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Procyanidins in *Lotus* L. genotypes grown in soil with different saturations of aluminum

Procianidinas em genótipos de *Lotus* L. cultivados em solos com diferentes saturações de alumínio

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Abstract

Condensed tannins are formed by monomers of procyanidins and prodelfinidins, where the proportion and concentration of their monomers varies according to the plant species and environmental conditions. In *Lotus* spp., condensed tannins prevent tympanism in ruminants that feed on them. This study aimed to evaluate the concentration of procyanidins and their monomers, catechin and epicatechin in the genotypes of *Lotus* L. grown in soil with different saturations of aluminum. A two-factor (genotype × Al saturation) assay was performed, where the genotypes São Gabriel, Ganador, and UFRGS (*Lotus corniculatus* L.); Serrano (*Lotus uliginosus*); and El Rincón (*Lotus subbiflorus*) were cultivated in soil with an Al saturation of 0-20 %. The procyanidins were evaluated using high-performance liquid chromatography, which was previously validated for catechin and epicatechin. The concentration of procyanidins and the proportion of epicatechin:catechin were affected by the genotype × environment interaction. In *L. corniculatus* and *L. subbiflorus*, the concentration of procyanidin was significantly higher when they were grown in the soil with an Al saturation of 20 % compared to that when they were grown in the soil with 0 % Al saturation, but the opposite effect was observed in *L. uliginosus*. The proportion of epicatechin:catechin decreased in plants grown in soil without Al, and only the UFRGS genotype maintained a similar proportion under both the soil acidity conditions. The predominant monomer was epicatechin, which varied from 57 % to 75 % according to the soil in which the plants were grown.

Key words: Catechin, birdsfoot trefoil, epicatechin, proanthocyanidins

Resumo

Os taninos condensados são formados por monômeros de procianidinas e prodelfinidinas, cuja proporção e concentração dos seus monômeros varia de acordo com a espécie vegetal e condições ambientais. Em *Lotus* spp., são essas substâncias que evitam o timpanismo em ruminantes que delas se alimentam. O objetivo deste trabalho foi avaliar a concentração de procianidinas e de seus monômeros, catequina e epicatequina, em genótipos de *Lotus* L. sob distintas saturações de alumínio no solo. O ensaio consistiu de um bifatorial (Genótipo x Saturação de Al), no qual os genótipos São Gabriel, Ganador e UFRGS (*L. corniculatus* L.), Serrano (*L. uliginosus*) e El Rincón (*L. subbiflorus*) foram cultivados em solo com 0 % e 20 % de saturação de Al. As procianidinas foram avaliadas por cromatografia líquida de alta eficiência,

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previamente validada para catequina e epicatequina. A concentração de procianidinas e a proporção epicatequina:catequina foram afetadas pela interação genótipo x ambiente. Em *L. corniculatus* e *L. subbiflorus* a concentração de procianidinas foi significativamente superior em solo com 20 % de saturação de Al em relação ao solo com 0 % de saturação de Al, mas o oposto foi observado em *L. uliginosus*. A proporção epicatequina:catequina reduziu nas plantas cultivadas em solo sem Al e apenas o genótipo UFRGS manteve proporção similar em ambas as condições de acidez do solo. A epicatequina foi o monômero preponderante, variando de 57 a 75 % de acordo com o solo em que as plantas foram cultivadas.

