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Trifolium pratense: Friable calli, cell culture protocol and isoflavones content in wild plants, *in vitro* and cell cultures analyzed by UPLC

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ABSTRACT

Trifolium pratense L., Fabaceae, is a rich source of isoflavones and has become the focus of several studies related to its phytoestrogenic activity. The aim of this study was to establish germination and cell cultures protocol for *T. pratense* and quantify isoflavones content in cell cultures, *in vitro* cultured and wild plants harvested in two different seasons. Murashige Skoog medium supplemented with naphthalene acetic acid and kinetin was able to produce the highest formation of friable calli. Calli cultures were analyzed qualitatively after 60 days of culture, and *in vitro* plants after 30, 45 and 60 days of cultivation. The chemical analysis was performed by ultra performance liquid chromatography, using the linearity curves of daidzein, genistein, formononetin and biochanin A as standards. The concentrations of isoflavones detected in wild plants were different in the two harvest periods and contrasted in content when compared to the *in vitro* plants. Cell cultures exhibited diverse profiles and concentration of isoflavones, none of which presented the isoflavonoid biochanin A. Pectinase was used to promote reduction of clumps and ended up altering the characteristics of secondary metabolites production in some cultures. Formononetin showed higher concentration in wild red clover samples (15.407 mg g^{-1}), and in the *in vitro* grown plants the highest concentration was daidzein (17.591 mg g^{-1}) at 60 days. The methods used for this research were effective, and the red clover plants of the analyzed variety can be cultivated *in vitro* aiming the commercial productivity by having contents greater than or equal to the wild plants in the periods studied, even without the use of elicitors during the cultivation.

Introduction

Trifolium pratense L., Fabaceae, popularly known as red clover, is a perennial plant rich in isoflavones such as daidzein (**1**), genistein (**2**), formononetin (**3**) and biochanin A (**4**) (Scheme 1). These isoflavonoids are biosynthesized through the phenylpropanoid pathway (Scheme 1), and these structures are produced mainly from intermediates naringenin and liquiritigenin. Genistein and daidzein are the initial metabolites produced by these two branches, respectively, and the enzyme isoflavone 4′-*O*-methyltransferase is the responsible for the origination of formononetin and biochanin A, respectively, by methylation of its 4′-OH (Du et al., 2010; Kanehisa et al., 2017). The therapeutical use of isoflavones is well known in relieving menopausal symptoms and preventing osteoporosis, benign prostatic hypertrophy, hormone replacement therapy, cardiovascular disease, hypertension and hormone-dependent tumors (Heinonen et al., 2002; Beck et al., 2005; Wuttke et al., 2006; Nissan et al., 2007; Ercetin et al., 2012; Ramos et al., 2012; Çölgeçen et al., 2014; Spagnuolo et al., 2014; Xu et al., 2015). The phytoestrogenic activity related to the isoflavones is based on the structural similarity to the steroidal estrogen 17-β-estradiol (**5**), acting as agonists or antagonists, in a dose-dependent manner (Wu et al., 2003).

Qualitative and quantitative analyses of isoflavones from *Trifolium* extracts are characterized by its variability, influencing directly in biological activity assays. Seasonal variations, production at specific stages of development, stress, nutrient availability or soil conditions are important factors that interfere in the chemical composition of plants, in general, becoming evident the necessity of chemical standardization of plant extracts. Thus, in the last decades, efforts have been made to minimize these chemical variabilities such as development of techniques of *in vitro* plant production. In these techniques, the production of plant metabolites can be controlled, reducing the interferences, predicting the mass content as

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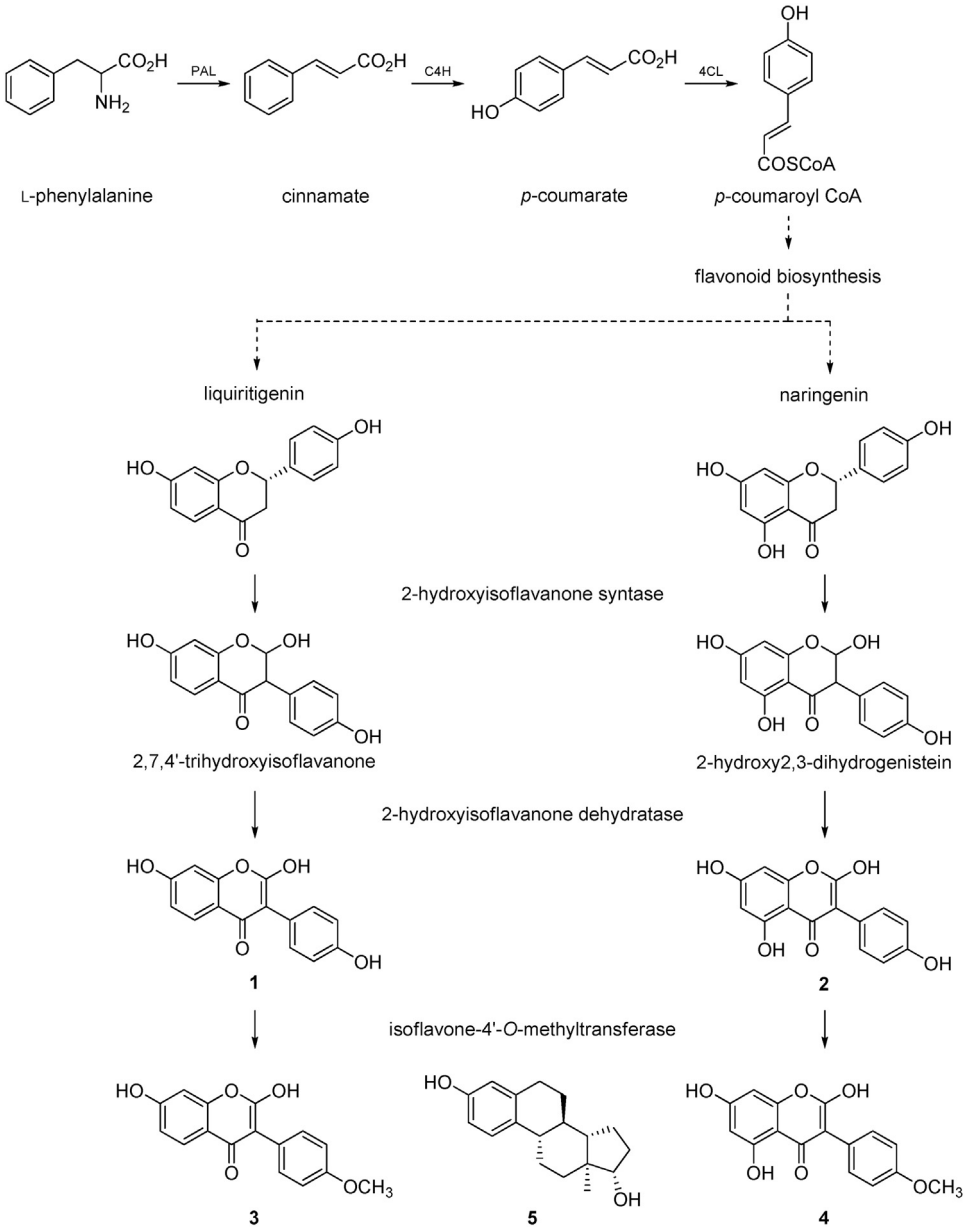

