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Article

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Influence of Phenology and Post-Harvest Processing of Vegetal Material on the Allelopathy of Annoni Grass (*Eragrostis plana*) Extracts

*Influência da Fenologia e do Processamento Pós-Colheita do Material Vegetal na Alelopatia de Extratos de Capim-Annoni (**Eragrostis plana**)*

ABSTRACT - The phenological stage, post-harvest processing and quantity of material can interfere in the allelopathic activity of extracts. The objective of this study is to test the allelopathic effect of aqueous extracts of the leaves of Annoni grass (*Eragrostis plana*), an invader of natural pastures. Twelve extracts were prepared by combining the phenological stage of the plants when harvesting the leaves (vegetative; flowering), post-harvest processing before extraction (fresh; dry at 40 °C), and the amount of plant material (5, 15, and 25 g 100 mL^{-1} distilled water). Two bioassays were conducted in a germination chamber, with evaluation of germination and growth of seedlings of lettuce (*Lactuca sativa*) and white clover (*Trifolium repens*). The extracts with higher allelopathic activity were further developed with 15 and 25 g of dried leaves and harvested in plants at the vegetative stage. Germination was attributed to being the greatest contribution to the divergence between the extracts. White clover was more sensitive to extracts, for which 50% to 67% of the extracts were effectively allelopathic (≥50% inhibition); in lettuce, between 8% and 58% of the extracts exhibited this potential. The phenological stage was the factor with a greater individual effect on the allelopathic activity on lettuce and white clover seedlings, and on lettuce germination. The post-harvest processing responded by the greater variation on germination and germination speed index of white clover. The sensitivity of the white clover to extracts of Annoni grass suggests a compromise of its establishment in pastures with the presence of the invader.

Keywords: South African lovegrass, aqueous extract, *Lactuca sativa,* methodology, *Trifolium repens.*

*RESUMO - O estádio fenológico da planta, a quantidade e o processamento do material vegetal são fatores que podem interferir na atividade alelopática dos extratos. Este trabalho teve como objetivo testar o efeito alelopático de extratos aquosos de folhas de capim-annoni (**Eragrostis plana**), invasora de pastagens naturais. Doze extratos foram elaborados, resultantes da combinação do estádio fenológico das plantas quando da colheita das folhas (vegetativo; florescimento), preparação do material antes da extração (fresco; seco a 40 °C) e quantidade de material vegetal (5, 15 e 25 g 100 mL^{-1} de água destilada). Foram conduzidos dois bioensaios, em câmara de germinação, com avaliação de germinação e crescimento de plântulas de alface (**Lactuca sativa**) e trevo-branco (**Trifolium repens**). Os extratos com maior atividade alelopática foram elaborados com 15 e 25 g de folhas secas oriundas de plantas em estádio vegetativo. A germinação foi o atributo com a maior contribuição para a divergência entre os extratos. O trevo-branco foi mais sensível aos extratos, para o qual 50% a 67% dos extratos foram efetivamente*

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alelopáticos (inibição ≥50%); na alface, entre 8% e 58% dos extratos exibiram esse potencial. O estádio fenológico foi o fator com maior efeito na atividade alelopática sobre plântulas de alface e trevo-branco e na germinação da alface. O processamento pós-colheita respondeu pela maior variação na germinação e no índice de velocidade de germinação do trevo-branco. A sensibilidade do trevo-branco aos extratos de capim-annoni sugere comprometimento de seu estabelecimento em pastagens com a presença da invasora.

Palavras-chave: capim-amoroso-sul-africano, extrato aquoso, *Lactuca sativa*, metodologia, *Trifolium repens*.

INTRODUCTION

Evaluation of allelopathic activity is generally performed using bioassays. However, no articles have been published that establish standard procedures for phytotoxic bioassays, including growth conditions or model species (Macías et al., 2000). Many laboratory bioassays have little or no relation to field interaction, which may be due to the dissimilarity between the laboratory bioassay and the natural conditions (Inderjit and Dakshini, 1995) among them, especially those relating to the extraction of extracts for use in bioassays.

There is interference in the bioactivity from the method of preparation of the plant extracts. Therefore there is a necessity for elaboration of efficient and reproducible protocols that can be routinely used in laboratories working with the effects of allelochemicals, so as to improve the conditions in which the tests are performed (Simões et al., 2013). The toxicity of allelopathic substances in the environment is a function of concentration, flux rates, age and physiological stage of the plant, climate, season and environmental conditions (Gniazdowska and Bogatek, 2005).