Palavras-chave: Catequina, cornichão, epicatequina, proantocianidinas

Introduction

Procyanidins (PCs) and prodelfinidins (PDs) are the units of proanthocyanidins, which are more commonly known as condensed tannins (CTs). The PCs are formed by monomers of catechin and epicatechin, and PDs are composed of epigallocatechin and gallocatechin (AOKI et al., 2000). Leguminous forages that have CTs are called "non-tympanic" because these compounds prevent tympanism. Tympanism, which is associated with factors that prevent the removal of gases produced during ruminal fermentation, occurs in ruminants (PAGANI, 2008). When the leaves, stems, and flowers consumed by the animals are disintegrated by chewing, CTs are released; they react and form complex soluble proteins and prevent the formation of foam in the rumen (BARRY, 1983). The levels of 2-4 % of CTs have been estimated to be required for the prevention of this nutritional disorder; further, CTs increase protein absorption in the small intestine and abomasum, which increases lactation, improves wool production and weight gain in sheep (WAGHORN et al., 1987, 1998; MOLAN et al., 1999), and promotes animal health by acting on gastrointestinal parasites (MINHO et al., 2010; YOSHIHARA et al., 2014).

Birdsfoot trefoil (*Lotus* spp.) are among the important non-tympanic leguminous forages worldwide, in particular, the common birdsfoot trefoil (*Lotus corniculatus* L.) and great trefoil (*Lotus uliginosus* Schkuhr.). Presence of CTs is considered the most important nutritional characteristic of the species belonging to this genus, since they prevents tympanism (LI et al., 1996), unlike alfalfa (*Medicago sativa* L.) and clover (*Trifolium* spp.) that lack CTs. Birdsfoot trefoil are distinguished ecologically by their greater tolerance to soil acidity than legumes, allowing greater success in improving natural pastures through overseeding without the need to incorporate limestone. The presence of CTs might be associated with the tolerance of *Lotus* spp. to aluminum (STOUTJESDIJK et al., 2001).

Genetic factors are known to be the main determinants of CT concentration in birdsfoot trefoil (ACUÑA et al., 2008), with records of 0.2-10.9 % of dry matter in these species (SIVAKUMARAN et al., 2006). Genotypic differences in these legumes also occur in terms of CT composition, since there is a predominance of PCs in common birdsfoot trefoil, especially epicatechin, whereas, in great trefoil, there is a predominance of PDs (FOO et al., 1997; MEAGHER et al., 2004). In southern Brazil, cultivation of common birdsfoot trefoil began in the 1950s; despite its importance, only one local study investigated the levels of CTs in the São Gabriel genotype and revealed surprisingly low CT concentrations between 0.34 % (great trefoil) and 1.99 % (common birdsfoot trefoil) (MORO et al., 2010).

The concentration of CT in birdsfoot trefoil varies according to the genotype (SIVAKUMARAN et al., 2006) and climate, soil (EHLKE; Le GARE, 1993), pasture management (BARRY; FORSS, 1983), and methodology used to determine these compounds (SCHOFIELD et al., 2001). Among the methods used for the analysis of chemical constituents of herbal extracts, high-performance liquid chromatography (HPLC) is considered suitable owing to the speed, sensitivity, specificity, efficiency, and excellent performance in the separation, isolation, and quantification of CTs (FALKENBERG et al., 2004). In addition, this method enables the manipulation of experimental selectivity by changing the characteristics of the mobile phase and retrieval and replication of previous analyses (AGUILAR, 2004).

The characterization of the monomers that form CTs in birdsfoot trefoil is critical for species improvement programs belonging to the genus *Lotus*, since these compounds ensure food security for ruminants by reducing the risk of tympanism. Furthermore, the characterization might clarify the possible relationships of CTs with the different responses of legume populations to soil acidity.

This study aimed to characterize the Brazilian genotypes of *Lotus* spp. (São Gabriel, UFRGS, and Serrano) at an unprecedented level and compare them with materials developed in Uruguay in order to evaluate the relationship between PC concentration and Al saturation levels in the soil. The study focuses on the following aspects: (a) Does Al saturation in the soil affect the PC concentration in the genotypes of *Lotus* L. and (b) Does Al saturation distinctly affects the concentration and proportion of the monomers that form PCs?

