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Genetic variability of isoflavones in the USDA red clover core collection

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Abstract: Red clover is one of the most utilized forage in agriculture and contains many of the isoflavones known for their human health benefits. The objectives of this study were: i) to quantify, using HPLC analysis, isoflavones in 77 accessions from the USDA core collection and a Brazilian line; ii) to verify possible relationships depending on their origin, improvement status or maturity type and; iii) to verify the seasonal variation. The isoflavone mean contents were 29.27 μg g^{-1} of dry material for daidzein, 163.69 μg g^{-1} for genistein, 11353.29 μg g^{-1} for formononetin and 6568.8 μg g^{-1} for biochanin A. Clustering was mainly influenced by the total amount of isoflavones and partially due to maturity type, improvement status and geographic origin. The seasonal evaluation demonstrated an increase of concentration during winter, and decrease during spring. These results highlighted accessions that can be used to develop new varieties with low or high isoflavones concentration.

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Introduction

Trifolium pratense L., Fabaceae (red clover), is one of the most utilized legume forage in the world (Bowley et al., 1984) and contains many isoflavones, mainly formononetin and biochanin A, as well as smaller amounts of daidzein and genistein. Many works using morphologic (Christie & Choo, 1991; Kouamé & Quesenberry, 1993), molecular (Greene et al., 2004; Sato et al., 2005; Dias et al., 2008a) and biochemical traits (Yu et al., 2001; Mosjidis & Klinger, 2006) have showed the high genetic diversity present in this species. All red clovers may be grouped into three divisions corresponding to early, medium and late maturity types (Bird, 1948; Taylor & Smith, 1995). Depending on the maturity type there are different usages. The late type varieties, or single cut, produce one cut in a year and the early type ones, or double cut, produce several times in a year (Taylor & Smith, 1995).

The red clover core collection of the National Plant Germplasm System of United States Department of Agriculture (NPGS-USDA) is composed by 85 accessions originating from 41 countries (Mosjidis & Klinger, 2006). This core collection was originated from the analysis of more than 800 accessions present in the Germplasm Resource and Information Network of the National Plant Germplasm System (GRIN-NPGS). The cluster analysis of these accessions using standardized values of fifteen morphological and physiological descriptors produced distinct groups that correspond to early, medium, and late maturity groups (Kouamé & Quesenberry, 1993). The accessions from the core collection were also classified by the GRIN-NPGS (http://www.ars-grin.gov/npgs/acc/acc_queries.html) on the basis of improvement status into cultivars, cultivated materials, breeding materials, landraces and wild populations (Mosjidis & Klinger, 2006) and also according to maturity into single-cut (late flowering type), double-cut (early flowering type) or other type.

Phytoestrogens are naturally occurring compounds found in plants to varying degrees. These compounds are non-steroidal polyphenolic plant metabolites that because of structural similarities to 17β-estradiol, have the ability to bind to estrogen receptors and so they may exert estrogenic and/or anti-estrogenic effects (Murkies et al., 1998). Studies of utilization of red clover by the pharmaceutical industry have pointed that dietary phytoestrogens (like isoflavones) play an important role in the prevention of menopausal symptoms (Beck et al., 2003; Lipovac et al., 2010), osteoporosis (Atkinson et al., 2004a), estrogen-related cancers (breast cancer, prostate cancer) (Atkinson et al., 2004b; Velentzis et al., 2008) and heart disease (Dixon, 2004; Cano et al., 2010). Future breeding activities need to take this into account, exploiting the wide range of genetic diversity present in this species in order to

develop new varieties to supply the demands from this new market (Greene et al., 2004). However, total and individual isoflavones concentration are affected by many factors including fertility management, plant maturity, tissue type, genetics, sampling methodology and seasonal variation (Papadopoulos et al., 2006; Booth et al., 2006).

The seasonal evaluation is a key point in isoflavones production to verify the optimal harvest time for highest or lowest isoflavones contents according to the intended use (forage or medicine). Despite the importance of the red clover core collection as source of germplasm in breeding programs, its biochemical diversity was never investigated before concerning the amount and the seasonal variation of isoflavones.

Considering all this points the aims of this study were to: i) evaluate the amount of four isoflavones (daidzein, genistein, formononetin and biochanin A) for the first time in the red clover accessions from the NPGS-USDA core collection, using a high-performance liquid chromatography (HPLC) method; ii) verify possible relationships between accessions depending on their origin, improvement status or maturity type by a cluster analysis; and iii) study the seasonal variation of isoflavones concentration in red clover accessions to verify the best harvest time in Brazil.

Materials and Methods

Plant material

For this study, a subset of 77 accessions from 35 different countries (one or two accessions for each country) from the red clover core collection of National Plant Germplasm System of the United States Department of Agriculture NPGS-USDA and one population (N°39) developed in Brazil were selected (Table 1). This Brazilian population was included in the analysis because it is a product of a breeding program aimed at creating adapted cultivars to southern Brazil, with a local seed production that is currently underway by the Federal University of Rio Grande do Sul (Crusius et al., 1999; Montardo et al., 2003).