The extracts can be prepared with growing amounts of fresh or dry plant material (Goetze and Thome, 2004; Marinov-Serafimov, 2010) from plants, at different stages of maturity. For the preparation of extracts, the classic recommendations include: use of water as a medium for extraction and the inclusion of species that may naturally be associated with the plant to be studied, instead of only using the species known to be sensitive to allelopathic compounds, which would help to generate data with more than one recipient species (Inderjit and Keating, 1999). Also, mixing the vegetable material with solvents from 30 minutes to several days of extraction or even the use of equipment such as an industrial blender, which sprays the vegetable parts and washes the cellular contents, could involve static or dynamic contact (Marques, 2005).

Among the main invaders of pastures, crop margins, highways, and rural roads, is Annoni grass (*Eragrostis plana*, Poaceae), and one of its main characteristics is an allelopathic action on other species (Favaretto et al., 2011; Ferreira et al., 2008). In the Pampa Biome alone, which is in the south of South America and whose area is close to 900,000 km^2, *Eragrostis* is the genus with the highest number of invasive species (Fonseca et al., 2013). In 2007, it was projected that in 2008 the scenario would see 2,200,000 ha infested by Annoni grass (Medeiros and Focht, 2007).

This weed has compounds, such as coumarin, phenolic acids (caffeic, ferulic, vanillic, *p*-coumaric), catechin, and epicatechin (Favaretto et al., 2015), with allelopathic action, whose concentration is affected by nitrogen fertilization and the phenological stage (Cecchin et al., 2017). From this an assumption can be made that the plant's allelopathy is dependent on several factors, and that it can probably be extended to other species.

In this study, we tested the hypothesis that the phenological stage of the plants when harvesting leaves, the post-harvest processing, and the amount of plant material used for extraction, are factors that interact and determine the degree of allelopathic activity of Annoni grass. For this, germination and seedling growth bioassays were performed with two target species: lettuce (*Lactuca sativa*) and white clover (*Trifolium repens*). White clover was chosen because it is the main legume forage used in oversowing on the natural pastures of the Pampa Biome, and lettuce because it is considered the main target species in allelopathy studies (Souza Filho et al., 2010).

MATERIAL AND METHODS

This study was carried out at the Laboratório Multidisciplinar Vegetal of Instituto de Ciências Biológicas of Universidade de Passo Fundo (UPF), Passo Fundo (28° 15' S, 52° 24' W), Rio Grande do Sul State, Brazil. The Annoni grass plants from which the material for the research was collected were present in an area of the secondary vegetation, which had a predominance of this grass (Scheffer-Basso et al., 2016) on the university campus. The soil of the area, classified as dystrophic Dark Red Latosol, was collected and analyzed (Tedesco et al., 1985), in triplicate, at the Laboratório de Solos/UPF. The soil presented the following attributes: 40.7% of clay; pH in water: 5.3; P: 6.6 mg dm^{-3}; K: 164 mg dm^{-3}; organic matter: 3.7%; Al: 0.3 $cmol_c$ dm^{-3}; Ca: 4.2 $cmol_c$ dm^{-3}; Mg: 1.9 $cmol_c$ dm^{-3}; base saturation: 43%; Al saturation: 4%; K saturation: 2.8%; S: 10 mg dm^{-3}; Mn: 30.9 mg dm^{-3}; Bo: 0.5 mg dm^{-3}; Zn: 1.20 mg dm^{-3}; Cu: 2.1 mg dm^{-3}.

For the two target species, lettuce and white clover, the bioassays consisted of a differential factorial design (2 x 2 x 3) + 1, in which 12 extracts of Annoni grass were tested in comparison to the control (distilled water). The experimental design was completely randomized, with four replications. The treatment was the result of a combination of the following factors: (a) phenological stage: vegetative (V); flowering (F); (b) post-harvest processing: fresh (FR); dry (D); (c) amount of plant material: 5, 15, and 25 g 100 mL^{-1}. Thus, from the combination of the abbreviations V, F, FR, and D with the numbers 5, 15 and 25, the extracts can be identified from the presentation and discussion of the results of the study.

The plants were harvested in two seasons according to the pre-determined phenological stage: for the vegetative stage, harvesting was done in mid-spring (October 22, 2013), and for the flowering stage the harvest was performed in summer, 71 days after the first harvest. The vegetative stage was considered to be one in which the plants were in active leaf emission and there was no evidence of tiller elongation. The flowering stage was one in which the plants exhibited at least one fully exposed panicle and with at least half of the spikelet in anthesis, based on the Zadoks growth scale (Zadoks et al., 1974).

