability of populations. In this study we evaluated (i) the size and number of seeds produced by aerial, subterranean and soil-surface flowers, (ii) the effect of seeds' scarification and depth of seed sowing at the germination of aerial and subterranean seeds; (iii) the weight and number of storage roots in plants derived from germination of aerial and subterranean seeds of *T. argentinense*.

## Material and Methods

Aerial inflorescences and mature subterranean legumes were collected from 25 plants of *T. argentinense* maintained in the field in an area of Departamento de Diagnóstico e Pesquisa Agropecuária (DDPA), Secretaria da Agricultura, Pecuária e Desenvolvimento Rural (SEAPDR), São Gabriel (50° 20' 11" S, 54° 19' 12" W), Rio Grande do Sul, Brazil, in December, 2012. Afterwards, the legumes were manually opened and the seeds collected and packed separately in bulk according to their origin (aerial *versus* subterranean) in small paper bags in a place at room temperature for later use in experiments. Soil-surface flowers were not collected because they were removed by insects.

In April 2013 (autumn), 25 aerial seeds and 25 subterranean seeds were scarified with sandpaper and germinated in Petri dishes on moistened filter paper. The seedlings germinated were transferred to plastic cups of 200ml filled with commercial substrate. In August, 2013 (winter), 24 plants of aerial origin and 24 plants of subterranean origin were transferred to an open area in four lines, distant 0.50 m between plants and 1 m between lines in a completely randomized design, aiming the collection of different types of seeds. Each plant was considered an experimental unit. Four plants of each origin did not persist after the transplantation to the field and the experiment was conducted with 20 plants of each origin. No chemical fertilization was applied and invasive plants were controlled by manual elimination. In October, 2013, a dry matter cover was placed on the experimental area to maintain humidity and to protect flowers and seeds at soil level from high temperatures. In November (days – 8<sup>th</sup>, 14<sup>th</sup> and 22<sup>nd</sup>) and December (days – 17<sup>th</sup> and 18<sup>th</sup>), 2013, the legumes at soil-surface were individually collected from the nine plants that produced these legumes (from a total of 40 plants that composed the experiment). In five of these plants, the legumes were manually opened and the seeds extracted, counted, measured with a graduated ruler. The other four plants were not evaluated because some mature legumes were damaged or removed by ants and this would impair the evaluation. In November (days – 14<sup>th</sup>, 22<sup>th</sup>, 30<sup>th</sup>) and December (days - 6<sup>th</sup>, 13<sup>th</sup>, 18<sup>th</sup>) the aerial inflorescences with mature legumes were collected individually and in December (days - 19<sup>th</sup> and 20<sup>th</sup>), the subterranean legumes were collected. The size of the three seed types produced by these five evaluated plants was determined by measuring 115 seeds of each type. Subsequently, the seeds were stored in bulk according to their origin (aerial, at soil-surface and subterranean).



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The influence of seed sowing depth and seeds' scarification at germination of seeds of *T. argentinense* was investigated in 2014 using seeds of the 2013 collection. In April, 2014, 20 seeds of aerial origin and 20 seeds of subterranean origin, not scarified and scarified with sandpaper were placed at 0 cm (soil-surface), 2.5 cm and 7.0 cm of depth in plastic cups of 300 ml. The plastic cups were filled with commercial substrate and disposed in plastic trays containing 10 cups, in a completely randomized design, in a total of 240 seeds (one seed per cup). Each tray with 10 cups was considered a repetition. During the day, the trays were maintained in an open area and at dusk (5 p.m.) they were transferred to a covered lightless room for preserve seeds at soil-surface and avoid cutting the seedlings. The cups were watered daily and the emergence of seedlings was registered at each three days, during a period of 90 days (from April, 1<sup>st</sup>, 2014 to July, 1<sup>st</sup>, 2014). The effect of scarification was also investigated in 2014, in 20 aerial and 20 subterranean seeds, scarified with sandpaper and not scarified, disposed in Petri dishes with moistened filter paper and maintained in a room with daily sun radiation. Each treatment (Petri dish) was replicated three times, in a total of 240 seeds. Germination was registered at each three days, during 90 days (from April 15<sup>th</sup>, 2014 to July 15<sup>th</sup>, 2014) and the seedlings removed to avoid the disturbance to other seeds.

To evaluate the number and weight of storage roots, the 22 plants derived from germination of aerial seeds and those 26 of subterranean origin (different depths) were transplanted to the field in August, 2014, in four lines, with 0.75 m between plants and 1.0 m between lines, in a completely randomized design. In January, 2015, the storage roots produced individually in 20 plants of aerial origin and 20 plants of subterranean origin were washed in running water, dried with paper towel, counted and weighed on a precision scale.