Material and Methods

The experiment was conducted in Passo Fundo, Rio Grande do Sul, (28º15′ S, 52º24′ W); this area is located at an altitude of 687 m. The climate is the humid fundamental type (f) and specific variety subtropical (CFA) (KUINCHTNER; BURIAL, 2001). The assay was two-factor (Genotype × Al saturation), in which the São Gabriel, Ganador, UFRGS (*L. corniculatus* L.), Serrano (*L. uliginosus*), and El Rincón (*L. subbiflorus*) genotypes were cultivated in soil with different Al saturation (0 %-20 %). The study was designed in a randomized block manner with four replications. The S. Gabriel, Ganador, UFRGS, and El Rincón seeds were donated by the Departamento de Plantas Forrageiras (Faculdade de Agronomia/UFRGS), and the Serrano seeds were donated by Epagri. Plants were cultivated for ten months between November 2011 and October 2012 in a screenhouse. During the cultivation period, the average temperatures ranged from 15.3°C to 27.7°C. The plants were grown in pots having height of 18 cm and diameter of 23 cm, containing 7 kg of dark Red Distrophic Latosol of the Unidade de Mapeamento Passo Fundo; the soil was sieved through a mesh of 1 cm.

Two Al saturation levels were obtained by mixing half of the soil with limestone, such that the water pH was 6.0 (CQFS-RS/SC, 2004) and Al toxicity was eliminated, which amounted to 9.9 $t \cdot ha^{-1}$ of limestone filler. Limestone was not applied to the other half; however, both the soils were fertilized with phosphorous and potassium to avoid the limitation of plant growth. In the soil containing limestone, the equivalent of 100 $kg \cdot ha^{-1}$ of P_2O_5 and 80 $kg \cdot ha^{-1}$ of K_2O was added; in the soil without lime, the equivalent of 25 $kg \cdot ha^{-1}$ of P_2O_5 and 20 $kg \cdot ha^{-1}$ of K_2O was applied. After three months of incubation, the soils were analyzed (TEDESCO et al., 1995); the following attributes were obtained: (a) soil with limestone: clay = 32.4 %; pH = 6.2; soil matric potential (SMP) index = 6.4; Al = 0 $cmol_c \cdot dm^{-3}$; H + Al= 2.8 $cmol_c \cdot dm^{-3}$; CEC= 13,7 $cmol_c \cdot dm^{-3}$; base saturation = 80 %; aluminum saturation = 0 %; K = 69.0 $mg \cdot dm^{-3}$; P = 5.1 $mg \cdot dm^{-3}$; (b) soil that did not receive limestone: clay = 37.1 %; pH = 4.9; SMP index = 5.0; Al = 2.5 $cmol_c \cdot dm^{-3}$; H + Al = 13.7 $cmol_c \cdot dm^{-3}$; CCT = 20.3 $cmol_c \cdot dm^{-3}$; base saturation = 32 %; aluminum saturation = 20 %; P = 4.3 $mg \cdot dm^{-3}$; K = 44.0 $mg \cdot dm^{-3}$.

The plots were seeded in November 2011 by planting four plants/pot $g \cdot L^{-1}$. The soil was inoculated 15 days after seeding by adding 250 $g \cdot L^{-1}$ *Mesorhizobium loti* solution at 250 $mL \cdot pot^{-1}$. The plants were maintained without water restriction. During the trial period, the plants were subjected to four cuts, which ensured that they permanently remained in the vegetative stage. The material harvested from the cuts was weighed and placed in a forced aeration incubator at 30°C for 72 h. Subsequently, the samples were weighed to determine the amount of dry matter by grounding in a Willie mill.