In each season, ten plants of similar size were cut close to the ground. The plants cut in the vegetative stage were not harvested at the flowering stage, because they did not sprout in time to reach the reproductive stage. After the leaves were harvested, the material was taken to the laboratory, where only the green leaves, without senescence signal, were separated, to prepare the extracts. The material was separated into two lots according to the post-harvest processing: for fresh plants, the plant material was immediately chopped into small pieces and immersed in distilled water, for extraction; the other lot was separated into five samples, to determine the dry matter content of the material. These samples were weighed, placed in perforated paper bags and kept for 72 hours in a forced air oven at 40 °C. After removing the samples from the oven, they were weighed to determine the dry matter content, estimated at 46%. This material was then chopped into slices and taken for aqueous extraction.

The preparation of the extracts was performed by the static maceration method, in which the vegetable material, previously chopped, was immersed in water, following the methodology generally used to obtain crude aqueous extracts (Soares and Vieira, 2000; Goetze and Thome, 2004; Marques, 2005; Marinov-Serafimov, 2010). For each post-harvest processing type (fresh or dry), 5, 15, and 25 g of the plant material were immersed in 100 mL of distilled water. The mixtures remained at room temperature (between 25 °C and 30 °C) under light, without agitation, for 24 hours, followed by filtration.

The bioassays were conducted in a seed germination chamber, with a constant temperature (20 °C) and a 12 hour photoperiod. The experimental units were crystal polyethylene boxes (Gerbox® type), lined with filter paper, containing fifty seeds of the target species arranged in an equidistant manner. The average germination of lettuce and white clover, according to the test performed by the Laboratório de Sementes/UPF, was 99% and 95%, respectively.

After the seeds were placed on the filter paper, the treatments were applied, adding 5 mL of the extracts or the distilled water (control). The seeds, and later, the seedlings were kept in the germination chamber for 15 days, in which daily evaluations of the germination were carried out for ten days, and the initial growth of the seedlings on the fifteenth day. The percentage of germination and the germination speed index (GSI) were evaluated.

Five seedlings of similar size, from each experimental unit, were evaluated for root and hypocotyl length. The classification of the allelopathic effect of the extracts on germination and seedling growth was based on Souza-Filho and Mourão (2010), with adaptation for this paper, in: (a) effective (≥50% inhibition); (b) potential (35%-49% inhibition); and (c) moderate (≤34% inhibition).

In order to verify the factors with the greatest contribution to the variation of the response to the extracts, analysis of variance was performed in the three-factorial model, in which the control treatment was not included. Later, to identify which extracts presented allelopathic activity, analysis of variance was performed with the differential factorial arrangement (2 x 2 x 3) + 1 (control), using the Dunnett's test, at 5% significance. In addition, a multivariate analysis was performed, taking into account the results of the two target species, to identify the attributes that had the greatest relative contribution in the divergence between the extracts, to determine the Mahalanobis-D2 distance, to illustrate and to examine the dissimilarity between the extracts, with the help of a dendrogram.

RESULTS AND DISCUSSION

Allelopathy bioassay with *Lactuca sativa*

The factors tested in this bioassay significantly affected, either the isolated, or interactively, the attributes of germination and seedling growth (Table 1). The phenological stage was the main cause of total variation in germination (34%), length of hypocotyl (74%) and root (49%). For the germination speed index (GSI), post-harvest processing was the factor that was the greatest representative of the total variation in the data (76%).

By comparison with the control treatment, the application of the Dunnett's test showed that only three of the 12 extracts reduced germination (Table 2). These extracts were prepared with leaves collected from plants at the vegetative stage, of 15 and 25 g (V-D-15; V-D-25; V-FR-25). The extract with higher allelopathic action was elaborated with 25 g of leaves previously dried, coming from plants at the vegetative stage (V-D-25). By the criterion of Souza Filho and Mourão (2010), only this extract would be effectively allelopathic (≥50% inhibition). The other two extracts, which significantly reduced seed germination in relation to the control ($p<0.05$), were moderately allelopathic (≤34% inhibition). The extracts V-D-15 and V-D-25 also affected the germination speed, culminating in a significant reduction of GSI; for germination and GSI, there was a convergence in the extract of higher inhibitory power (V-D-25) (Table 2). However, the GSI was reduced by extracts V-D-5 and F-D-25, which, in turn, did not interfere with germination.