The 78 accessions were planted in a greenhouse (in the same day) using a complete randomized design with five repetitions (plants) per accessions. From each accession, seeds were germinated in Petri dishes with watered filter paper and after 15 days were planted in plastic pots (750 mL capacity). The plants were inoculated with 1 mL of a solution of *Rhizobium leguminosarum* bv. *trifolii* in aqueous solution (concentration of 10^9 cells mL^{-1}). After one month the plants were transferred to pots of 5 kg soil capacity. The greenhouse was located at Porto Alegre, Rio Grande do Sul, Brazil (30º01'59" S, 51º13'48" W and elevation 10 m). Irrigation was applied to maintain soil moisture through the growing season. From each accession three leaves were collected from five plants. At the harvest moment, all plants were at the vegetative stage.

The accessions used for the seasonal study (N°29-PI 419550, N°44-PI 179146 and N°72-PI 376880) were selected randomly. They were planted as described above and placed outside of the greenhouse in a field at weather conditions. Harvests were realized always from the same five plants (three leaves were collected from each adult plant) one month after the start of the season, the plants were already adults (about 12 months) at the first harvest season. Plants were evaluated for four different isoflavone aglycones: daidzein, genistein, formononetin, and biochanin A.

Assays

HPLC analysis: Analyses were performed on a Waters Alliance 2695 chromatograph and a UV detector (UV/VIS Waters 2487). The system was equipped with a C18 reverse-phase column (Nova-Pak, 4 µm, 3.9 x 150 mm) with guard-column and operated at room temperature.

The sample preparation and HPLC method used in this work were previously validated by Ramos et al. (2008). One harvest was made, where three leaves from each five plants were pooled, resulting in 15 leaves from each accession, and dried in an oven at 100 °C during 1 h, after that, the plant material was ground with a mortar and pestle. Ten mg of plant material were extracted with 4 mL of 6M HCl and incubated at 100 °C for 15 min with magnetic agitation in a water bath. After cooling, the residue was filtrated and washed with methanol (5 mL). The extract was transferred to a 10 mL volumetric flask that was filled up with distilled water. Before HPLC injection the extracted was filtrated using a 0.45 µm membrane (Ramos et al., 2008).

Elution of isoflavones was performed using a linear gradient system. The mobile phase consisted of acetonitrile:water:trifluoroacetic acid (20:80:0.01; v/v/v) (A) and acetonitrile:trifluorocetic acid (100:0.1; v/v) (B). The gradient profile was: 0-10 min from 0 to 40% B, 10-11 min 40% B, 11-12 min from 40 to 100% B. At the end each run, 6 min of 100% A was used to restore the initial conditions. The flow-rate was 0.7 mL/min. The wavelength of detection was 260 nm.

Statistical analysis

Isoflavones quantification: Analysis of variance was carried out, with data from all accessions and all isoflavones using PROC GLM of SAS software (SAS Institute, 2001). The model applied to the greenhouse data analysis was: $Y_{ij}=\mu+pi+E_{ij}$, where Y_{ij} is the value

Table 1. Passport data of 78 red clover accessions from NPGS-USDA core collection.

Number	Accession	Country	Type[a]	Improvement Status[b]	Group[c]
1	PI 237705	Denmark	Late	Cultivar	III
2	PI 196424	Denmark	Late	Cultivar	III
3	PI 217507	Denmark	Other	Cultivar	III
4	PI 237714	Denmark	Late	Cultivar	II
5	PI 235847	Sweden	Early	Cultivar	III
6	PI 235854	Sweden	Late	Landrace	III
7	PI 235870	Sweden	Early	Landrace	II
8	PI 229799	Finland	Late	Cultivar	III
9	PI 236455	Finland	Late	Cultivar	III
10	PI 310459	Switzerland	Early	Cultivar	II
11	PI 266047	Poland	Late	Cultivar	II
12	PI 384058	Poland	Early	Cultivar	III
13	PI 294481	Austria	Other	Cultivar	III
14	PI 318888	Hungary	Late	Landrace	III
15	PI 315522	Italy	Other	Uncertain	III
16	PI 249870	Greece	Other	Wild	III
17	PI 253583	Spain	Early	Wild	III
18	PI 188680	France	Other	Landrace	II
19	PI 207972	France	Early	Uncertain	III
20	PI 201191	Netherlands	Early	Uncertain	III
21	PI 204506	Turkey	Early	Uncertain	II
22	PI 204507	Turkey	Early	Uncertain	III
23	PI 371959	Bulgaria	Other	Cultivar	III
24	PI 294797	Bulgaria	Other	Landrace	III
25	PI 251564	Serbia	Other	Cultivated	III
26	PI 207520	Afghanistan	Other	Uncertain	III
27	PI 228160	Russia	Other	Cultivar	III
28	PI 345675	Russia	Other	Cultivated	II
29	PI 419550	Japan	Early	Breeding Material	I
30	PI 184960	Australia	Early	Uncertain	II
31	PI 187284	United Kingdom	Early	Cultivar	II
32	PI 306188	United Kingdom	Early	Cultivar	II
33	PI 315534	Canada	Other	Cultivar	III
34	PI 295355	USA	Other	Landrace	I
35	PI 230229	USA	Early	Landrace	I
36	PI 302421	Colombia	Early	Cultivar	II
37	PI 304842	Chile	Early	Cultivar	II
38	PI 226952	Ethiopia	Early	Wild	II
39	EEA/UFRGS	Brazil	Lated	Breeding material	II
40	PI 314840	Norway	Other	Cultivar	III
41	PI 188905	Sweden	Early	Cultivar	II
42	PI 235867	Sweden	Other	Landrace	III
43	PI 310465	Switzerland	Early	Uncertain	II
44	PI 179146	Switzerland	Early	Cultivated	II

Continuation of Table 1. Passport data of 78 red clover accessions from NPGS-USDA core collection.