Scheme 1. Biosynthetic route of isoflavonoids showing the general phenylpropanoid metabolic pathway and the isoflavonoid pathway with the best known isoflavone molecules: daidzein (**1**), genistein (**2**), formononetin (**3**), and biochanin A (**4**). The steroidal estrogen 17β-estradiol (**5**) is drawn for molecular comparison related to the isoflavonoid biological activity. Adapted from Du et al., 2010; Kanehisa et al., 2017. Abbreviations: PAL phenylalanine ammonialyase, C4H cinnamate-4-4hydroxylase, 4CL 4-coumarate CoA ligase.

well as the improvement of metabolites production aiming the use in drugs manufacture with standardized composition (Verpoorte et al., 2002; Booth et al., 2006).

Cell culture is an alternative to produce metabolites on a large scale with high commercial value, yielding significant quantities in small spaces, with different lineages for specific molecules. Also, it is possible to maintain these cultures for long periods, without excessive maintenance required; it is feasible to elicitate the cultures to improve productivity of target metabolites, and the chemical extraction could also be simplified by the fact that cellular aggregates or cells in suspension are less chemically complex than plants (Satdive et al., 2015).

Protocols for *in vitro* germination and cell culture are necessary to develop biotechnological methods. In this scenario, the objective of this study was to establish such protocols for *in vitro* germination and suspended cell cultures *using T. pratense* var. URS-BRS Mesclador and to quantify isoflavone production of *in vitro* and wild plants.

Materials and methods

Plant material

The seeds of *Trifolium pratense* var. URS-BRS Mesclador, Fabaceae, were donated by Dr. Miguel Dall'Agnol (Faculty of Agronomy, UFRGS). The wild plants were collected in the same location in two different months: sample 1 (May 2015) and sample 2 (December 2015). The harvest occurred at Faculty of Agronomy, Federal University of Rio Grande do Sul (Porto Alegre, Brazil) in the latitude: −30.0331 and longitude: −51.23 [err: ±29946 WGS84]. The voucher specimen was deposited in the Herbarium Alarich Rudolf Holger Schultz-HAS, in the Museum of Natural

Sciences—Zoobotanic Foundation of Rio Grande do Sul, under registration HAS 87114, number 4291 (10/14/1986).

Disinfestation and germination

After receiving the seeds, the disinfestation protocol started using ethanol 70% (v/v) (1 min, under stirring), sodium hypochlorite 11% active chlorine (Mazzarollo Química Ltd., Gravataí, Brazil) with detergent (two drops per 100 ml of solution) (5 min, under stirring) and washed three times with ultrapure steriled water (Milli-Q®, Merck KGaA, Darmstadt, Germany), in laminar flow. The sterile seeds were left on sterile filter paper for 30 min and then inoculated in MS medium (Murashige and Skoog, 1962) supplemented with 100 mg l^{-1} inositol, 1.5 g l^{-1} phytagel® (Sigma, St. Louis, MO, USA), sucrose 30 g l^{-1} (Sigma, St. Louis, MO, USA), thiamine, nicotinic acid and pyridoxine at 0.5 mg l^{-1} (Sigma, St. Louis, MO, USA), glycine 1 mg l^{-1} (Sigma, St. Louis, MO, USA) and *Plant Preservative Mixture*™ (PPM, Plant Cell Technology, Washington, DC, US) at 0.2 g l^{-1}. The culture medium used was MS; 1/2 MS and 1/4 MS medium salts (macro- and micronutrients). The pH was adjusted to 5.8 and autoclaved for 20 min at 121 °C and 1 atm.

Subsequent to inoculation, the seeds were taken to dark environment where they remained for 15 days and then transferred to light (daylight, Philips, USA) (photon flux of 40 μmol m^{-2} s^{-1}, 16 h photoperiod), 25 ± 2 °C, for another 15 days. After the seventh and the thirtieth days of cultivation, the cultures were evaluated for percentage of contamination (efficiency of the disinfestation) and germination in the different mediums used.

