Optimization of enzymatic parameters for the production of formononetin from red clover (Trifolium pratense L.) through a response surface methodology

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ABSTRACT
Trifolium pratense, is a forage found worldwide, but it is negatively impacted by the clover root borer, Hylastinus obscurus. Methanolic extraction has been reported for isolating formononetin from vegetal tissues, with an antifeeding effect on H. obscurus. However, this methodology is time-consuming and also extracts other secondary metabolites, whereas enzymatic assays can provide higher specificity. Hence, the objective of this work was to determine the optimal conditions in pH, temperature, and incubation time for the activity of isoflavone synthase via a response surface model. Once these parameters were optimized, the concentration of formononetin in cultivars and experimental lines of T. pratense was evaluated enzymatically. The results showed that the best condition for developing the enzymatic assay was pH 9.1 with an incubation at 34.5 °C for 155 min. The formononetin content fluctuated between 0.74 and 1.96 mg/g of fresh weight, where Precoz-3, Precoz-1, and Superqueli-INIA presented the highest production.

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1. Introduction

In Chile, red clover (Trifolium pratense L.) is a valuable resource in both the animal and seed industries, exporting 700 to 1,700 tons of seed per year and recording more than 100,000 hectares under cultivation. This legume is a bi-annual perennial species harvested two to three times per season, producing its highest forage yield in the year after being established; after this time, it has a lower yield (Steiner and Alderman 2003). One of the main factors limiting its performance is attack from the clover root borer (Hylastinus obscurus Marsham) (Coleoptera: Curculionidae), which is considered the most significant pest of this legume in the world (Quiroz et al. 2017).

Control of this insect is very laborious due to the underground nature of the pest. To date, there is no effective chemical or biological control (Andersch et al. 2010). Previous studies have evaluated the response of the root borer to compounds present in red clover; the results showed that the insect responds to chemical stimuli from the plant (Tapia et al. 2007; Quiroz et al. 2018). Quiroz et al. (2017) showed that the isoflavonoids formononetin and genistein present in red clover generate an antifeeding effect in this curculionid, where formononetin has the highest activity, suggesting that this secondary metabolite could be used to increase the persistence of red clover plants.

The role of flavonoids is widely known in plant-insect interactions because they modulate ingestion and the development of oviposition in insects (Simmonds, 2001). Under this premise, it is postulated that the stimulation of its biosynthesis via isoflavone synthase activity optimization could have a role in the defense mechanism of the plant at the time and place of the attack. An environmentally friendly pest control method that promotes the biosynthesis of formononetin in red clover is needed. This is the first study reporting a RSM approach in the optimization of an enzymatic assay for evaluating formononetin in red clover. Such work is the first step in the generation of new cultivars of red clover with optimal levels of formononetin for the control of H. obscurus.
2. Result and discussion

2.1. Protein analysis

The maximum concentration of total proteins was obtained at pH 7.6 (2.18 mg/mL) (Table S1). Electrophoresis analysis developed under denaturation conditions (12% SDS-PAGE) showed that all samples, regardless of the pH of the buffer used to perform the protein extraction had the same protein profile. In addition, all samples showed a predominant band between 48-63 kDa in relation to the weight marker (Figure S1). This band of interest had an approximate molecular weight of 59.5 kDa. Similarly, Pičmanová et al. (2013) reported a molecular weight of 59.4 KDa for isoflavone synthase (IFS) from *Pisum sativum* L. (CYP93C18). When this IFS was introduced into *Arabidopsis thaliana*, the isoflavone genistein and its derivatives tectorigenin and biochanin A were detected in the overexpressing lines. This result corroborates the presence of IFS in red clover samples responsible for initiating the biosynthetic pathway of isoflavonoids. The next step was to improve the enzymatic assay for the determination of the isoflavonoid formononetin in different red clover cultivars.