Number	Accession	Country	Type[a]	Improvement Status[b]	Group[c]
45	PI 234925	Switzerland	Early	Wild	III
46	PI 239696	Switzerland	Early	Uncertain	III
47	PI 632214	Not available	Early	Uncertain	II
48	PI 255894	Poland	Late	Cultivar	III
49	PI 225119	Germany	Early	Cultivar	III
50	PI 187008	Germany	Other	Not available	III
51	PI 234836	Germany	Early	Wild	II
52	PI 318887	Hungary	Early	Landrace	III
53	PI 418889	Italy	Early	Wild	III
54	PI 419294	Greece	Early	Wild	III
55	PI 220856	Portugal	Early	Uncertain	II
56	PI 311492	Spain	Late	Uncertain	II
57	PI 307948	Spain	Early	Wild	III
58	PI 189174	Netherlands	Other	Cultivar	III
59	PI 187224	Belgium	Early	Cultivar	II
60	PI 234448	Belgium	Late	Cultivar	III
61	PI 205313	Turkey	Early	Uncertain	II
62	PI 120105	Turkey	Early	Landrace	II
63	PI 171870	Turkey	Early	Uncertain	III
64	PI 314487	Georgia	Other	Wild	III
65	PI 315533	Bulgaria	Early	Cultivar	III
66	PI 228365	Iran	Early	Uncertain	III
67	PI 250899	Iran	Early	Wild	III
68	PI 401469	Romania	Early	Cultivar	III
69	PI 232941	Hungary	Early	Cultivar	III
70	PI 440737	Russia	Early	Wild	III
71	PI 419565	Japan	Early	Breeding Material	III
72	PI 376880	New Zealand	Early	Cultivar	III
73	PI 306185	United Kingdom	Early	Cultivar	III
74	PI 286116	Canada	Early	Cultivar	II
75	PI 286222	Canada	Other	Cultivar	II
76	PI 306677	Ecuador	Early	Landrace	III
77	PI 449326	Chile	Early	Landrace	III
78	PI 271627	India	Early	Uncertain	III

[a]Other: would not have a consistent response across tested locations (NPGS-GRIN); [b]Classification according to the NPGS-GRIN; [c]Group obtained by the cluster analysis based on the isoflavone amounts; [d]Maturity in the southern Brazilian conditions.

recorded on the plant from population *i* in repetition *j*, μ is the overall mean, pi is the fixed population effect, and E_{ij} represents the random error present in the *i* population on the *j* repetition. Furthermore, two analysis of variance for the amounts of isoflavones were carried out following the NPGS classification. Thus, the 78 accessions of red clover were classified into (1) cultivar, (2) cultivated materials, (3) breeding materials, (4) landraces and (5) wild populations for the improvement status and for the maturity type into (1) early flowering type, (2) medium flowering type and (3) late flowering type. The ANOVA model applied for each analysis was the same used for the population analysis.

The Euclidean distance matrix based on the standardized values for the amounts of isoflavones was calculated between the 78 accessions and a cluster analysis was performed using the unweighted pair-group procedure with an arithmetic mean (UPGMA) as described by Sneath & Sokal (1973). A cophenetic

matrix was calculated from the tree distance matrix. The reliability of the dendrogram generated by the clustering analysis was tested by computing Mantel test statistics (Mantel, 1967) for the correlation between the Euclidean distance matrix and the cophenetic matrix. The mean Euclidean distance (6.62) was considered as the cutting point for the dendrogram analysis. A principal component analysis (PCA) was performed using the standardized values for all isoflavones. All analyses were performed using NTSYSpc version 2.1 program (Rohlf, 2000).

Seasonal variation

Using the PROC GLM of SAS software (SAS Institute, 2001), the analyses of variance for all isoflavones were performed on each of the three accessions comparing the data from all seasons. The model applied for each of the three accessions in the data analysis was the same used for the greenhouse analysis. The season's amounts of isoflavones in each accession were compared using the post hoc Tukey HSD test of means.

Results

Most isoflavones in red clover are malonated and glycosylated with only a small percentage present in the aglycone form (Edwards et al., 1997; Toebes et al., 2005; Sivesind & Seguin, 2005). Therefore, the quantification of isoflavones in this study represents the total isoflavone concentration in red clover, including malonyl, glycosylated and aglycone forms. The aglycone contents across the 78 accessions (Table 2) varied from 0.00 to 137.91 μg g^{-1} of dry material (DM) for daidzein, 14.70 to 516.91 μg g^{-1} of DM for genistein, 452.97 to 28 548.65 μg g^{-1} of DM for formononetin and 2 199.02 to 15 670.39 μg g^{-1} of DM for biochanin A. The total isoflavone concentration ranged between 9.81 and 36.36 mg $g^{-1}$of dry red clover leaves.