Maintenance, induction of friable calli, and initiation of cell culture

The seedlings that emerged after 30 days of seed inoculation were transferred to semisolid media containing the same MS medium base (Murashige and Skoog, 1962), plus inositol 100 mg l^{-1}, agar 5.5 g l^{-1}, sucrose 30 g l^{-1}, solution of thiamine, nicotinic acid, and pyridoxine at 0.5 mg l^{-1}, glycine 2 mg l^{-1}, and PPM 0.2 g l^{-1} and activated charcoal 5 g l^{-1} with the same pH and autoclaving procedure used previously. These *in vitro* cultured seedlings were collected at 30, 45, and 60 days in culture for quantitative analysis.

The callogenesis induction was carried out in *T. pratense* foliar (0.5 × 0.5 cm, lacerated diagonally, three times) and stem explants (0.5 cm). They were placed into culture medium with MS medium macronutrients (50 ml l^{-1}) (Murashige and Skoog, 1962), inositol 100 mg l^{-1}, thiamine 0.1 mg l^{-1}, nicotinic acid and pyridoxine at 0.5 mg l^{-1}, casein 1 g l^{-1}, glycine 2 mg l^{-1}, phytagel® 2 g l^{-1}, sucrose 30 g l^{-1}, pH adjusted to 5.5.

Three growth regulator (GR) combinations were added to the medium, according to previous studies in our laboratory (data not shown): callogenesis induction medium I (CIM-I)-without regulators; CIM-II-naphthalene acetic acid (NAA) 0.1 mg l^{-1} + 6-benzylaminopurine (BA) 0.5 mg l^{-1}, and CIM-III-NAA 2.5 mg l^{-1} + kinetin 5 mg l^{-1}, all GR from Sigma (St. Louis, MO, USA). The sterilization procedure was realized using 0.22 μm Millipore membrane (Merck, Germany), the GR were added after autoclaving the medium and poured into sterile and disposable Petri dishes. The parameters evaluated were: callogenesis percentage (for stem and leaf explants) and calli friability (da Silva, 2012) after 30 days in culture.

Calli induced in the semi-solid medium were transferred to liquid medium for the development of suspended cell cultures. Cell suspensions were initiated by transferring 0.5 g of friable calli to 30 ml of liquid medium in Erlenmeyer flasks (125 ml), in a shaker (100 rpm), in the dark, at 25 ± 2 °C. 1/2 MS medium was used with the addition of inositol 50 mg l^{-1}, sucrose 15 g l^{-1}, thiamine, nicotinic acid, and pyridoxine at 0.25 mg l^{-1}, glycine 0.5 mg l^{-1}, PPM 0.2 g l^{-1}, adenine 50 mg l^{-1}, and casein 1 g l^{-1}. The pH was adjusted to 5.5, and autoclaving was performed under the same conditions as above. The medium was changed every 4 weeks. Pectinase from *Aspergillus niger* (Sigma Aldrich®) 0.005% (v/v) was membrane sterilized and incorporated in half of the cultures after the medium has been autoclaved (Mustafa et al., 2011). These samples code with an "*E*" in the name of the line. This medium was used in the first four weeks of culture, the exchange was performed every 2 weeks, and then pectinase was withdrawn from the remaining subcultures until 60 days of culture was completed.

Subcultures were performed by transferring 30 ml of fresh medium into the culture and distributing them in two new flasks. Compared with weighting or pipetting, the liquid transfer technique is simpler (less tools used), faster, with less chances of contamination, and stress of the cells in this initial phase of cultivation, but presents low reproducibility (Mustafa et al., 2011).

Extraction and chemical analysis of isoflavones

The method applied for isoflavone quantification by ultra performance liquid chromatography (UPLC) was adapted from the literature (Galland et al., 2014) and the samples were compared to standards of the isoflavones daidzein (**1**), genistein (**2**), formononetin (**3**), and biochanin A (**4**).

The cell cultures were filtered using filter paper (Whatman No. 1), and the liquid medium was lyophilized. The *in vitro* plants were separated from the culture medium and the whole plant was lyophilized, as well as the wild plant samples and used in its entirety for the analysis.

Approximately 100 mg of *T. pratense* plant dried material, which was powdered using liquid nitrogen was extracted using 1.5 ml of extractive solution (methanol:water:acetone, 40:32:28, v/v and 0.05% trifluoracetic acid) by sonication in an ultrasonic bath for 20 min at 25 kHz, at 4 °C. The samples were centrifuged at 20 000 × *g*, for 20 min and the extraction process repeated once. The two resulting supernatants were pooled, and the solvent eliminated in a rotary evaporator and then lyophilized. The dry pellet was dissolved in a solution of acetonitrile:water (1:1, v/v) and filtered in 0.22 μm membrane.

UPLC quantitative analysis

The samples produced according to the methodology described above using *T. pratense* samples were injected in a Waters Acquity® UPLC equipped with a Waters Photodiode Array Detector. The separation of the compounds was achieved in a reverse phase column (Waters Acquity UPLC® BEH C18; 1.7 μm; 2.1 × 50 mm), injection volume of 3 μl in a flow rate of 0.3 μl min^{-1}, at 40 °C, and a linear gradient of water:formic acid (100:0.1; v/v) (A) and acetonitrile:formic acid (100:0.1, v/v) (B) as follow: 0–2 min 5% B, 2–4 min 10% B, 4–17 min 40% B, 17–21 min 100% B, and 21–23 min 100% B. The wavelength selected was 260 nm.

Linearity curves, using the isoflavone standard isoflavones, were prepared in triplicate and constructed with six different concentrations on each curve. The concentration range used were: 1.5–5.5 μg ml^{-1} for daidzein (**1**), 4.3–216.4 μg ml^{-1} for genistein (**2**), 76.3–225 μg ml^{-1} for formononetin (**3**), and 9.4–357.2 μg ml^{-1} for biochanin A (**4**). The linear equation and the determination coefficient (R^2) were calculated individually for each curve. The samples were injected in triplicate and the limits of quantification (LOQ) and detection (LOD) also were established at a signal-to-noise ratio (S/N) of 10 and 3, respectively.