Hypocotyl elongation was inhibited only by extracts that affected germination (V-D-15; V-D-25; V-FR-25). Inhibition ranged from 48% to 92%, indicating that such extracts were effectively allelopathic on shoot growth of lettuce seedlings (Table 2). Already, the root elongation was affected

Table 1 - Mean squares of the sources of variation and significance by the F test, for germination (G), germination speed index (GSI), length of hypocotyl (H) and primary root (R) of lettuce (*Lactuca sativa*), in the allelopathy bioassays of aqueous extracts of Annoni grass (*Eragrotis plana*). Passo Fundo, RS, 2016

Sources of variation	DF	G	GSI	H	R
Phenological stage (PS)	1	5985.33**	354.20**	2786.88**	1968.36**
Post-harvest processing (PP)	1	2760.33**	4855.35**	154.49*	1147.41**
Amount of plant material (PM)	2	3394.75**	659.51**	313.48**	442.12**
PS*PP	1	1121.33**	244.52*	25.96 ns	298.69**
PS*PM	2	2269.98**	22.31 ns	343.18**	39.33 ns
PP*PM	2	1178.08**	163.75*	73.54 ns	39.34 ns
PS*PP*PM	2	634.08**	0.52*	5.96 ns	24.45 ns
Error	36	70.50	51.77	34.11	20.64
Total	47				

ns= non-significant ($p>0.05$); * significant ($p<0.05$); ** significant ($p<0.01$).

Table 2 - Effect of the phenological stage, post-harvest processing, and amount of plant material on the allelopathic activity of aqueous extracts of Annoni grass (*Eragrostis plana*) tested in bioassays of seed germination and seedling growth of lettuce (*Lactuca sativa*). Passo Fundo, RS, 2016

Phenological stage/Post-harvest processing	Amount of plant material (g 100 mL^{-1})		
	5	15	25
	Germination (%)		
Flowering/fresh	100	99	99
Flowering/dry	99	94	89
Vegetative/fresh	97	89	*74 (24)
Vegetative/dry	97	*72 (26)	*16 (84)
Control= 98%			
	Germination speed index		
Flowering/fresh	41	41	37
Flowering/dry	31	26	*15 (63)
Vegetative/fresh	42	40	33
Vegetative/dry	*23 (43)	*16 (60)	*2 (95)
Control= 40			
	Hypocotyl length (mm)		
Flowering/fresh	27.6	34.1	32.8
Flowering/dry	29.9	33.2	25.1
Vegetative/fresh	25.8	15.9	*11.4 (57)
Vegetative/dry	22.3	*13.6 (48)	*2.1 (92)
Control= 26.4 mm			
	Primary root length (mm)		
Flowering/fresh	29.9	28.4	*16.2 (40)
Flowering/dry	*15.3 (43)	*9.5 (64)	*5.5 (79)
Vegetative/fresh	*14.7 (45)	*4.6 (83)	*1.8 (93)
Vegetative/dry	*5.7 (79)	*0.9 (97)	*0.1 (99)
Control= 26.8 mm			

** Value differs from the control by the Dunnett test ($p<0.05$). Values in parentheses indicate the percentage of inhibition relative to the control (distilled water).

by most of the extracts, of which three were potentially allelopathic (35%-49% inhibition) and seven were effectively allelopathic (≥50 inhibition). The three extracts with greater inhibitory action (≥93%) on the primary root were the same ones that showed effective allelopathic action on the germination and the only extracts that significantly reduced the seedling shoot.

For the four attributes evaluated in this bioassay, the extract with greater allelopathic power was the result of the V-D-25 combination. On the other hand, extracts made with fresh leaves, from flowering plants and in the two smaller amounts of plant material (5 and 15 g) did not affect seed germination and seedling growth. The other extracts showed variability regarding the character on which they were allelopathic (Table 2).

Allelopathy bioassay with *Trifolium repens*

Similar to the data obtained using lettuce as a bioindicator, the phenological stage, the post-harvest processing, and the amount of plant material showed a significant effect on seed germination and seedling growth of white clover (Table 3). The post-harvest processing explained the greatest variation in germination (49%) and GSI (69%). For the hypocotyl, the highest representation of the total variation was the phenological stage (53%), followed by the amount of vegetal material (19%). For the root elongation this variation was due to phenology and post-harvest processing, which, together, accounted for 38% of the variation in this character.