The data about the size and number of aerial seeds, at the soil surface and the subterranean ones, the number of seeds germinated in Petri dishes and the number and weight of storage roots derived from the germination of two types of seeds were submitted to the analysis of variance (ANOVA). The germination of aerial and subterranean seeds in different depths, scarified and not scarified was outlined in a factorial 2x2x3. The data were submitted to analysis of variance and the averages compared using the Tukey test at 5% of significance. The statistical analysis were carried out using the JPM software (V.12, SAS Institute Inc., Cary, NC, USA).

## Results and Discussion

The five plants evaluated produced 115 seeds in the soil-surface, 171 subterranean seeds and 199 aerial seeds (115 from which were included in size determination). There was no difference between the average size of seeds produced at soil-surface (0.11 cm) and the aerial seeds (0.10 cm) in *T. argentinense*. Nevertheless, both types of seeds were significantly smaller than the subterranean seeds (0.14 cm) (Table 1; Fig. 1a). Although



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discreet, the flowers at soil-surface exhibited numerically a lower production of seeds (23.00) in relation to the ones produced by the aerial (39.80) and subterranean flowers (34.20), but without statistical difference (Table 1).

**Table 1.** Averages for size and number of different seeds in *T. argentinense*.

Variable	Aerial seeds	Soil-surface seeds	Subterranean seeds	<i>P</i> value
Seed size (cm)	0.10 a	0.11 a	0.14 b	<.0001
Seed number	39.80 a	23.00 a	34.20 a	0.1689

Values in each row followed by the same letters do not differ significantly (Tukey test, at  $P = 0.05$ ).

In general, the germination of both types of seeds was low. Only 22 aerial seeds and 26 subterranean seeds of a total of 120 seeds of both types germinated. The aerial and subterranean scarified and non-scarified seeds and at 2.5 cm of depth germinated better than both types of scarified seeds and non-scarified at soil-surface and at 7.0 cm (Table 2) in each replicate with 10 seeds. There was significant interaction ( $P=0.0002$ ) between depth and scarification in the germination of seeds. The scarified seeds (aerial and subterranean) and at 2.5 cm of depth had significantly better average germination (7.5) than the scarified and non-scarified seeds at soil-surface (1.50 and 0.75) and at 7.0 cm (0.25 and 0.50), respectively in each replicate with 10 seeds (detailed data not shown). No interaction occurred between scarification and the type of seeds ( $P=0.8663$ ) and between the depth and the type of seed ( $P=0.7087$ ).

**Table 2.** Average germination of aerial and subterranean seeds in each replicate at different depths in *T. argentinense*.

Depth	Seed type	
	Aerial	Subterranean
Soil-surface	0.75c	1.50 b,c
2.50 cm	4.25 a,b	4.50 a
7.00 cm	0.50 c	0.50 c

Values followed by the same letters in each row are not significantly different from each other (Tukey test, at  $P=0.05$ ). It was analyzed 40 seeds of each type and at each depth (each replicate=10 seeds).



In Petri dishes, from 120 aerial seeds, 55 germinated (53 scarified and two non-scarified), while from 120 subterranean seeds, 64 germinated (57 scarified and seven non-scarified). On average, in each replicate (Petri dishes) with 20 seeds, the aerial scarified seeds (18.00) and subterranean scarified (19.00) had greater germination in relation to the non-scarified seeds (0.66 and 1.66, aerial and subterranean, respectively). Variation for both types of scarified seeds was not significant. When non-scarified, aerial and subterranean seeds did not differ from each other and had low germination (Table 3).

**Table 3.** Average germination in each replicate of different types of scarified and non-scarified seeds in *T. argentinense*.

Seed type	Treatment	
	Scarified seeds	Non-scarified seeds
Aerial	18.00 a	0,66 b
Subterranean	19.00a	1.66 b

Values followed by the same letters in each column are not significantly different from each other (Tukey test, at  $P=0.05$ ). Each replicate=20seeds.

In field conditions, the plants originated from germination of subterranean seeds produced on average storage roots significantly heavier (41.44 g) than the ones derived from germination of aerial seeds (27.22 g) ( $P<.0001$ ), and in greater number (27.72 and 14.60, respectively) ( $P<.0001$ ) (detailed data not shown).

