Differential formononetin content in cultivars and experimental lines of red clover (Trifolium pratense L.) plants affect the feeding behaviour of Hylastinus obscurus (Coleoptera: Curculionidae)

[Contenido diferencial de formononetina en cultivares y líneas experimentales de plantas de trébol rosado (Trifolium pratense L.) afecta la conducta alimenticia de Hylastinus obscurus (Coleoptera: Curculionidae)]

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Abstract: Red clover (Trifolium pratense L.) is a perennial plant widely used as a forage resource for several animals. This plant is the exclusive host of Hylastinus obscurus (Marsham) which causes irreparable damages to the root system affecting their persistence. It has been reported that the presence of the isoflavonoid formononetin in roots of red clover could act as an antifeedant on H. obscurus. There are not studies related to the formononetin content in red clover roots to the antifeedant effect elicited by experimental lines and cultivar of red clover. Six red clover genotypes were investigated in both formononetin content and their respective antifeedant action. The results showed to Saboron High and Superqueli-INIA with both the highest formononetin content in red clover roots and antifeedant effect, allowing to suggest that this secondary metabolites could be used as a chemical factor for red clover plants. Moreover, a rapid methodology for searching red clover genotypes with high formononetin content is reported.

Keywords: Formononetin; Trifolium pretense; Isoflavonoids; Hylastinus obscurus; Antifeedant effect.

Resumen: El trébol rosado (Trifolium pratense L.) es una planta perenne ampliamente utilizada como fuente de forraje de variados animales. Esta planta es el exclusivo hospedero de Hylastinus obscurus (Marsham) el cual causa irreparables daños al sistema radical afectando seriamente su persistencia. Se ha reportado que la presencia del isoflavonoide formononetina en raíces de trébol rosado podría actuar como antialimentario sobre H. obscurus. Actualmente no existen estudios que relacionen el contenido de formononetina en raíces de trébol rosado con el efecto antialimentario elicitado por líneas experimentales y cultivares de trébol rosado. Seis genotipos de esta leguminosa fueron evaluados en cuanto a su contenido de formononetina y actividad antialimentaria. Los resultados mostraron que los cultivares Saboron High y Superqueli-INIA presentaron altos niveles de formononetina en sus raíces y efecto antialimentario sobre H. obscurus, lo que permite sugerir que este metabolito secundario podría ser usado como factor químico para incrementar la persistencia de plantas de trébol rosado. Además, se informa una metodología rápida para la búsqueda de genotipos con altos contenidos de formononetina.

Palabras clave: Formononetina; Trifolium pratense; Isoflavonoides; Hylastinus obscurus; Efecto antialimentario.
INTRODUCTION
Red clover (Trifolium pratense L.) is an important Leguminosae used as grazing food for cattle and livestock in temperate regions and has a high potential as silage for milk production due to its high protein quality. It is a significant resource in Chile for animal production and seed industry, representing nearly 15% of the total sown pastures and 60% of the forage seed exports (Ortega & Levío, 2011). Moreover, red clover is used as an herbal medicine for several diseases (Lin et al., 2000; Wu et al., 2003). The low persistence of this species is one of the main problems originated by biotic and abiotic factors, determining a half-life of two or three seasons (Cuevas & Balocchi, 1983; Ortega, 1996; Taylor & Quesenberry, 1996; Rhodes & Ortega, 1996; Rhodes & Ortega, 1997; Steiner & Alderman; 2003; Ortega et al., 2012a; Ortega et al., 2012b; Ortega et al., 2014). Pests and diseases constitute the main biotic factors affecting the survival of red clover plants. Among the abiotic factors pH, soil fertility, climatic conditions and management conditions have been mentioned as the most important parameters related to red clover yielding (Ortega et al., 2014). Ortega (1996) reported the low persistence of red clover in southern Chile, and one of the main factors associated to this decline is the root borer Hylastinus obscurus (Marsham) (Coleoptera: Curculionidae) (Carrillo & Mundaca, 1974; Quiroz et al., 2017). This insect produce a significant damage on the radical system when both larvae and adults build galleries inside the roots of the plant (Aguilera et al., 1996). In Chile, it has been estimated that the infestation of this root borer can reach between 70% and 100% of the plants at the second season. Forage yield can be reduced in 5.5% when an average of 1.5 insect/plant are found in 2-3 year-old pastures (Aguilera et al., 1996). As our knowledge, there is no an effective control for this pest, and pesticides have not been successful in controlling borer infestations and only crop rotation is used to decrease the damage (Aguilera et al., 1996). In this scenario, alternative strategies for controlling this pest have been investigated such as the potential use of semiochemicals identified from the insect-plant relationship (Quiroz et al., 2005; Tapia et al., 2005; Tapia et al., 2007; Manosalva et al., 2011; Palma et al., 2012; Parra et al., 2013; Ortega et al., 2014; Toledo et al., 2014; Quiroz et al., 2017). Different class of compounds have been identified from red clover plant roots (Manosalva et al., 2011; Quiroz et al., 2017), belonging to fatty acids and shiquimate biosynthetic routes. Recently, Quiroz et al. (2017) reported the presence of four isoflavonoids in roots of two Chilean red clover cultivars, QuiñequeLI-NIA and Superqueli-NIA. Antifeedant bioassays indicated that the addition of formononetin and genistein to an artificial diet decreased the feeding behavior of H. obscurus. This work pretend to answer if there are any relation between formononetin content in red clover roots and the feeding behavior of H. obscurus.