The germination of white clover was significantly reduced by eight of the 12 extracts, of which seven were effectively allelopathic (Table 4). Of the four extracts that were innocuous to

Table 3 - Mean squares of the sources of variation and significance by the F test, for germination (G), germination speed index (GSI), length of hypocotyl (H) and primary root (R) of white clover (*Trifolium repens*), in the allelopathy bioassays of aqueous extracts of Annoni grass (*Eragrotis plana*). Passo Fundo, RS, 2016

Source of variation	DF	G	GSI	H	R
Phenological stage (PS)	1	11844.08**	53.98*	445.53*	146.25*
Post-harvest processing (PP)	1	24390.08**	2866.47*	127.55*	145.77*
Amount of plant material (PM)	2	8130.33**	803.42*	157.77*	65.12*
PS*PP	1	2002.08**	16.31^{ns}	0.01^{ns}	11.32*
PS*PM	2	185.33*	128.40*	0.29^{ns}	1.00^{ns}
PP*PM	2	234.33**	126.73*	41.85*	2.33^{ns}
PS*PP*PM	2	2792.33**	133.68*	65.34*	7.68*
Error	36	42.47	12.68	4.20	2.08
Total	47				

ns= non-significant ($p>0.05$); * significant ($p<0.05$); ** significant ($p<0.01$).

Table 4 - Effect of the phenological stage, post-harvest processing, and amount of plant material on the allelopathic activity of aqueous extracts of Annoni grass (*Eragrostis plana*) tested in bioassays of seed germination and seedling growth of white clover (*Trifolium repens*). Passo Fundo, RS, 2016

Phenological stage/Post-harvest processing	Amount of plant material (g 100 mL^{-1})		
	5	15	25
	Germination (%)		
Flowering/fresh	92	87	89
Flowering/dry	*72 (23)	*18 (81)	*4 (86)
Vegetative/fresh	85	*34 (64)	*17 (82)
Vegetative/dry	*32 (66)	* 4 (86)	*3 (97)
Control= 94%			
	Germination speed index		
Flowering/fresh	25	*19 (34)	*17 (41)
Flowering/dry	*9 (69)	*2 (93)	*1 (97)
Vegetative/fresh	35	*11 (62)	*6 (79)
Vegetative/dry	*8 (72)	*0.5 (98)	*0.4 (99)
Control = 29			
	Hypocotyl length (mm)		
Flowering/fresh	11.2	9.8	11.3
Flowering/dry	14.9	*6.7 (39)	*1.8 (84)
Vegetative/fresh	8.4	*5.1 (54)	*1.5 (84)
Vegetative/dry	*5.3 (52)	*0.1 (99)	*0.1 (99)
Control = 11.0 mm			
	Primary root length (mm)		
Flowering/fresh	9.2	7.6	*6.9 (32)
Flowering/dry	*6.9 (32)	*2.7 (73)	*0.6 (94)
Vegetative/fresh	*5.7 (44)	*2.9 (71)	*1.6 (84)
Vegetative/dry	*2.7 (73)	*0.1 (99)	*0.1 (99)
Control = 10.1			

** Value differs from the control by the Dunnett test ($p<0.05$). Values in parentheses indicate the percentage of inhibition relative to the control (distilled water).

germination, fresh leaves from flowering plants were used in three (F-FR-5; F-FR-15; F-FR-25), regardless of the amount used in their elaborated. The other non-allelopathic extract was also prepared with fresh leaves and from plants in the vegetative stage, but with the least amount of plant material (V-FR-5). The GSI was reduced by the majority of the extracts, except those elaborated in less proportional amount of fresh leaves (F-FR-5; V-FR-5).

Hypocotyl elongation was negatively affected by most extracts, except for those produced with fresh leaves from flowering plants, regardless of the amount used (F-FR-5; F-FR-15; F-FR-25); most of them were prepared with 5 g of plant material, except with dry leaves and plants at the vegetative stage (Table 4). Six extracts were effectively allelopathic (≥50% inhibition) and one was potentially allelopathic (35%-49% inhibition) for this attribute.

The primary root of white clover was more sensitive than the hypocotyl to the action of the extracts, as only two of them did not inhibit root elongation (Table 4). Two extracts (F-D-5; F-FR-25) were moderately allellopathic (≤34% inhibition), one extract (V-FR-5) was potentially allelopathic, and seven extracts (V-FR-15; V-FR-25; V-D-5, V-D-15; V-D-25; F-FR-15; F-FR-25) were effectively allelopathic for this attribute.

Joint analysis of the target species

White clover was more sensitive than lettuce, as there was an inhibitory effect of 58% (hypocotyl length), 67% (germination), and 83% (GSI and root length) of the extracts (Table 4). In the lettuce, only 25% of the extracts significantly decreased germination and hypocotyl elongation, and 33% reduced the GSI. Only for the growth of lettuce root, that action was similar to the one observed in the white clover, with allelopathic action of 83% of the extracts (Table 2).

