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Original article

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

Andressa Reis, Marina Scopel, José Angelo Silveira Zuanazzi*

Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

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

* Corresponding author.
E-mail: zuanazzi@ufrgs.br (J.A. Zuanazzi).

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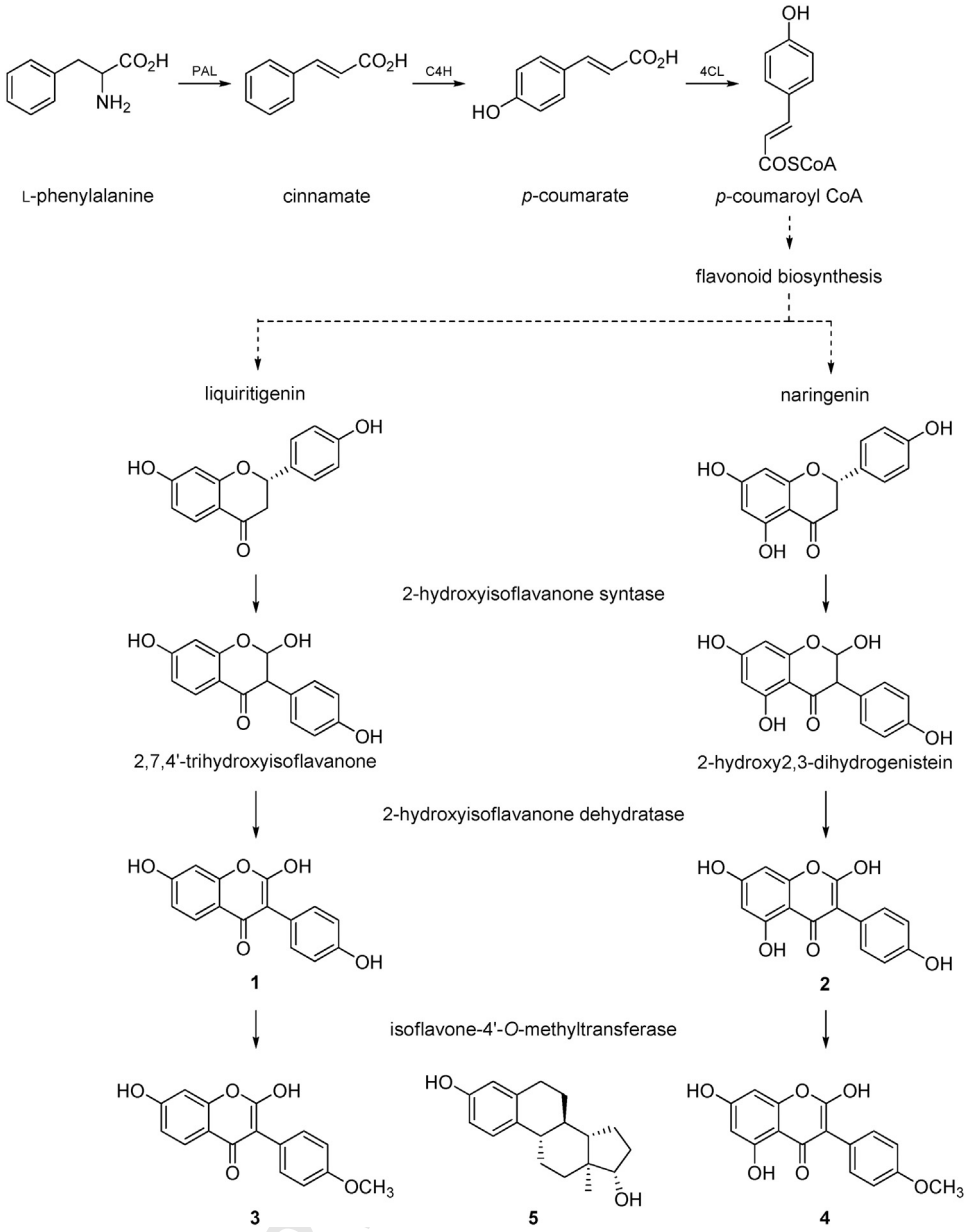

Scheme 1. Biosynthetic route of isoflavonoids showing the general phenylpropanoid metabolic pathway and the isoflavonoid pathway with the best known isoflavone molecules: daidzein (**1**), genistein (**2**), formononetin (**3**), and biochanin A (**4**). The steroidal estrogen 17β-estradiol (**5**) is 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.

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 *T. 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).

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