Table 1
Percentage of germination and contamination in *Trifolium pratense* seeds after 30 days of cultivation.[a]

Medium	Contamination (%)	Germination (%)
MS	11.5 B	29.0 B
1/2 MS	18.5 A	83.5 A
1/4 MS	0 C	80.0 A

[a] Averages with different letters, differed statistically according to the Tukey test (5%).

Statistical analysis

The germination experiments were completely randomized, composed of three different concentrations of culture media and 15 replicates per treatment, considering each plate containing 10 seeds in an experimental unit. The callogenesis induction experiments were performed in duplicate, with 17 replicates per tissue and treatment. Cell suspensions were run in duplicates for each cell line. The replicates were combined prior to extraction and analysis of isoflavones. The significant differences of each treatment were determined with an analysis of variance followed by the Tukey test at 5% probability level using Winstat 1.0 Software (Machado and Conceição, 2002).

Results and discussion

Disinfestation and germination

The disinfestation protocol has been proved effective in the sterilization of *T. pratense* seeds (Table 1). The highest percentage of contamination occurred in the seeds placed in the 1/2 MS medium, followed by the 1 MS medium. No contamination was detected in the 1/4 MS medium. An up to 10% degree of contamination demonstrates the efficiency of the disinfestation process and allows us to obtain good results in plant cultivation. It is important to note that the higher the concentration of active chlorine, the higher the pH of the disinfecting solution (which should be between 5 and 8). Alcaline pH favors the development of some bacterial defense mechanisms and even in high concentrations of active chlorine, the action is not efficient (Donini et al., 2005; Braga et al., 2015). In our experiment, it was observed that even with elevated active chlorine concentration (11%), the medium containing 1/4 MS, sucrose 7.5, and 25 mg l^{-1} inositol did not present microbial contamination. Lower availability of carbohydrates seemed to also be one of the factors responsible for the success of the 1/4 MS medium.

Seed germination was evaluated at seventh and thirtieth day after inoculation. Little was detected in the 7-day timeframe, thus, in Table 1 it was only presented the results related to the 30 days evaluation. The mean values differed statistically, and the best results were found in the media containing 1/2 MS and 1/4 MS, respectively, with a germination percentage of the inoculated seeds at 83.5 and 80%. Studies with murmuru (*Astrocaryum ulei*) showed that at low concentrations of sucrose, good percentages of germinated plants are obtained, due to the fact that the species use the embryo reserves themselves for emission of the plumule and radicle, without the need for energy source supplementation (Pereira et al., 2006; Reis et al., 2008).

For some species like *Pyrostegia venusta* (Braga et al., 2015), there is a greater variability of behavior in relation to the water availability. Researchers observed that the germination success is entirely related to the water availability of the medium in *Vigna subterranea* (L.) seeds, modifying agar concentrations and fixing the macro- and micronutrients strengths during the germination (Koné et al., 2015).

Table 2
Percentage of callogenesis and friable calli in *Trifolium pratense* explants after 30 days of treatment.[a]

Medium	GR (mg l^{-1})			Callogenesis %	Friability %	
	NAA	BA	Kinetin		Leaves	Stem
CIM-I	–	–	–	1.64 C	0	0
CIM-II	0.1	6.0	–	28.7 B	0	0
CIM-III	2.5	–	0.5	42.28 A	21.0 A	33.0 A

[a] Averages with different letters, differed statistically according to the Tukey test (5%).

Maintenance, induction of friable calli, and initiation of cell culture

In the maintenance media, the use of activated charcoal was employed due to the hyperhydricity of the plants. A reduction of vitrification has been observed after the addition of this component in some experiments with onion crops shoots (species-dependent results) and artichoke (no reversal using charcoal). As for *Picea abies* and *Sequoia sempervirens*, the charcoal induced this condition (Pan and van Staden, 1998; Klenotičová et al., 2013).

After 30 days of inoculation, in media with different concentrations of phytohormones, the stems and leaf explants were evaluated for the percentage of calli formation and the calli friability (Table 2). In the medium without hormones, the callogenesis was practically null in the two evaluated periods. Thereby, the best results were in the CIM-III medium (NAA and kinetin) for both the induction of callogenesis and friable calli formation, even though the last did not differ statistically for stems and leaves.

In studies with calli for red clover regeneration, culture media containing 2,4-dichlorophenoxyacetic acid (2,4-D) and NAA or cytokinin (kinetin) (Horvath Beach and Smith, 1979; Khanlou et al., 2011) provided better results using the Gamborg B5 base medium (Gamborg et al., 1968). The composition differs from the MS considering the nitrogen and phosphorus concentrations. In experiments conducted by Kokina et al. (2005), the use of MS medium added by 2,4-D promoted the formation of large regenerative calli using smaller sizes explants.

Fig. 1A and B shows the pictures of the explants after the 30-day treatment in the CIM-I and CIM-II media. The leaves and stems presented some morphological modification with formation of hair and slight darkening in the stem explant. In the CIM-II medium, the explants showed hair formation and conservation of the chlorophyll pigments in the leaves and necrosis spots in the leaf tissue (Fig. 1C); the stems displayed a large browning area, with cell formation surrounding the material (Fig. 1D). The calli formed were homogeneous, with a light-yellow coloration and no regeneration ability under this cultivation conditions. Some studies suggest that there are variants of calli cultures that are no longer able to undergo differentiation in intact plants. However, these cell lines may be useful, even in their undifferentiated form seeking the *in vitro* production of desirable metabolites (Skirvin, 1978).

In the calli formation, the cell growth occurred in the central part of the leaf explants (Fig. 1E) when the explants were cultivated in CIM-III medium. Also, the increased number of cells fully covered the leaf explant (Fig. 1F). For stem explants on CIM-III medium, their cell growth started at the edge of the explants (Fig. 1G) and expanded across the entire area, hiding the stem (Fig. 1H). Calli from leaf explants took longer to present the same size as stem calli at the end of 30 days.