The highest toxicity, which resulted in 99% inhibition, occurred under allelopathic action of the V-D-25 extract, on the root of the both target species, hypocotyl length and germination speed index of white clover (Tables 2 and 4). However, the root and hypocotyl elongation of white clover was also inhibited under the action of the extract V-D-15 (Table 4), which reinforced the indication of its greater sensitivity in relation to lettuce. The only innocuous extracts for both species were elaborated with fresh leaves and harvested in flowered plants, regardless of the amount of vegetal material used in their elaboration, but only for germination and hypocotyl elongation.

The most potent character contributing to divergence between extracts was the germination of white clover (Table 5). Together, the percentages of germination of lettuce and white clover accounted for 65% of the divergence between treatments.

The Mahalanobis distance matrix (D2) revealed maximum divergence (D2=378.73) between F-FR-5 and V-D-25 extracts and minimum between F-FR-5 and F-FR-15 (D2=7.62) extracts, consistent with the configuration of the dendrogram (Figure 1). The innocuous extracts on any attribute and the extracts with less inhibitory action are in group I, which include those formulated with fresh leaves originating from flowered plants (Table 6). In group II are the extracts that do not affect the germination and seedling growth of lettuce, but effectively inhibit all attributes in the white clover. In group III are the extracts with the highest allelopathic activity, as all of them effectively inhibit the germination, the GSI, and the growth of white clover, with variable allelopathic activity on lettuce. To this group belong all the extracts elaborated with 15 or 25 g of leaves harvested in plants in the vegetative stage.

Table 5 - Relative contribution of the characters to the divergence (Singh, 1981) between aqueous extracts of Annoni grass (*Eragrostis plana*) tested in bioassays of seed germination and seedling growth of lettuce (*Lactuca sativa*) and white clover (*Trifolium repens*). Passo Fundo, RS, 2016

Character	Contribution (%)
White clover germination	47.77
Lettuce germination	17.42
Primary root length of lettuce seedlings	12.75
Hypocotyl length of lettuce seedlings	11.51
Primary root length of white clover seedlings	4.73
Germination speed index of white clover	3.72
Hypocotyl length of white clover seedlings	1.57
Germination speed index of lettuce	0.53