The information about the production of seeds by flowers at soil surface in *T. argentinense* increases the knowledge about the species and indicates the allocation of reproductive resources for a fourth reproductive strategy, besides the production of aerial seeds, subterranean seeds and storage roots (CONTERATO et al., 2013) to ensure the species survival (Fig. 1). Seeds of aerial and subterranean flowers are produced under different environmental conditions and therefore it is reasonable to expect them to differ in several characteristics as size, mass and number (BASKIN; BASKIN, 2014). In this study, the flowers above the soil (aerial and at soil-surface) produced seeds lower than the ones with the flowers under the soil (subterranean) (Table 1). Kumar et al., (2012) suggested that while the smallest size can be an adjustable arrangement in aerial seeds, the greatest size of geocarpic seeds is more probable to be a positive feature in drier environments,



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because the larger seedlings could be capable to emerge from greater depths in the soil. In amphicarpic species without vegetative propagation (WEISS, 1980; CHEPLICK; QUINNN, 1982; KAUL et al., 2002) the plants produced by larger subterranean seeds were more vigorous and had better competitive ability than those originated from aerial propagules. Nevertheless, in other amphicarpic species with (CONTERATO et al., 2013; NIKOLIC et al., 2005) or without additional vegetative propagation (Siebold and Zucc.) H. Gross (KIM et al., 2016), this clear relation was not observed, supporting what was observed by Cheplick (1987) that amphicarpy is a highly variable phenomenon.

The flowers at soil-surface showed a discrete tendency to a lowest production of seeds in relation to the aerial and subterranean flowers, in agreement with the reduced number of anthers and lower production of pollen grains by these flowers (CONTERATO et al., 2013), as was found in *Commelina benghalensis* L.(KAUL et al., 2002). From the population point of view, production of seeds at soil-surface will be lower as only about 20% of the plants (nine out of 40 plants) produced this type of seed, while aerial and subterranean seeds were produced by all the plants. As in several amphicarpic species, *T. argentinense* produces more aerial seeds, whose function is to increase the species distribution in its habitat, while subterranean seeds serve mainly to ensure the species survival (CONTERATO et al., 2013). Although not very numerous, seeds at soil-surface may increase the number of offspring and population genetic variability due to the allogamy of aerial flowers (CONTERATO et al., 2013). These seeds may also partly compensate flowers, fruits and seeds loss due to herbivory and excessive trampling, and also be a way to reinforce local adaptation together with subterranean seeds and storage roots, as they are more protected of risk of chance dispersion.

Flowers at soil-surface, possibly cleistogamous (CONTERATO et al., 2013) produced legumes quite similar to the subterranean legumes (Fig. 1f). The legumes at soil-surface were produced only by some plants, throughout some stolons and in some nodes of these stolons that did not produce storage roots, that is, in some nodes that did not “root” (CONTERATO et al., 2013, Fig.1h). Nevertheless, several nodes that did not root did not produce flowers at soil-surface either and, thus, these results should be treated with caution, because they were obtained in only one year of observation. Further work is in progress, trying to elucidate if these flowers are really cleistogamous and produced at soil-surface or if they are so placed because they were not “buried” due to the non-rooting of the nodes.



**Figure 1.** Reproductive versatility in *Trifolium argentinense* Speg. (a) aerial inflorescence with chasmogamous flowers; (b) subterranean immature legumes; (c) storage roots and subterranean mature legumes; (d) plant with young flowers at soil-surface (arrow); (e) stolons with flowers and young legumes at soil-surface (arrow); (f) aerial, soil-surface, subterranean seeds and legumes (from left to right); (g) subterranean legumes between 2.5-3.0 cm depth; (h) young plant with numerous storage roots (arrow).





The maximum germination occurred in aerial and subterranean seeds scarified and sowed at 2.5 cm of depth (Table 2), a strong indicative that the seeds have some type of dormancy and that depth can influence in the relative success and establishment of *T. argentinense* population, probably due to a more favorable regime of moisture compared to soil-surface. In *Emex spinosa* (L.) Camb., more than 78% of the subterranean achenes and 60% of the aerial achenes emerged from 1-4 cm deep (WEISS, 1980), similar to what was obtained by Javaid and Tanveer (2014) for aerial seeds buried at 2 cm depth. In *Amphicarpum purshii* Kunth the average depth of 3.5 cm of subterranean seeds coincided with the depth of seeds from which the plants derived presented larger growth in height, biomass and reproductive performance (CHEPLICK; QUINN, 1987). Similar situation could be occurring in *T. argentinense*, maybe to protect the subterranean seeds from intense trampling, and lack of moisture on the soil surface of this region of Rio Grande do Sul in the end of spring (period of seeds maturation), thus ensuring a better chance for these seeds to retain viability and germinate. Observations at the field suggest that *T. argentinense* is unlikely to produce subterranean seeds at a depth greater than 3.0 cm (Fig. 1f). The poor germination of aerial and subterranean seeds scarified, and those at soil-surface in *T. argentinense* suggests that both types of seeds may not germinate under limited conditions of moisture, as in Rio Grande do Sul the species grows in the humid fields of South Campanha and South Coast (KAPPEL, 1967). In Uruguay it is restricted to humid soils until flooded (SPERONI; IZAGUIRRE, 2003).