It is known that increasing auxin levels generally promote an intensification in friability and a reduction in cell differentiation. This can be explained by friability tendency of the cells to round off and separate just after division, promoting a rupture of the cell–cell contacts, which could contribute to the differentiation mediated by neighboring cells (Williams et al., 1990).

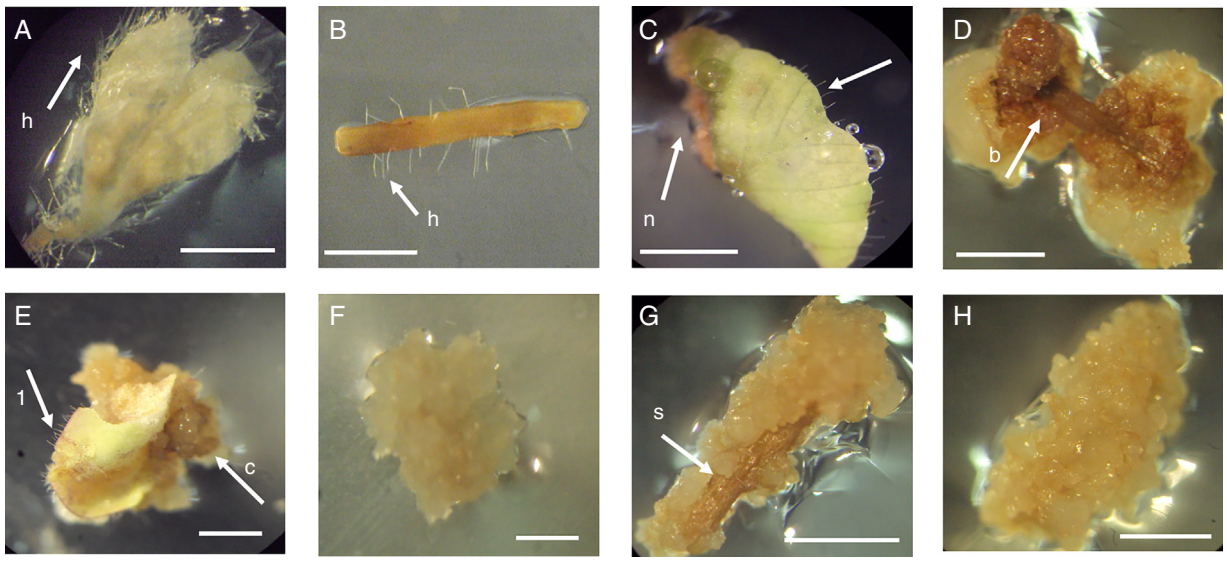

Figure 1. *Trifolium pratense* explants after 30 days inoculation. Medium CIM-I: (A) leaf explants; (B) stem explants; Medium CIM-II: (C) leaf explants; (D) stem explants. The arrows indicate some spots where the cells are covering the explant. Medium CIM-III: Leaf explants; (E) the arrows show cells and explant; (F) the callus cells completely covered the explant; stem explants: (G) part of the explant can still be visualized; (H) the caulinar explant was totally hidden by the cells. Abbreviations: h, hair; n, necrosis; b, browning; c, cell formation; l, leaf explant; s, stem explant. Scale bars represent 0.5 cm.

Similar results were reported in studies using calli from leaves, epicotyl, and cotyledonary explants from *T. pratense* grown in MS and PC-L2 medium. These cell formations presented different colors, and the most friable were the ones with yellow coloration. Also, reports have shown that the addition of NAA to the culture medium can be quite effective in the production of phenolic compounds and their correlates in the calli (Çölgeçen and Toker, 2008; Ercetin et al., 2012).

From the 45th day in culture, the friable calli were transferred to the liquid culture media under agitation, where they remained for 30 days, until suspension. According to the different calli that originated them, we obtained distinctive cell cultures, with diverse colorations (from light yellow to brown). Also, there was a variability in the coloring of the medium without and with pectinase, and this enzyme can break down the pectin in the cell wall, releasing cells more easily than just by shaking them in the medium. Cell cultures in *T. pratense* (tetraploid variety) were previously performed using Gamborg medium with the addition of 2,4-D and BA and culture intervals similar to those we used in this study (Kasparová et al., 2006; Kašparová et al., 2012).

Extraction and chemical analysis of isoflavones

The analysis was carried out with aglycone and *O*-conjugated forms of the isoflavones. The quantification was performed using daidzein (**1**), genistein (**2**), formononetin (**3**), and biochanin A (**4**) standards, and the peaks in the samples were identified according to the retention time and UV profile of these molecules.

The calculations to determine isoflavonoid concentration in the *in vitro* cultured samples after 30, 45, and 60 days of cultivation and wild plants were performed after triplicate injections on UPLC, using the linear equations for each standard isoflavone curve: daidzein: $y = 5266.7x + 1741.7$ ($R^2 = 0.9974$); genistein: $y = 15264x - 47\,994$ ($R^2 = 0.99956$); formononetin: $y = 6068.4x + 43\,924$ ($R^2 = 0.99788$); and biochanin A: $y = 7798.4x + 23\,175$ ($R^2 = 0.9998$).

The LOD and LOQ calculated for each isoflavone were 0.19 and 0.58 μg ml^{-1} for daidzein, 0.69 and 2.08 μg ml^{-1} for genistein, 4.73 and 14.34 μg ml^{-1} for formononetin, and 2.94 and 8.90 μg ml^{-1} for biochanin A, respectively.