The lowest total isoflavone concentration was found in a landrace from Hungary (N°14-PI318888) while the highest total isoflavone concentration was founded in a landrace from United States of America (N°35-PI230229) (Table 2). Interestingly, the two accessions classified by the NPGS-USDA as tetraploid (N°40-PI314840 and N°11-PI266047) presented no differences in total isoflavone contents when compared to the diploid ones.

Analysis of variance within each type of analysis (population, improvement status and type of maturity) indicated significant differences between accessions for the isoflavones with only exception for biochanin A considering the improvement status and the type of maturity (Table 3).

The mean Euclidean distance between the 78 accessions was 6.62 and ranged from 0 to 17.58. The dendrogram based on the Euclidean distance matrix indicated the clustering of the accessions in three main groups (Figure 1). The cophenetic correlation, i.e., the correlation between the cophenetic matrix and the distance matrix, was r=0.88, indicating a good fit to the dendrogram derived from the cluster analysis. The means and standard deviation of isoflavones concentration on the three groups found with cluster analysis are reported in Table 4.

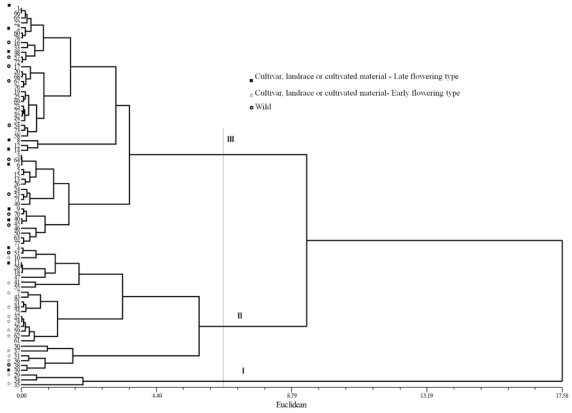

Figure 1. Dendogram of the 78 red clover accessions revealed by UPGMA cluster analysis of Euclidean genetic distance. The thin vertical line indicates the cut point (mean genetic distance).

Group I was composed by three accessions (Figure 1) that showed the highest content of daidzein and formononetin and presented the lowest content of genistein (Table 2). This group was mainly characterized by the highest total isoflavone content. Most accessions present in this group were classified by NPGS-USDA as landraces (N°34-PI295355 and N°35-PI230229) and breeding material (N°29-PI419550) with early flowering maturity types.

Group II was composed by 26 accessions, mostly cultivars or cultivated material that showed the highest genistein content and the second high total isoflavone content. Most accessions present in this group were also classified as early maturity type according to NPGS-USDA (Table 1). The Brazilian population (N°39) is present in this group with high total isoflavones content.

Finally, group III gathered the largest number of accessions (49) characterized by the lowest contents of daidzein, biochanin A, formononetin. This group was mainly characterized by the lowest total isoflavone content. Interestingly, almost all accessions classified as wild with early maturity (N°17, 45, 53, 54, 57, 67, 70) from the red clover core collection were gathered in this group (Table 1, Figure 1). It is important to

Table 2. Isoflavone means and standard deviation for the 78 red clover accessions.[a]