2.2. Enzyme assay optimization

Optimization of an enzymatic bioassay considers the study of at least three parameters: pH, temperature, and incubation time (Scopes 2002). When studying the activity of a certain enzyme, assays are generally carried out under totally different conditions from those found in nature; however, it should be possible to extrapolate to the activity expected to occur in vivo with a complete study of the parameters that affect the enzyme, considering that one of the most carefully maintained constants is the pH of the internal medium in multicellular organisms. Here, a few tenths of variation in pH can become incompatible with life since small local variations in pH can lead to marked alterations in enzyme activity (Scopes 2002). This work proposes the application of an experimental response surface model (RSM) design to optimize the enzymatic assay conditions (pH, temperature, and incubation time) of the isoflavone synthase involved in the biosynthesis of formononetin in red clover. The highest production of formononetin occurred at pH 9.1 regardless of the temperature (Figures S2A–S2D); at 40 °C (Figure S2E), the highest production of the isoflavonoid was observed at pH close to 7.6. Under the experimental conditions studied, the maximum amount of formononetin was observed at pH 9.1, 33.5 °C, and 135 min of incubation (Table S2, 238%). Figure S3A shows a border condition marked by temperature (27–40 °C), pH (7.1 to 9.1), incubation time (30–240 min), and an increase in formononetin production (0.52 to 238.05%). However, after the optimization process (Figure S3B) carried out through the experimental design program, the following ideal conditions were suggested: pH 9.1, 34.5 °C, and 155 min, which were applied for protein extraction. Different parameters for determining the isoflavone synthase activity have been reported in the literature. Hagmann and Grisebach (1984) and Kochs and Grisebach (1986) performed protein extraction using buffer at pH 7.5, and the enzyme assay was incubated at 30 °C for 25 min. Sawada et al. (2002) studied the key amino acid residues necessary for aryl migration of the enzyme isoflavone synthase at 30 °C.
and an incubation time of one hour at pH 7.5. Sreevidya et al. (2006) studied the metabolic engineering of rice with soy isoflavone synthase to promote the expression of the nodulation gene in *Rhizobium*. They carried out enzymatic tests using buffer at pH 8.2 for protein extraction and incubated for 12 h at room temperature. Li et al. (2016) used pH 8.0 buffer with 20 min of incubation at 37°C when studying an alternative pathway for formononetin biosynthesis in *Pueraria lobata* (Fabaceae). The fact that there is significant variability of parameters used for assessing enzymatic activity of IFS makes it possible to suggest that the optimization of these parameters, via RSM, is a valuable tool for developing enzymatic bioassays.

Enzymatic assays were performed using an equivalent of 1 mg/mL of CE protein, incubated for 155 min at 34.5°C, and pH 9.1. Analysis of formononetin content in the extracts supplemented with the substrate liquiritigenin (Figure S4) indicated that the experimental lines Precoz-3 and Precoz-1 had the highest content of the isoflavonoid (1.96 ± 0.14 mg/g and 1.89 ± 0.06 mg/g, respectively). The lowest content corresponded to cultivars Intermedio-1 (0.78 ± 0.03 mg/g) and Quínequeli-INIA (0.74 ± 0.02 mg/g). Superqueli-INIA was the best red clover cultivar, showing 1.78 ± 0.10 mg/g of formononetin content.

Previous studies have reported the formononetin content in foliage and roots of red clover. These studies have applied direct chemical extraction of this isoflavonoid from lyophilized material via 80% methanol (Quiroz et al. 2017; 2018). The content (mg/g DM) of formononetin, determined by HPLC-UV (Figure S5), present in the polar fractions obtained from the foliage of cultivars and experimental lines of 2-yr-old red clover plants showed similar formononetin content in Superqueli-INIA and Redqueli-INIA, 1.189 and 1.097 mg/g DM, respectively (Quiroz et al. 2018). We report here for the first time formononetin determination via an enzymatic bioassay. Our results are similar to those reported by Quiroz et al. (2018), where Superqueli-INIA and Redqueli-INIA showed formononetin amounts of 1.78 and 1.61 mg/g DM, respectively. This result is relevant because the enzymatic bioassay is faster and less expensive than the classical extraction by organic solvents; it is also more specific.

Several studies have shown that the genotype of the red clover has a major impact on outcomes (Saviranta et al. 2008). Thus, cultivar selection is essential to obtaining the highest isoflavonoid yield (Oleszek et al. 2007).

Our results suggest that cultivars or experimental lines of red clover that produce a high content of formononetin could have an antifeedant effect on *H. obscurus*. Thus, they may be great candidates to further enhance isoflavonoid biosynthesis because they provide natural protection against the root (Barbour et al. 1991; Dakora 2000). These results constitute the first step in developing a strategy focused on producing cultivars with a high potential for biosynthesized formononetin. The next step is the characterization of the isoflavone synthase responsible for the biosynthesis of formononetin in red clover.

### 3. Experimental

See supplementary material.
4. Conclusions

The determination and production of formononetin in red clover (Trifolium pratense L.) was optimized via an enzymatic method. The best conditions to perform the assay were protein extraction at pH 9.1 and incubation at 34.5 °C for 155 min. Production of the isoflavonoid formononetin in the cultivars and experimental lines was found over a range of 0.74 and 1.96 mg/g. Precoz-3 and Precoz-1 had the highest production, and the Quiñeque-liquini-INA cultivar showed the lowest production of this secondary metabolite. These results constitute an important step in the design of a plant metabolic engineering strategy (Wang et al. 2011) for obtaining red clover plants enhanced in the production of formononetin through overexpression of this enzyme by regulation of the expression of its protein-coding gene or inhibition of the enzyme responsible for formononetin degradation.

Disclosure statement

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