The retention time observed for each isoflavone peak were 10.598 min for daidzein (**1**), 12.648 min for genistein (**2**), 14.744 min for formononetin (**3**), and 17.541 min for biochanin A (**4**). Due to the variability presented in the production of the various types of isoflavones in this species, the initial analysis of the plants was performed to determine the existence of these molecules in the wild red clover, *in vitro* plants, and in the cell cultures.

UPLC isoflavones quantitative analysis

The chromatograms corresponding to the *T. pratense* var. URS-BRS Mesclador (wild sample) and *in vitro* grown plants presented different characteristics (Fig. 2), and it was not possible to observe chromatographic peak corresponding to daidzein (**1**) and genistein (**2**) in one of the samples (Wild 2) (Table 3). The presence of constant fluctuation in the concentration of the isoflavones (Spagnuolo et al., 2014) led us to believe that the growing conditions, the metabolic balance, the environment, or even the internal signaling processes that support the biosynthesis did not favored these two specific molecules.

The wild samples were harvested in different seasons of the year, although the collect site and the cultivar were the same. The Wild 1 sample was taken in May (2015 – Autumn in South Hemisphere), and the Wild 2 sample, in December (2015 – Summer in South Hemisphere). Through the web site of the National Institute of Meteorology of Brazil (Ministério da Agricultura Pecuária e Abastecimento, 2015), it was possible to observe changes in the temperatures, maximum (23 and 29 °C, for Samples 1 and 2, respectively) and minimum (13 and 17 °C), hours of sunlight (5.24 and 7 h), and the amount of rain (140 and 100 mm).

In experiments done in North Hemisphere, researchers found that the isoflavones in red clover flowers are better produced at high temperatures and for the above-ground parts of the plant, the biosynthesis is stimulated when there is a higher incidence of rainfall (Booth et al., 2006). Soybean isoflavones (*Glycine max* (L.) Merr.) had their daidzein and genistein content stimulated using irrigation systems, with maximum isoflavone content in plants collected in the coldest and rainy season (Bennett et al., 2004).

The isoflavonoid that presented the highest concentration in the wild-red clover samples (Table 3) was formononetin

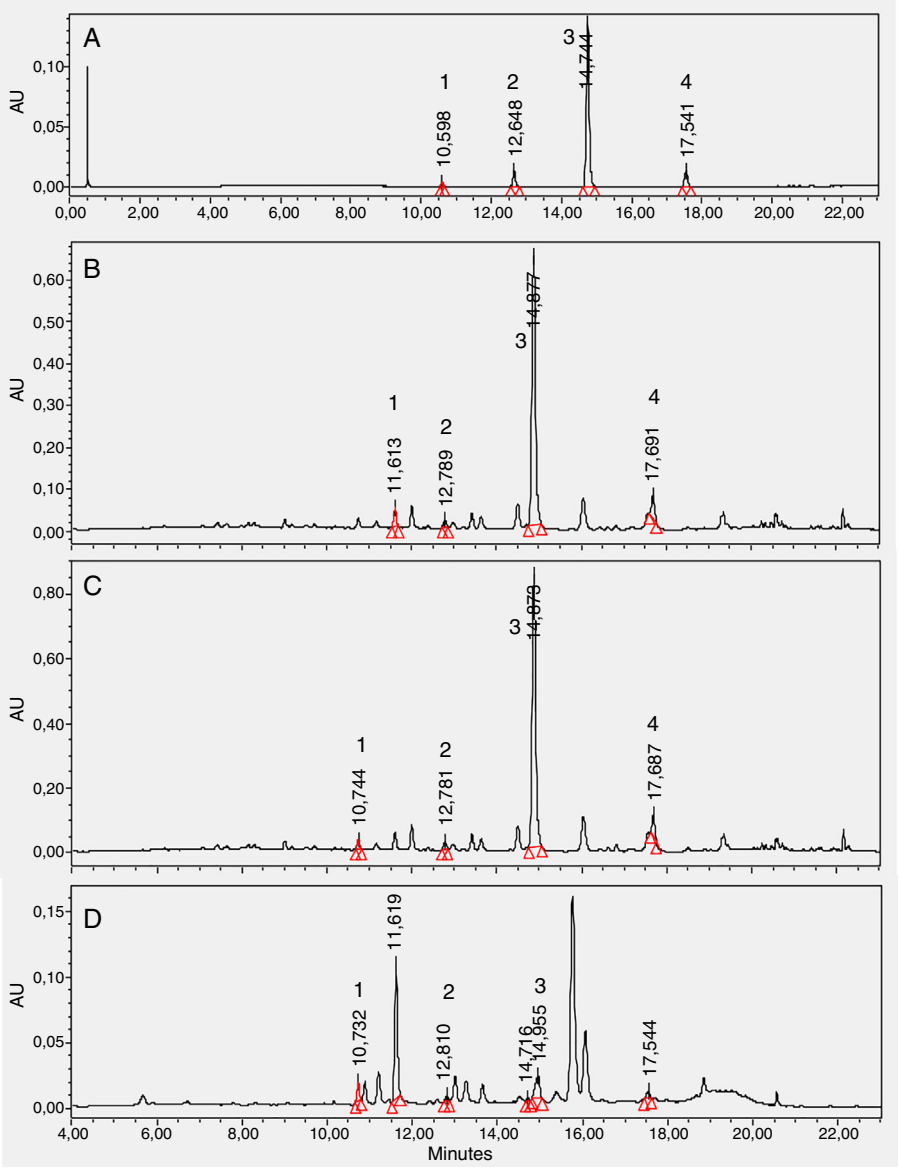

Figure 2. Chromatogram of the mix of isoflavones used as standard and the samples of *Trifolium pratense* in the different types. (A) Standard: 1 (daidzein) 10598; 2 (genistein) 12648; 3 (formononetin) 14744; 4 (biochanin A) 17541; (B) *In vitro*: (1) 11613; (2) 12789; (3) 14877; (4) 17691; (C) Wild: (1) 10744; (2) 12781; (3) 14873; (4) 17687; (D) Cell culture: (1) 10732; (2) 12810; (3) 14955.