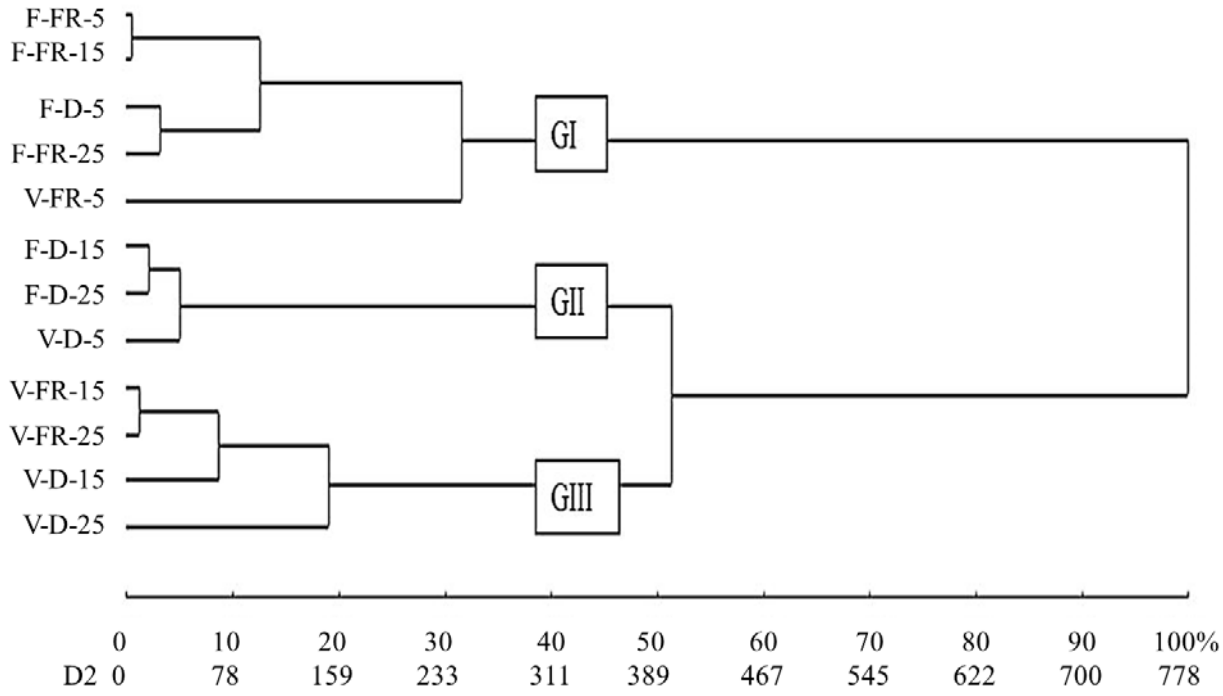

Figure 1 - Dendrogram of Ward, based on Mahalanobis distances matrix (D2), among 12 aqueous extracts elaborated with leaves of Annoni grass (*Eragrostis plana*) from plants in vegetative (V) or flowering (F) stage, fresh (FR) or dry (D), and increasing amounts in the extraction (5, 15 and 25 g 100 mL^{-1}), based on germination, germination speed index, length of hypocotyl and primary root of (*Lactuca sativa*) and white clover (*Trifolium repens*).

Table 6 - Mean of germination (G), germination velocity index (GSI), length of hypocotyl and primary root of lettuce (*Lactuca sativa*) and white clover (*Trifolium repens*) of the main groups formed by the dendrogram from Ward's method, composed of aqueous extracts of Annoni grass (*Eragrostis plana*) elaborated with leaves from plants in vegetative (V) or flowering (F) stages, fresh (FR) or dry (S), and increasing amounts in the extraction (5, 15, and 25 g 100 mL^{-1}). Passo Fundo, RS, 2016

Groups of extracts	Character							
	$G^{(1)}$ (%)	$G^{(2)}$ (%)	$GSI^{(1)}$	$GSI^{(2)}$	$H^{(1)}$ (mm)	$H^{(2)}$ (mm)	$R^{(1)}$ (mm)	$R^{(2)}$ (mm)
I (F-FR-5; F-FR-15; F-FR-25; F-D-5; V-FR-5)	98.6	84.8	38.4	21.0	30.0	11.3	20.9	7.3
II (F-D-15; F-D-25; V-D-5)	91.2	10.8	20.5	1.4	29.1	4.31	7.5	1.6
III (V-FR-15; V-FR-25; V-D-15; V-D-25)	62.8	14.0	23.2	4.4	10.8	1.7	1.9	1.1

(1) Lettuce; (2) White clover.

In this study, it was tested the hypothesis that the phenological stage, previous preparation of the material to be extracted and amount of plant material are factors that interact and determine the allelopathic effect of Annoni grass on the germination and initial growth of seedlings of lettuce and white clover. The goal was to identify the allelopathic extracts, rather than to verify the response of the target plants in terms of absolute values of germination or seedling growth. To obtain such information, the Dunnett's test ($p<0.05$) was applied.

In addition, the allelopathic extracts were classified as proposed (effective: ≥50% inhibition; potential: ≥35% inhibition) by Souza-Filho and Mourão (2010), but specifying the amplitude of inhibition percentage for potentially allelopathic extracts (35%-49%). A new category was included in the extracts classification, adopting the term 'moderate' for the extracts with inhibition control below ≤34%, provided they showed a significant difference ($p<0.05$) in relation to the control (distilled water).

In order to reinforce the reproducibility of the results, it was planned to test allelopathy on more than one target species, as the closer the bioassay is to reality, the better are the chances of the conclusions approaching the natural condition (Silva et al., 2009). The use of more than one species allows better scaling of the actual allelopathic potentialities of the donor species (Souza Filho et al., 2010). In this study, the white clover was chosen because it is the main legume that is oversown in the natural pastures of Pampa Biome, with the purpose of reducing the seasonality of the production and increasing the nutritive value.

The choice of the host species in tests of allelopathy is an aspect that can hinder the interpretation of results, especially if there is dormancy (Silva et al., 2009), which causes unevenness in root extrusion. In addition, species with slow root growth rate (RGR) may be more susceptible to allelochemicals than those with a higher RGR. Lettuce is one of the species most used for bioassays of this nature, for its ready and uniform germination. However, because it is extremely sensitive to the effects of both crude extracts and allelochemicals, it may lead to overestimation of the phytotoxic activity or may even induce phytotoxicity, where in reality it does not exist or is inexpressive (Souza-Filho et al., 2010).

White clover, which is a domesticated species, with commercial seed production and without dormancy, was adequate for the bioassay, as it had an average germination time of 2.4 days. The germination of the two target species, considered by the results of the control treatment (Tables 2 and 4), was close to 100%, which demonstrates the validity of the comparison of the results obtained with the application of the extracts.