Number	Daidzein[b]	Genistein[b]	Formononetin[b]	Biochanin A[b]	Total[c]
1	17.70±5.53	131.30±2.53	9 785.96±3.31	2 499.55±4.10	12.43
2	25.87±8.86	9.52±6.41	9 061.74±3.93	3 751.38±5.04	12.85
3	24.28±1.36	19.68±7.47	12 200.17±5.46	3 591.37±7.59	15.84
4	30.94±7.78	100.41±1.99	12 598.38±0.77	7 539.74±0.44	20.27
5	16.43±3.72	150.18 ±4.66	7 176.57±3.74	9 135.45±3.82	16.48
6	21.46±8.86	122.35±4.26	10 970.80± 6.58	4 691.05±6.65	15.81
7	52.53±1.39	77.79±4.00	15 621.64±0.98	8 804.96±1.30	24.56
8	7.87±3.25	78.19±6.20	6 568.33±2.59	3 806.78±3.34	10.46
9	22.20±3.09	96.27±1.02	8 018.49±1.22	8 710.42±1.23	16.85
10	21.37±9.92	118.21±6.04	10 163.32±1.47	9 505.79±0.88	19.81
11	24.15±5.87	115.03±3.57	14 076.68±2.44	6 838.78±0.26	21.05
12	9.11±7.97	59.62±2.64	6 479.65±3.57	3 466.56±5.18	10.01
13	19.58±6.75	49.52±7.45	7 907.60±3.81	8 129.54±2.05	16.11
14	7.28±4.93	33.76±7.84	5 595.14±0.66	4 178.28±7.03	9.81
15	35.72±12.40	85.69±0.92	11 410.87±4.22	4 869.87±4.36	16.40
16	0	99.57±6.21	578.22±7.17	10 813.49±6.29	11.49
17	0	149.77±3.79	5 243.77±10.71	7 755.45±3.74	13.15
18	0	217.61±3.02	9 368.94±3.96	11 432.91±3.61	21.02
19	22.33±5.79	51.60±11.36	9 234.70±3.75	4 717.85±2.01	14.03
20	22.22±3.30	17.14±8.38	9 460.43±4.02	4 004.19±5.54	13.50
21	25.81±6.18	130.28±6.45	13 867.67±3.39	9 923.49±5.04	23.95
22	4.26±3.52	76.55±9.84	8 724.34±4.88	3 725.11±8.74	12.53
23	17.89±8.63	39.76±8.49	9 781.59±5.81	4 109.10±5.14	13.95
24	41.80±3.62	160.15±4.90	8 970.84±0.83	6 073.66±5.47	15.25
25	18.92±6.71	46.29±6.09	9 805.80±1.66	4 330.47±3.95	14.20
26	72.75±3.04	39.15±3.29	452.97±1.43	15 670.39±1.41	16.24
27	21.36±8.06	75.75±2.24	8 451.93±0.76	5 340.85±0.01	13.89
28	37.98±0.90	125.92±1.79	14 916.18±3.83	5 977.54±1.86	21.06
29	71.37±3.16	226.10±5.31	25 026.62±5.91	9 272.23±7.59	34.60
30	67.59±8.26	128.01±7.81	16 160.09±8.86	11 478.19±8.25	27.83
31	23.11±4.96	334.80±5.98	14 167.54±2.07	12 591.14±3.65	27.12
32	33.08±0.79	104.06±5.55	12 632.00±5.34	10 337.33±6.17	23.11
33	20.32±4.96	206.47±6.11	9 268.17±2.84	2 199.02±3.61	11.69
34	91.31±0.14	78.71±5.36	23 461.82±5.37	9 950.20±1.76	33.58
35	102.91±7.96	73.58±6.57	28 548.65±3.04	7 612.77±2.98	36.36
36	21.40±2.54	278.40±2.34	11 672.11±2.16	14 908.10±0.15	26.88
37	53.77±9.20	171.44±3.36	18 023.39±2.14	11 247.64±2.75	29.50
38	42.61±8.48	76.07±7.77	18 338.64±7.15	9 417.67±8.05	27.87
39	36.78±2.29	138.60±6.96	16 376.52±3.04	11 144.73±4.28	27.70
40	5.31±5.96	124.72±5.71	13126.22±1.78	3708.69±1.14	16.96
41	6.73±3.67	315.21±5.43	13726.68±8.84	5045.36±7.11	19.09
42	32.01±9.90	160.31±5.89	10713.98±4.88	2948.10±9.05	13.85
43	3.19±14.10	516.80±8.80	19234.79±11.05	4974.13±9.28	24.73
44	4.03±14.24	286.93±4.21	18602.98±7.58	5020.09±6.04	23.91

Continuation of Table 2. Isoflavone means and standard deviation for the 78 red clover accessions.[a]

Number	Daidzein[b]	Genistein[b]	Formononetin[b]	Biochanin A[b]	Total[c]
45	6.11±4.11	239.24±8.33	11325.43±2.10	5610.23±11.71	17.18
46	10.07±7.69	327.15±1.00	8345.16±9.14	9344.65±7.27	18.03
47	25.55±3.82	160.51±3.12	16181.23±1.31	5446.30±2.71	21.81
48	11.42±9.68	156.20±13.17	9838.78±14.46	1967.64±8.23	11.97
49	22.40±3.23	153.65±3.65	11778.49 ±13.35	3180.85±4.67	15.14
50	29.04±10.98	380.90±9.78	12836.81±3.86	4487.31±5.20	17.73
51	21.31±2.61	347.44±10.32	12348.43±3.37	7608.48±3.93	20.33
52	9.44±11.47	195.38±1.12	10774.09±9.45	2796.69±10.52	13.78
53	14.33±7.20	161.14±11.15	10292.76±5.68	4633.65±5.40	15.10
54	54.74±10.67	108.65±13.15	10198.99±8.84	3988.89±8.55	14.35
55	11.72±13.32	179.57±1.76	12948.83±1.08	5515.34±3.16	18.66
56	0.55±14.66	329.70±2.07	2705.39±12.35	20145.27±9.37	23.18
57	28.21±12.78	312.28±10.38	6466.00±10.11	5200.73±12.50	12.01
58	8.15±6.10	164.62±0.38	9682.74±11.97	4751.97±11.75	14.61
59	54.52±10.64	261.88±6.53	14894.87±7.94	7778.15±8.60	22.99
60	53.26±9.07	298.56±3.16	8172.88±4.01	4214.03±5.22	12.74
61	24.18±1.35	190.98±6.10	16674.89±1.29	6584.11±3.62	23.47
62	46.10±2.83	305.74±1.97	10991.52±0.85	11478.19±8.25	22.82
63	6.09±5.83	166.61±2.72	12849.86±4.63	4382.20±6.72	17.40
64	20.84±3.77	124.24±6.35	9080.60±3.95	6596.83±3.55	15.82
65	13.37±13.15	136.49±3.57	8946.91±8.27	3484.12±12.96	12.58
66	40.74±12.32	119.71±0.25	8727.61±12.28	3562.58±12.25	12.45
67	73.84±10.09	406.60±14.59	9429.42±11.14	3501.50±10.89	13.41
68	19.63±1.37	186.01±10.17	8242.93±5.70	5097.21±9.15	13.55
69	47.43±14.40	192.41±9.14	8857.70±10.11	5019.37±5.45	14.12
70	35.91±13.07	339.56±2.69	7969.84±5.04	8421.07±8.87	16.77
71	23.99±10.91	143.87±13.07	9252.61±4.18	5601.70±3.47	15.02
72	40.25±1.19	87.03±7.70	7539.25±0.26	2781.34±0.79	10.45
73	31.71±11.87	151.53±4.94	12465.50±1.15	4518.29±2.32	17.17
74	16.43±14.03	70.47±9.20	15956.81±11.18	7039.93±14.19	23.08
75	33.00±7.36	292.36±13.15	15302.95±6.99	8504.21±12.01	24.13
76	32.41±11.80	89.27±10.31	9122.64±7.74	4089.31±4.80	13.33
77	41.71±3.74	134.96±11.23	10858.07±3.79	6455.88±2.05	17.49
78	30.66±6.90	100.67±2.60	9243.96±7.53	3319.91±7.94	12.70