The plant material was extracted with 50 % methanol at a 1:10 (plant:solvent) proportion at room temperature (±20°C) for seven days. The extracts were filtered, concentrated in a rotary evaporator at a temperature not exceeding 60°C, and weighed to calculate the amount of dry extract per amount of dry matter of each plant. Solutions of 10 $mg \cdot mL^{-1}$ of dry extract in HPLC-grade methanol were prepared, filtered through a 0.45 µm membrane, and injected into the Flexar LC Perkin Elmer HPLC system (Burnsville, MN, USA) equipped with a Flexar LC binary pump, a Flexar PDA detector, and an autosampler, where the data of the peak areas were integrated into the Chromera Workstation software. A reverse-phase Brownlee C18 column (250 mm × 4.6 mm, 5 µm) was used for the quantification of PC according to prior validation (CHINI, 2013). The best elution of the peaks was achieved by using a mobile phase consisting of acetonitrile and water (proportion, 18:82 v/v) acidified with phosphoric acid to pH 3, with a flow rate of 1.0 $mL \cdot min^{-1}$. The injection volume was 20 µL, and injections were made in triplicate (GIRARDI et al., 2014).

The results of the chemical analysis and production of dry matter yield by the plants were subjected to analysis of variance, and the means were compared using Student's *t* test (DMS) by using the statistical program SISVAR (FERREIRA, 2011).

Results and Discussion

The genotype × environment interaction significantly affected the PC concentration (catechin + epicatechin). Except Serrano (*L. uliginosus*), the other genotypes exhibited significantly higher concentrations of this component in the presence of Al (Table 1). This result is consistent with previously reported findings on this genre of legumes (ACUÑA et al., 2008). The concentration of CT in common birdsfoot trefoil is controlled by additive genetic effects, indicating that mass selection would be effective to select genotypes with low or high content of CTs (MILLER; EHLKE, 1994). The fact that Al did not affect the production of dry matter eliminates the possibility that the differences in PC concentrations might have been caused by the dilution effect (Table 2).

Table 1. Concentration of procyanidins (PC) in *Lotus* L. genotypes under different Al saturations.

Species	Genotype	0 % Al	20 % Al
		PC (% of DM)	
L. corniculatus	UFRGS	0.53 cB	2.31 aA
L. corniculatus	Ganador	0.75 aB	1.50 bA
L. corniculatus	São Gabriel	0.70 bB	1.12 cA
L. subbiflorus	El Rincón	0.23 dB	0.90 dA
L. uliginosus	Serrano	0.70 bA	0.56 eB

Means followed by the same lowercase letter, in the column and uppercase on the line, don't differ by Student's t-Test (P>0.05). C.V (%) = 2.17.

The presence of CT in birdsfoot trefoil is unquestionable, since it is a characteristic of the genre. However, there are differences in the concentration of CTs in the germplasm, because they are affected by environmental conditions and methodology used. In the common birdsfoot trefoil, Sivakumaran et al. (2006) obtained between 1.56 % and 1.8 % of PC, which exceeds the values observed in this study. Barry and Forss (1983) found concentrations of 8 %-11 % in the New Zealand cultivar of *L. uliginosus* (cv. Maku), which was grown in acid soil without fertilizer, and 2 %-3 % in high-fertility soil. By using 33 samples of the same species evaluated in this study, Kelman and Tanner (1990) found that, despite most samples showing an increased content of these compounds (CT = 3.4 %) when grown in soil without limestone (pH = 4.3; Al = 4.2 µg with 0.01 M $CaCl_2 \cdot g^{-1}$), the reduction in content to 2.9 % was found in plants grown in soil with limestone (pH = 5.2; Al = 0.2 µg with 0.01 M $CaCl_2 \cdot g^{-1}$); these findings were not sufficient to indicate significant differences between the treatments.

Table 2. Dry matter yield obtained from four cuts in *Lotus* L. genotypes under different Al saturations.

Species	Genotype	0 % Al	20 % Al
		-------------------- DM (g pot^{-1}) -----------------	
L. corniculatus	UFRGS	27.4^{ds}	21.2^{ds}
L. corniculatus	Ganador	24.4	22.0
L. corniculatus	São Gabriel	28.0	18.3
L. subbiflorus	El Rincón	19.5	20.4
L. uliginosus	Serrano	20.2	21.5

ds= don't differ significantly by Student's t-Test (P>0.05).