The factors tested (phenological stage, post-harvest processing, and amount of plant material) interacted and affected the germination and growth of lettuce and white clover seedlings (Tables 1 and 3). However, the contribution of each factor to the allelopathic activity showed variation between the target species and/or attributes evaluated. There was a convergence of results with regard to the effect on the growth of seedlings of both species, for which the phenological stage was the factor that contributed most to the results of the allelopathic activity. The same was observed for lettuce germination, but for the white clover it was the post-harvest processing that caused the greatest variation in this characteristic, as well as for the GSI.

The susceptibility of the white clover to the action of foliar extracts of Annoni grass confirmed the previous results (Favaretto et al., 2011), showing that the establishment of this legume can be compromised in pastures with the presence of the invasive plant. In addition to the damage to germination and the initial growth of seedlings, the allelochemicals of the weed may have a negative impact on the biological nitrogen fixation (Gniazdowska and Bogatek et al., 2006) of the white clover, which may reduce its competitiveness and persistence. This species was more sensitive than the lettuce to the extracts, both in relation to the effect on germination and seedling growth (Table 4), indicating genetic differences, as they were submitted to the same treatments and experimental conditions. In the lettuce, the germination was less affected by the extracts in relation to the seedling growth, which is usually more commonly reported in literature. In laboratory conditions, during bioassays, the impact of allelochemicals is more pronounced on seedling growth due to the direct contact of seedlings with the extracts (Marinov-Serafimov, 2010).

The phenological stage of Annoni grass was seen to be a determinant in its allelopathic activity; this had already been verified by Cecchin et al. (2017). This factor has also been shown to be a determinant in sorghum allelopathy (*Sorghum* spp., Poaceae) (Marchi et al., 2008) and fenugreek (*Trigonella foenum-graecum*, Fabaceae) (Omezzine et al., 2014). Therefore, there is compliance with what is attested about the influence of plant maturity on chemical composition, and consequently, on allelopathy, in response to metabolic changes. When young plant residues are added to the soil, toxic substances are produced relatively early in the decomposition process, but also disappear rapidly (Khalid et al., 2002). When mature plants are incorporated into the soil, decomposition is slower and toxicity remains high for a longer period.

In the same amount of vegetal material, the drying of leaves competed to obtain extracts with greater allelopathic activity in relation to fresh leaves. The extracts were more harmful (Tables 2 and 4) on the germination and growth of the seedlings with the same amount of vegetal material extracted, but with previous drying, it partially eliminated the humidity of the leaves. This was reported in extracts of plants with allelochemical potential, such as rosemary

(*Rosmarinus officinalis*) (Zortéa et al., 2015), tobacco (*Nicotiana tabacum*), and eucalyptus (*Eucalyptus grandis*) (Goetze and Thomé, 2004). In this study, there are indications that allelochemicals of Annoni grass were not inactivated with drying at 40 °C, in addition to the dilution factor, as, in order to obtain the same proportional amount of plant material with fresh material, a greater amount of leaves is needed.

With regard to the amount of plant material used in the extraction, it was evident that, with increasing content, there was a reduction in germination and seedling growth compared to the control (Tables 2 and 4). In a similar study, Marinov-Serafimov (2010) observed the inhibitory effect of aqueous extracts of weed material on germination and growth of soybean (*Glycine max*), pea (*Pisum sativum*), and vetch (*Vicia sativa*). Similar to our results, there was a genotypic effect regarding the sensitivity to the extracts, with the pea species being the most affected.

The results verified in this study reinforce the importance of establishing a methodological standard for allelopathy bioassays, as differences in phytotoxicity are observed when considering, for example, phenological stage, quantity and post-harvest processing of the plant material, and target species. The methodological standardization would allow, in addition to better efficiency, greater reliability in the comparison between research experiments.

The findings of this work support the hypothesis that the allelopathic effect of leaf extract of Annoni grass is dependent on the phenological stage of plants, the post-harvest processing, and amount of plant material used for extraction, which act together. The inhibitory effect on germination and seedling growth of lettuce and white clover validate previous evidences on the allelopathic potential of this grass (Favaretto et al., 2011, 2015; Cechin et al., 2017). The extraction in water, through which the extracts were made, reproduces, in part, the natural condition of the plants, assuming that, by decomposition and leaching, the leaves are naturally detached from the Annoni grass or cut by the mechanical grazing of the pastures, and will be a source of allelochemicals.

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