Temperature and luminosity are environmental signs regulating germination, species distribution and ecological interaction (EBRAHIMI; ESLAMI, 2012). As during the present experiment there were no hydric restriction, osmotic and saline stresses and daytime temperature was similar to that endured by the species in natural conditions, the poor germination of aerial and subterranean seeds, scarified and not scarified and at 7 cm of depth can be related to the absence of luminosity. However, an insufficient quantity of energetic reserve of seeds (small seeds and with little endosperm) should also be considered, since in nature subterranean seeds are formed just below the soil-surface (2-3 cm) (Fig. 1g). Apart from this, it should be investigated if the dimorphic seeds of *T. argentinense* require light for germination, as some species are insensitive to while other are inhibited by light (BEWLEY; BLACK, 1994). In *Emex spinosa* and *E.australis* emergence percentage of aerial seeds also decreased after 4 cm of depth, although some seeds emerged at 10 cm of depth (JAVAID; TANVER, 2014).

The expressive germination in Petri dishes of scarified seeds of both types in relation to the seeds non-scarified (Table 3), showed that scarification increased the germination, possibly due to the breakage of dormancy in the seeds. Besides it is hardly probable that this elevated proportion of the two seed types, mainly the aerial seeds, may survive and germinate in the natural pastures of Rio Grande do Sul, where the aerial



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legumes and the seeds are heavily grazed and/or trampled. Therefore, seedlings of subterranean seeds would be in greater frequency in field than the ones of aerial seeds. In *T. polymorphum*, the aerial and subterranean seeds had low germination when scarified mechanically, but an additional cold treatment proportionate an improvement in germination, mainly of subterranean seeds, although the authors did not mention anything regarding dormancy of seeds or viability of them (SPERONI et al., 2014). The extension of pastures between 34°S and 30°S where *T. argentinense* (CONTERATO et al., 2013) and also *T. polymorphum* occur is not considered an extreme environmental condition leading to severe reproductive instability (SPERONI et al., 2014). Therefore, the production of dimorphic seeds in *T. argentinense*, differently from other amphicarpic plants (WEISS, 1980; CHOO et al., 2014) does not necessarily conducts to great differences in germination. The germination of both aerial and subterranean seeds is important in a forage species such as *T. argentinense* because legume persistence is not only influenced by grazing mismanagement and other environmental stresses but also by the inherent regeneration ability of the plant (SCHULTZE-KRAFT et al., 1997).

The allocation of resources for the vegetative growth was influenced by the origin of the plants. Plants of subterranean seeds produced more and heavier storage roots than those originated of aerial seeds, similar to what was observed in *Centrosema rotundifolium* where plants of subterranean origin tended to allocate more resources for vegetative growth (NIKOLIC et al., 2005). Nevertheless, plants of *T. argentinense* resulting from aerial seeds that have grown with commercial substrate produced more storage roots than the ones of subterranean seeds, but there was no difference when the plants of both types were grown from the regrowth of storage roots (CONTERATO et al., 2013). Therefore, some influence of soil type cannot be excluded. In *C. rotundifolium* production of aerial seeds and formation of storage roots in soils with metals were favored at the cost of production of subterranean seeds (SCHULTZE-KRAFT, 1997). In field conditions, the greatest number of roots produced by plants of subterranean origin can indicate a greater size of these plants in relation to the ones originated from aerial seeds, since storage roots are formed in stolons.

Amphicarpic *T. argentinense* invests firstly in asexual reproduction via production of storage roots (Fig. 1d). As verified in other species, this partition of resources indicates that asexual reproduction is highly competitive (YANG; KIM, 2016) and contributes mainly for population local growth through short distance distribution (XIÃO et al., 2011) and high resilience following conditions of drought, herbivory and other stresses (OTT; HARTNETT, 2011). Apart from this, the regrowth of storage roots ensures the cloning of specific genotypes and enables the plants to persist vegetatively year after year without the necessity of regeneration by seeds (CONTERATO et al., 2013) in unfavorable environments for sexual reproduction. However, long periods of asexual reproduction can reduce the capacity of dispersion of species and affect genetic diversity inside the



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population (MCLELLAN et al., 1997), which *T. argentinense* overcomes by combining amphicarpic and vegetative reproduction.

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