Of the genotypes of common birdsfoot trefoil investigated in this study, São Gabriel showed the least variation in PC content when grown in soil with Al. The opposite was noted for UFRGS, which showed quadruple amount of polyphenols in soil with Al compared to that when it was cultivated in soil without Al (Table 1). Since previous studies have shown that this genotype has more Al tolerance than other genotypes of common birdsfoot trefoil (Draco and São Gabriel) (SANTOS et al., 2007, 2008; JANKE et al., 2010), the findings of this study indicate a possible association between tolerance to acidity and production of PCs in this species. If such a relationship is confirmed, the catechin and epicatechin contents could be used as chemical markers for Al tolerance. This is supported by the fact that, because CTs are involved in vacuolar storage of Al in plant cells, the species that synthesize these compounds also have greater tolerance to this element. Stoutjesdijk et al. (2001) observed complexation of Al by CT at the apex of the roots in great trefoil, suggesting a possible protective effect of these compounds. Malmir et al. (2009) indicated that, among various cultivars of sorghum (*Sorghum* sp.), the most tolerant to Al was the one that showed a higher concentration of CTs. These results corroborate earlier observations of many leguminous shrubs and trees adapted to acidic soils that have high CT levels (LASCANO; CARULLA, 1992).

The genotype, El Rincón (*L. subbiflorus*) showed similar behavior to the genotypes of the common birdsfoot trefoil: an increase in PC in response to the saturation of Al (Table 1). This was also observed by Kelman and Tanner (1990), who used a sample of that originated from Denmark, which had CT levels of 2.4 % in soil with limestone and 3.9 % in soil without liming. However, for Serrano, which had a behavior opposed to those of the other genotypes (Table 1), the results obtained in this study showed a decrease in CT in plants grown in acidic soil. This could be because, in great trefoil (*L. uliginosus*), such compounds are predominantly formed by PD (epigallocatechin and gallocatechin) (McNABB et al., 1997); however, this aspect was not assessed in this study. Previous studies have shown a PC:PD ratio of 16:84 (SIVAKUMARAN et al., 2006) and

19:81 (MEAGHER et al., 2004) in great trefoil, whereas this ratio was 60:40 (SIVAKUMARAN et al., 2006) and 84:16 for the common-trefoil (MEAGHER et al., 2004).

Thus, the changes in CTs of great trefoil might be regulated by the variation in PD, indicating the need for the quantification of gallocatechin and epigallocatechin in future studies. Separate quantification of the monomers that form CTs by HPLC in *Lotus* spp. would enable collection of more relevant information and allow a better characterization of the germplasm of these legumes in genetic improvement programs. The benefits of the presence of CTs in birdsfoot trefoil are related to not only the prevention of tympanism, but also the increase in ruminant livestock production. Moderate CT concentrations can be used to increase the efficiency of protein digestion and improve animal health, thereby allowing the production of more sustainable grazing systems (MIN et al., 2003).

The effect of Al saturation on the concentration of catechin and epicatechin was different in the genotypes evaluated in this study (Table 3). Except for El Rincón, which was grown in soil without Al, epicatechin was the predominant monomer and varied from 57 % to 75 % according to the quantity of Al in the soil. The predominance of this monomer in common birdsfoot trefoil was observed by Foo et al. (1996), who found 67 % of epicatechin of the total proanthocyanidins, i.e., of the total CTs in the species, about 30 % epigallocatechin and less quantities of catechin and epiafzelechin were found. This information, combined with the findings of other studies (MEAGHER et al., 2004; SIVAKUMARAN et al., 2006), reinforces that our findings might allow the investigation of a relationship between Al saturation level in the soil and CT concentration in the legumes. However, the same does not hold true for the great trefoil, since PC levels were lower in the CTs of this species.

Table 3. Concentration of procyanidins,catechin and epicatechin monomers in *Lotus* L. genotypes under different Al saturations.