[a]Results present the mean of isoflavones quantification per population analyzed in triplicate±Standard Deviation (%); [b]Concentration in μg g^{-1} of DM; [c]Concentration in mg g^{-1} of DM.

note that the most part of cultivars or landraces from Northern Europe (N°1, 2, 6, 8, 9, 14, 40, 48) with late flowering behavior were also present in this group (Table 1). According to PCA analysis 72.08% of the total variation in red clover accessions is explained by two first principal components. The major sources of diversity along the first principal component (loading in parentheses) were total of isoflavones (0.875), formononetin (0.827) and daidzein (0.787). The equivalent source of variability for the second principal component was genistein (0.870) and biochanin A (0.573).

Seasonal evaluation results are presented at Table 5. Results showed that the total concentration of isoflavones is lower during spring (when the plants are at reproductive stage), and higher when the plant is at vegetative stage (during autumn and winter); however this difference is not significant for all populations evaluated.

Table 3. Means, standard deviations (SD) and analysis of variance for amounts of isoflavones, of 78 accessions of red clover by accession, improvement status and type of maturity.

Isoflavone	Mean[a]	SD	Means squares[b]		
			Accessions	Error	F
Daidzein	29.27	21.47	1370.62	12.05	113.70
Genistein	163.69	106.87	33811.84	368.92	91.65
Formononetin	11353.29	4745.34	66601586.07	759104.84	87.74
Biochanin A	6568.80	3434.07	35168422.86	254905.94	137.97
Total	37.05	6.09	108.29	1.66	65.24
			Improvement status	Error	F
Daidzein			2951.32	415.59	7.10
Genistein			36415.99	10984.27	3.32
Formononetin			151976743.16	20256923.62	7.50
Biochanin A			11411411.76 NS	11799486.13	0.97
Total			186.94	34.40	5.44
			Type of maturity	Error	F
Daidzein			1344.52	452.91	2.97
Genistein			77017.04	10852.94	7.10
Formononetin			143121285.70	21474025.59	6.66
Biochanin A			136161.29 NS	11893747.40	0.01
Total			147.98	36.05	4.10

[a]Concentration in μg g^{-1} of DM; [b]All mean squares were significant at $p \leq 0.05$ except those indicated as NS.

Table 4. Means of the four isoflavones contents and total isoflavone for each group.

Group	Daidzein [a]	Genistein [a]	Formononetin [a]	Biochanin A [a]	Total [b]
I	94.53	126.13	25679.03	8945.07	34.84
II	27.63	206.70	14136.63	9087.98	23.46
III	24.34	142.04	9005.86	5086.42	14.26

Table 5. Results for seasonal evaluation for each accession.[a]

PI179146	Daidzein[b]	Genistein[b]	Formononetin[b]	Biochanin A[b]	Total[b]
Summer	0.03±11.6a	0.11± 0.71a	8.52±4.74a	6.60±12.64a	15.28a
Autumn	0.00b	0.13±2.64a	8.94±11.08a	4.75±10.87b	13.82a
Winter	0.00b	0.20±6.78b	11.26±7.02b	5.76±6.96a	17.22a
Spring	0.00b	0.07±12.02a	7.27±5.65a	5.28±8.79a	12.63a
PI376880	Daidzein[b]	Genistein[b]	Formononetin[b]	Biochanin A[b]	Total[b]
Summer	0.02±11.27a	0.09±3.07a	8.85±0.98a	6.11±2.88a	15.07a
Autumn	0.00b	0.25±3.65b	9.99±11.24b	4.65±14.62b	14.90a
Winter	0.00b	0.23±2.63c	13.23±7.36c	2.87±14.13c	16.33a
Spring	0.00b	0.30± 3.40b	6.84±7.76b	3.33±5.23c	10.47b
PI419550	Daidzein[b]	Genistein[b]	Formononetin[b]	Biochanin A[b]	Total[b]
Summer	0.00a	0.13±5.24a	10.81±9.24a	3.76±8.04a	14.69a
Autumn	0.00a	0.16±7.31b	9.86±4.64a	2.52±4.66b	12.54b
Winter	0.00a	0.26±1.02c	10.12±4.51a	2.94±2.34bc	13.33ab
Spring	0.05±4.36b	0.06±8.30d	9.22±2.41a	3.27±1.88c	12.60b

[a]Results present the mean of isoflavones quantification per population analyzed in triplicate±Standard Deviation (%); [b]Concentration in mg g^{-1} of DM. means followed by different letters are statistically different (α=0.05).