Table 3
Isoflavone quantification in wild and *in vitro* cultivated plants of *Trifolium pratense* (mg g^{-1} dry material).

Samples	Daidzein	Genistein	Formononetin	Biochanin A	Total isoflavone
In vitro—30 days	0.229 ± 0.012 Cf	0.173 ± 0.0002 Af	9.086 ± 0.144 Cb	0.582 ± 0.053 Cef	10.071 ± 0.065 D
In vitro—45 days	1.361 ± 0.030 Bde	0.419 ± 0.1982 Aef	8.762 ± 0.100 Cb	1.800 ± 0.763 Ad	11.414 ± 0.334 D
In vitro—60 days	17.591 ± 0.213 Aa	0.113 ± 0.0070 Bf	3.774 ± 0.319 Dc	2.053 ± 0.189 Ad	23.535 ± 0.129 A
Wild (1)	0.368 ± 0.0 Cd	0.198 ± 0.0295 Ad	15.407 ± 1.113 Aa	0.763 ± 0.113 BCcd	16.515 ± 0.535 B
Wild (2)	Cd[a]	Bd[a]	12.689 ± 0.073 Bb	1.832 ± 0.073 Ac	14.521 ± 0.042 C

[a] Trace or peak not identified in the sample. Results are the mean of three replicates ± SD. Different letters indicate significant differences at $p \leq 0.05$ by Tukey's for the same isoflavone (capital letters) and for the same culture treatment (small letters).

(15.407 and 12.689 mg g^{-1}) and, in smaller quantities, biochanin A (1.832 mg g^{-1}). Slightly different results were reported in *T. pratense* flowers, in which formononetin was the major isoflavone, followed by genistein and biochanin A and daidzein (Ercetin et al., 2012). In another study, comparing the content of different organs in wild plants, the standards were detected in all of the organs, except daidzein which was not found in flowers. Daidzein and genistein were also in lower concentrations compared to other compounds (Saviranta et al., 2008).

In the wild samples evaluated (Table 3), it is believed that the biosynthesis of isoflavones is directed to methylated metabolites such as formononetin and biochanin A, due to its higher concentration in both samples. Biosynthetically, there is a possibility of bioconversion using 4′-*O*-demethylation of biochanin A and

formononetin, to form genistein and daidzein (Scheme 1), respectively. In agreement with our data, a survey using distinctive organs of red clover determined that biochanin A and formononetin were in greater concentration, with larger production in the leaves (Wu et al., 2003).

After the *in vitro* plants analysis, the samples grown for 60 days showed the maximum in the total amount of isoflavones (Table 3). Daidzein (17.591 mg g^{-1}) presented the uppermost concentration, at 60 days in culture plants, followed by formononetin (9.086 and 8.762 mg g^{-1}), in plants cultivated by 30 and 45 days. The similar pattern of concentration for the isoflavones at the 30 and 45 days changed when compared to the 60 days' samples. This fact demonstrates that even under laboratory conditions, there is a change in the biosynthesis outlines of these molecules, suggesting the need for long-term studies to better examine these modifications.

Studies with calli of *T. pratense* reported that the concentration of some molecules decreases from the third subculture, most of the time, agreeing with our results. However, an increase in the concentration of formononetin and genistein is usually detected, differing from what occurred in our plants (Ercetin et al., 2012). Studies prove that some hypocotyl lesions can cause a dramatic rise in the level of daidzein in soybean leaves (Morris et al., 1991), what may have happened during the subcultures.

Comparing each isoflavone (Table 3), wild plants had the best results only for formononetin (15.407 mg g^{-1}). For daidzein, genistein, and biochanin A, the greater production was visualized in the *in vitro* cultures. The total isoflavone content presented an increase in concentration over time for the *in vitro* grown plants. This data was similar to the wild red clover leaves analyzed by Tsao and co-workers (2006). The fact that *in vitro* plants have a higher concentration, even without the elicitation involved in the process, may be due to the modification in the culture process that occurs and to the stress that the plants are exposed when they grow inside of an *in vitro* vessel (Georgiev et al., 2010).

The calli were induced using the aerial part of the plant and, seeking to achieve suspension cultures, pectinase was added to reduce clumps and to produce a homogeneous material. The pectinase can disturb the external cell walls and in a low concentration break the aggregates by dilution of the pectins (Mustafa et al., 2011). Additionally, because of the formation of cell wall fragments, this enzyme can act as elicitors (Verpoorte et al., 1999). Considering this, they were used only in a half of the cultures.

In the analysis performed with the cell cultures (Table 4), isoflavone biochanin A was not observed in any of the samples. Some cultures presented only one of the four isoflavones analyzed, *Tp1E* (formononetin) and *Tp4E* (daidzein), and in others, the metabolite formononetin was not detected. Comparing the samples cultured with or without pectinase (Table 4), the best results were found in lineages that used the enzyme, as in the line *Tp1E* which reached the highest levels detected (formononetin 0.91 mg g^{-1}), followed by *Tp2E* (formononetin 0.67 mg g^{-1}) and by *Tp6E* (daidzein 0.61 mg g^{-1}).

The statistical analysis to determine which line best produced each isoflavone individually (Table 4) has shown that for daidzein and formononetina, the samples with pectinase were superior (*Tp6E* and *Tp1E*, respectively). For genistein, the results were determined in samples with (*Tp6E*) and without (*Tp1* and *Tp2*) the use of the enzyme.