Species	Genotype	0 % Al	20 % Al
		---------------------Catechin (% of DM)---------------	
L. corniculatus	UFRGS	0.14 eB	0.59 aA
L. corniculatus	Ganador	0.17 dB	0.33 bA
L. corniculatus	São Gabriel	0.24 aB	0.27 cA
L. subbiflorus	El Rincón	0.22 bB	0.30 dA
L. uliginosus	Serrano	0.20 cA	0.18 eB
Species	Genotype	--------------------Epicatechin (% of DM)------------	
L. corniculatus	UFRGS	0.40 dB	1.72 aA
L. corniculatus	Ganador	0.57 aB	1.17 bA
L. corniculatus	São Gabriel	0.45 cB	0.90 cA
L. subbiflorus	El Rincón	0.01 eB	0.60 dA
L. uliginosus	Serrano	0.49 bA	0.38 eB

Means followed by the same lowercase letter, in the column and uppercase on the line, don't differ by Student's t-Test (P>0.05). C.V(%): catechin= 3.6 %; epicatechin= 2.7 %.

With the exception of Serrano, acidic soil was found to promote a significant increase in catechin and epicatechin. El Rincón showed distinct behavior from the other genotypes, since it contained epicatechin traits when grown in soil without Al, indicating its potential to synthesize this monomer in acidic soil. Because of the different quantities of PC monomers, the epicatechin:catechin proportion was affected by the saturation of Al in the soil (Table 4). Only UFRGS did not respond to this factor and maintained a similar proportion of these monomers in both the conditions of acidity in the Al soil. The epicatechin:catechin proportion decreased in the other genotypes when grown in soil without Al, suggesting the importance of considering the soil factors while analyzing these compounds.

Table 4. Epicatechin:catechin ratio (E:C) in *Lotus* L. genotypes under different Al saturations.

Species	Genotype	0 % Al	20 % Al
		E:C	E:C
L. corniculatus	UFRGS	74:26 bA	75:25 bA
L. corniculatus	Ganador	76:24 aB	78:22 aA
L. corniculatus	São Gabriel	65:35 dB	77:23 aA
L. subbiflorus	El Rincón	1:99 eB	68:32 cA
L. uliginosus	Serrano	70:29 cB	68:32 cA

Means followed by the same lowercase letter, in the column and uppercase on the line, don't differ by Student's t-Test (P>0.05). C.V(%): 5.2 %.

Thus, the fact that only UFRGS maintained the epicatechin:catechin proportion in both soil acidity conditions might be related to the selection process to which it was submitted. This genotype is preferred as forage material owing to its higher tolerance to Al (SANTOS et al., 2007; JANKE et al., 2010). Among the possible causes of Al tolerance is the amount of polyphenols, such as catechins that can detoxify this element by chelation (CHEN et al., 2009). Previous studies have shown that the UFRGS genotype has surpassed other birdsfoot trefoil genotypes regarding the exudation of organic acids and radical growth and accumulation of tannins after treatment with Al (PAVLOVKIN et al., 2009; PAL'OVE-BALANG et al., 2012).

This study indicates the value of *Lotus* L. species as enhanced leguminous plants of the natural pastures in southern Brazil, especially owing to their high tolerance to acidity and requirement of low soil fertility compared to clovers. However, further studies are warranted to analyze PCs in these plants for their application in genetic enhancement programs. Future studies need to analyze the interaction between PCs and the Al in *Lotus* L.; this might indicate that PCs are the possible indicators for tolerance.

Conclusions

1. The concentration of procyanidins and their monomers-catechin and epicatechin-in *Lotus* spp. varies according to the genotype and Al saturation in the soil.

2. In soils with Al saturation, there is an increase in the concentration of procyanidins in *L. corniculatus* and *L. subbiflorus* and a decrease in procyanidin concentration in *L. uliginosus.*

3. Epicatechin is the main forming unit of procyanidins in *Lotus* spp. grown in soil with Al saturation.

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