Discussion

Genetic diversity for isoflavones and cluster analysis

In our study, we analyzed 78 populations of red clover and evidenced the high significant variation for the isoflavone contents taking into account individual populations and different improvement status and maturity types.

The means of total isoflavone concentration reported here (Table 2) were higher than results found in previous studies. Sivesind & Seguin (2005), related isoflavone concentrations in ten red clover cultivars in Quebec, ranging from 8 923 to 12 753 μg g^{-1} of DM. Booth et al. (2006) also found similar isoflavones concentrations ranging from 3 070 to 10 210 μg g^{-1} of DM when analysing only one red clover cultivar.

It is important to note that individual isoflavones concentration, and total isoflavone concentration varied considerably among the accessions of the red clover core collection analysed in our study. Similarly, high genetic variability for the amounts of isoflavones was also found in other works using cultivars (Sivesind & Seguin, 2005, Tsao et al., 2006, Papadopoulos et al., 2006) and non cultivated populations of red clovers (Vetter. 1995; Oleszek et al. 2007). This variability is important to select accessions with high isoflavones content to be used by the pharmaceutical industry or accessions with low content to be used as forage plants. The variation of isoflavone content can be explained by the fact that the isoflavones concentration in legumes is controlled by genetic and environmental factors. Isoflavones are constitutively found in legumes, but their concentrations often increase in response to biotic and abiotic stresses (Sivesind & Seguin, 2005).

Concerning the different ploidy levels present in the red clover core collection, similar results were found by Tsao et al. (2006) when comparing total isoflavones contents in diploid and tetraploid cultivars.

All the characters that showed genetic variation contributed to structure the diversity in this subset of the red clover core collection, as found in the principal component analysis and in the hierarchical cluster.

The 78 populations were separated into three distinct clusters, mainly characterised by differences in the total of isoflavones, formononetin, daidzein and partially due to genistein. The groups with the highest total of isoflavones were mainly composed by landraces, cultivar or cultivated materials classified by NPGS as early flowering types. Some accessions (N°29, 30, 32, 74) present in these groups were evaluated for maturity type by Dias et al (2008a) in the same Brazilian region and were also classified as early flowering types.

Surprisingly, these cultivars, landraces or cultivated material normally used as forage (Figure 1), presented the highest total concentration of isoflavones (Table 2). However, in general isoflavones concentration is not a problem for large ruminants and little research has been conducted on breeding for lower isoflavones content in forage breeding programs in United States (Taylor, 2008). Contrary to large ruminants, high levels of isoflavones have been showed to cause infertility in ewes (Mcdonald, 1995; Moorby et al., 2004) and low phytoestrogens cultivars have been released in Australia and New Zealand (Oram, 1990; Rumball et al., 1997).

Finally, the largest group characterized by the lowest total isoflavone content was mainly composed by wild accessions with early flowering behavior and cultivars from Northern Europe classified as late flowering. However, it is important to note that the same wild accessions when analyzed by Dias et al (2008a) in the same Southern region of Brazil presented a late flowering behavior.

Although no clear geographic separation between groups was demonstrated, clustering highlighted some late flowering cultivars from Northern Europe that were almost all present in the Group III which presented the lowest isoflavones content (Table 1) (Figure 1). Taylor & Smith (1995) described that distinct plant types of red clover have evolved through natural selection and in Europe these types are largely distributed according to latitude, with the late flowering types more common to north of 60° latitudes. These cultivars from Northern Europe were also grouped and showed late maturity in recent studies realized in the same region of Brazil using morphological, biochemical and molecular markers (Dias et al., 2008a; Dias et al., 2008b).

So, differences in the total of isoflavones seems to be influenced by type of maturity of cultivars with the early types showing the highest contents and partially due to improvement status and geographic origin with the accessions classified as wild and late flowering cultivars from Northern Europe showing the lowest content of isoflavones.

Several works have demonstrated that isoflavones composition is similar among different red clovers cultivars. But, the total concentration of isoflavones differed significantly between cultivars (Sivesind & Seguin 2005, Tsao et al., 2006). The red clover core collection is formed mainly by cultivars or cultivated material. Thus the high level of variation in total isoflavone concentration between cultivars could explain this partially structurxation related to improvement status. Moreover, the breeding schemes and the synthetic structure of the red clover cultivars with several parental families maintain a large within cultivar variation (Taylor, 2008).

Papadopoulos et al. (2006) showed in their study that total and individual isoflavone concentration in red clover plants presented a high genetic variability among

thirteen red clover cultivars, and suggest that selecting individual plant phenotypes for high isoflavones contents would be highly effective for cultivars development. Specific cultivar recommendations could thus be made depending on if concentrations are to be maximized or minimized depending on the intended use (Sivesind & Seguin, 2005).