The use of pectinase to improve the production of secondary metabolites was already employed with *Lithospermum erythrorhizon*, *Polygonum tinctorium*, and *Solanum melongena* cells. In these cases, the action was effective increasing the yields of the molecules (Imoto and Ohta, 1988; Hiroshi et al., 1990; Kim et al., 1997). In strawberry cell cultures, the consumption of pectinase was responsible for the production of anthocyanins and other phenylpropanoid metabolites, presenting a good correlation between cell-aggregate formation and secondary metabolism; also, it was suggest that the size of the cells can modify the metabolite accumulations (Edahiro and Seki, 2006). *Nicotiana tabacum* cells demonstrated browning after adding pectinase to the culture medium (Negrel and Javelle, 1995), similar to our findings.

Regarding the total isoflavone content (Table 4), the cell lines *Tp1E, Tp2E*, and *Tp6E* (0.91, 0.84, and 0.89 mg g^{-1}, respectively) were the highest producers, with all treatments using pectinase, although the isoflavone profile was diverse, presenting one (formononetin), three (daidzein, genistein and formononetin), and two chemical types (daidzein and genistein) as main molecules.

The presence of isoflavonoids was tested in the cell culture medium, but it was not detected. Thus, it can be assumed that in the cell lines produced in the experiment, there was no transference of the analyzed molecules from the plant cells to the culture medium or they were properly degraded. Even isoflavone-producing cell cultures may not generate one of the phytoestrogens synthesized by the plants, as it happened in these *T. pratense* cultures, in which biochanin A was not observed. Studies related to the production of phytoestrogens in cell cultures of *Psoralea corylifolia* and *T. pratense* have been reported showing dependence of induction on cell growth and plant hormone elicitation, such as IAA (3-indoleacetic acid), NAA (1-naphthaleneacetic acid), kinetin, or 2,4-D (2,4-dichlorophenoxyacetic acid) (Çölgeçen et al., 2014; Satdive et al., 2015).

The chemical differences in the extract compositions of the samples may have been caused by defense responses, which induce the biosynthesis of diverse metabolites even in the same cell lines, generated by cell wall degradation enzymes (Negrel and Javelle, 1995). Also, the production of some compounds in cell cultures can be almost absent due to their origin from undifferentiated cells. These cells lack specific metabolites by the need for tissue-specific biosynthesis. It is demonstrated that sequential biosynthetic enzymes can occur in different cell types, with the need to translocate route intermediates between cells, emphasizing the complex cellular biology necessary in the biosynthesis of these molecules (Facchini, 2001; Ziegler and Facchini, 2008).

Conclusions

The *in vitro* cultures showed the production of the isoflavonoids with qualitative differences during the subcultures. Then, even under controlled conditions, there were modifications in their metabolism, being necessary long-term studies to obtain the isoflavones' kinetic curve. The wild plants analyzed showed variable individual isoflavone contents.

According to the media used, which best favored the formation of friable calli and that later demonstrated quality and applicability in the initiation of cell cultures using *T. pratense* leaves and stems as explants was the CIM-III. All cell lineages produced were able to biosynthesize isoflavonoids, presenting qualitative (molecules) and quantitative (concentrations) differences between them. Also, the cultures profile detected was quite different from that determined in wild plants and with an isoflavones concentration much lower than those found in these and in the *in vitro* grown plants.

The *in vitro* grown plants at the end of 60 days in culture presented superior concentration of total isoflavones than those produced by wild plants, the profile of these molecules was different as well. Thus, the methods used in this research were effective and this cultivation can represent a viable source for the production of these molecules in a controlled environment aiming commercial productivity.

Table 4
Isoflavone quantification in cell culture lineages of *Trifolium pratense* (mg g^{-1} dry material).

Samples	Daidzein	Genistein	Formononetin	Total isoflavone content
Tp 1	0.13 ± 0.01 Cfg	0.25 ± 0.002 Ade	Dk[a]	0.38 ± 0.01 B
Tp 2	0.06 ± 0.01 Dhij	0.27 ± 0.001 Ad	Dk[a]	0.32 ± 0.01 BC
Tp 3	0.11 ± 0.003 Cfg	0.11 ± 0.009 BCDfg	Dk[a]	0.23 ± 0.004 CD
Tp 4	0.02 ± 0.001 Ejk	0.11 ± 0.03 BCDfg	0.09 ± 0.016 CDfgh	0.30 ± 0.01 BC
Tp 5	0.04 ± 0.01 Dijk	0.10 ± 0.004 CDfgh	0.13 ± 0.006 Cfg	0.28 ± 0.001 BCD
Tp 6	0.22 ± 0.02 Be	0.08 ± 0.004 Dghi	Dk[a]	0.26 ± 0.013 BCD
Tp 1 E	Fk[a]	Ek[a]	0.91 ± 0.02 Aa	0.91 ± 0.01 A
Tp 2 E	0.11 ± 0.02 Cfg	0.12 ± 0.017 BCfg	0.67 ± 0.04 Bb	0.84 ± 0.01 A
Tp 3 E	0.06 ± 0.02 Dhij	0.11 ± 0.0004 BCDfg	Dk[a]	0.16 ± 0.01 DE
Tp 4 E	0.06 ± 0.002 Dhij	Ek[a]	Dk[a]	0.06 ± 0.001 E
Tp 5 E	0.05 ± 0.01 Dij	0.14 ± 0.001 Bf	Dk[a]	0.19 ± 0.01 CDE
Tp 6 E	0.61 ± 0.01 Ac	0.28 ± 0.004 Ad	Dk[a]	0.89 ± 0.01 A

[a] Trace or peak not identified in the sample. Results are the mean of three replicates ± SD. Different letters indicate significant differences at $p \leq 0.05$ by Tukey's for the same isoflavone (capital letters) and for the same culture treatment (small letters).

Authors' contributions

AR contributed by running the laboratory work, design of the study, chromatographic and data analysis, and the draft of the paper. MS contributed to chromatographic analysis and reading the manuscript. JASZ contributed to the drafting and critical reading of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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