Since all red clover accessions evaluated here were grown in greenhouse under the same environmental conditions, the differences between cultivars in total isoflavone concentration could be explained by different genetic backgrounds. Genetic variation has been found to play an important role in isoflavones production in red clover (Sivesind & Seguin 2005, Tsao et al., 2006).

Some reasons may account for this high amounts of isoflavones found in the early flowering types of cultivars, landraces or cultivated materials of red clover. First, early and late types of red clover could show different peaks of isoflavones in the moment of evaluation, due to differences in the growth rate between them. Second, the major type of red clover grown worldwide is the early flowering and much of the increased longevity in newly released red clover cultivars is at least partially due to improved disease resistance. (Quesenberry & Casler, 2001; Taylor, 2008). Finally, breeding to improve disease and insect resistance could indirectly rise isoflavonoid amounts since several classes of isoflavonoids acts like phytoalexins and have been showed to be important in pathogen resistance (Dixon, 2001; Samac & Graham, 2007).

He & Dixon (2000) showed that the constitutive over expression of isoflavone *O*-methyltransferase (IOMT) gene in transgenic alfalfa (*Medigaco sativa*) resulted in more rapid and increased production of the phytoalexin pterocarpan and medicarpin after infection by *P. medicaginis*, resulting in amelioration of symptoms. Other interesting questions could arise concerning this difference found in the amounts of total isoflavones between the early and late flowering cultivar, cultivated, and landraces materials. As isoflavones are precursors for these protective compounds (phytoalexins), the differences in the total amounts could influence in the disease susceptibility. Disease sensitive and resistant plant genotypes may be characterized, among other traits, by their response time: those plants that are able to synthesize phytoalexins faster will be able to control the disease in the early stages (Macias et al., 2007).

Ohberg et al. (2005) found differences in disease resistance between the early and late types of Swedish red clover cultivars, with the late types showing more resistance to *Sclerotinia trifoliorum* (Clover Rot) than the earlier ones. It would be important to test this hypothesis of different disease resistance in other diseases and red clover cultivars from various geographic regions.

Although differences between early and late types of red clover seems to be important to various agronomic traits as showed by Bird (1948), none work that evaluated the amounts of isoflavones in red clover cultivars took this into account. It would be interesting to compare early and late cultivars of red clover from various regions to better understand this difference in amount of isoflavones found here.

Finally, some accessions present in groups I and II (N°21, 29, 35, 36, 37, 38) were also analyzed by Dias et al. (2008a) and were classified as highly persistent and high-yielding in the Southern Brazilian conditions. Since these accessions are probably better adapted to Brazilian conditions and contains high contents of isoflavones, they could be used in future breeding scheme aiming to create cultivars for industry use combining persistency, yield and high amounts of isoflavones.

Seasonal evaluation

These results present that it is possible to harvest, at the weather condition tested, at any season. It is also important to highlight that is time saving to harvest at vegetative stage once that is known that leaves have a greater concentration of isoflavones than flowers, and the cultivation period is smaller (Sivesind & Seguin, 2005; Booth et al., 2006; Tsao et al., 2006).

Contradictory results exist concerning the seasonal variation of isoflavones in red clover. This is partly due to fluctuations in chemical content according to geographic location, differing photoperiods of sunlight, weather, soil fertility, and partly because of differences in the sensitive and specificity of older analytical chemical methods used to quantify isoflavones (Vetter, 1995). To our knowledge no study, before our, has been reported for seasonal variation in isoflavones amounts in the Brazilian conditions. It is known that in order to achieve exact results, about seasonal variation of plant metabolites, at least a five year data comparison would be need. Nevertheless, some preliminary considerations may be started after one-year experiment. Our results showed that the vegetative stage is the best period to harvest the plant, in order to obtain higher amounts of isoflavones, once is at this stage the plant has a slight higher concentration of these compounds, and there is no need to wait until the plant blossom, this conclusion is in agreement with other studies about seasonal evaluation of isoflavones in red clover (Sivesind & Seguin, 2005; Booth et al., 2006; Tsao et al., 2006), but these studies did not take into account the seasons, but the reproductive stage of the plant, and they were realized at North America. The use and harvest of vegetative material would maximize the isoflavone concentration of red clover once we have only

leaves that have greater concentration of isoflavones, and we do not have to waste time separating the flowers (which require manual labor, increasing production costs) that have smaller isoflavones concentration and would dilute our amount of isoflavones.

In this study we confirmed the high variation for the amount of isoflavones in red clover, analysing for the very first time the core collection at the same environment, using the same sample method and tissue type. Clustering was mainly related to differences in the total of isoflavones and partially related to maturity types of red clover, improvement status and geographic origin.

Some of accessions evaluated here could be selected by breeding programs, once the results highlighted accessions from red clover core collection with low and high individual isoflavone contents that could be used to produce cultivars for forage or pharmaceutical use. We also detected some promising populations for red clover breeding in Brazil showing higher amounts of isoflavones.

Results from seasonal evaluation suggested that for the Brazilian conditions, the vegetative stage is the best time for harvest in red clover accessions, with higher amounts of isoflavones. Findings in this study therefore provide important information for further studies on the utilization of red clover as a source for phytomedicines, nutraceuticals and functional foods, or forage use.

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