MATERIALS AND METHODS
Plant Samples
Three red clover (Trifolium pratense L.) Chilean cultivars with different degrees of persistence (QuiñequeLI-NIA, Redqueli-NIA and Superqueli-NIA), one foreign cultivar (Sabtoron High) with high formononetin content and two experimental lines (Syn II Int 4 and Syn II Int 5) were sown at Centro Regional de Investigación INIA Carillanca (Vilcún, Chile). These plants were established in September 2015 under irrigated conditions at a seeding rate of 12 kg ha⁻¹. The cultivars were distributed in a randomized complete block with three replicates. Each plot size was 1.8 x 7 m. Fertilization consisted of 150 kg of P₂O₅ ha⁻¹ and 100 kg of K₂O ha⁻¹. Weed control was performed only manually. The whole plants were sampled in October 2017 (third cut) at flowering stage with a sufficient amount of soil to avoid root damage and kept to low temperature with liquid nitrogen until freeze-drying at -55 °C.

Insect
Red clover roots infested with adult H. obscurus were collected in January 2018 from a red clover field at Centro Regional de Investigación INIA Carillanca (Vilcún, Chile). Plants were extracted with both aerial and radical parts, and they were put into a paper bags and transferred to the laboratory. The insects were gently removed from the roots and were maintained in Petri dishes at 4°C with pieces of red clover roots. Prior to each bioassays, curculionids were deprived of food (24 h) and only insects that were able to walk were selected and used for the feeding assay (Quiroz et al., 2017).

Feeding behavior
Feeding experiment with adult H. obscurus were carried out using the same system reported by Faccoli
& Schlyter (2007) but with a modification: the artificial diet was replaced by lyophilized roots. Approximately 0.5 g of lyophilized root of each cultivar or experimental line of red clover were deposited separately inside Eppendorf tubes (10 mm diameter x 35 mm length). Then, a pre-weighed H. obscurus (iw) was introduced into each tube. The Eppendorf tube was kept in a vertical position and once closed, the plastic cap was made a small hole to allow ventilation of the insects. H. obscurus was allowed to feed for 3 d under darkness at room temperature. The insects were weighed again (fw) at the end of the experiment (Toledo et al., 2014). Weight gain was calculated as the difference between the final weight (fw) at the time of observation and the initial weight (iw) of each H. obscurus (Singh & Johnson, 2013). Ten replicates were carried out for each of the six experimental lines and cultivars.

**Formononetin analysis**

The extraction of the isoflavonoids was carried out following the methodology reported by Quiroz et al. (2017) with some modifications. Red clover root or foliage tissue (20 mg) was lyophilized, milled and extracted with 80% MeOH (1.4 mL) for 15 m using an ultrasonic bath in dark at room temperature to obtain a polar fraction of isoflavonoids. The extract was centrifuged at 3,000 rpm for 30 m. An aliquot of 30 μL was taken from the supernatant and diluted until 1.5 mL with 80% methanol and it was stirred in a vortex by 1 min.

Formononetin content was determined by HPLC. Samples (20 μL) were injected into a Shimadzu HPLC (LC-20A Prominence, Kyoto, Japan) equipped with a Kromasil 100-5C18 column (300 x 4.6 mm I.D.; particle size 5 μm). The analysis was isocratic and the mobile phase was composed by a mixture of acetonitrile and water (50:50) at a flow rate of 1 mL min⁻¹. The detection was performed at the preferred wavelength of 254 nm. The standard solutions for the calibration curve were prepared using a pure formononetin standard (Sigma Aldrich, 99% purity). Standard and samples were filtered before injection using 0.45 μm membrane.

**Statistical analysis**

The statistical software Statistix 10 (Tallahassee, Florida, United States of America) was used to analyze the data. H. obscurus feeding bioassay and formononetin content were analyzed by ANOVA tests (P ≤ 0.05), and statistical differences among groups were determined by Fisher’s LSD tests.

**RESULTS**

The recoveries of formononetin standard was 95%. The intra-day relative standard deviation ranged from 0.7 to 1.1 and for inter-day repetitiveness was lower than 0.5%.

Table No. 1

<table>
<thead>
<tr>
<th>Cultivar/Experimental Line</th>
<th>Formononetin content (mg/g DM)</th>
</tr>
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<tbody>
<tr>
<td>Sabtoron High</td>
<td>3.101 ± 0.540 a</td>
</tr>
<tr>
<td>Syn II Int 5</td>
<td>2.318 ± 0.324 b</td>
</tr>
<tr>
<td>Syn II Int 4</td>
<td>1.394 ± 0.435 c</td>
</tr>
<tr>
<td>Quiñequeili-INIA</td>
<td>1.400 ± 0.142 c</td>
</tr>
<tr>
<td>Superqueili-INIA</td>
<td>1.189 ± 0.187 c</td>
</tr>
<tr>
<td>Redqueili-INIA</td>
<td>1.097 ± 0.081 c</td>
</tr>
</tbody>
</table>

Tables No. 1 and No. 2 shows the formononetin content in foliage and roots of red clover plants respectively. Superqueili-INIA and Sabtoron High showed the highest amount of formononetin (0.225 ± 0.030 and 0.204 ± 0.014 mg/g DM, respectively) in roots and Syn II Int 5 showed the lowest formononetin content (0.095 ± 0.040 mg/g DM). The other two Chilean cultivars, Redqueili-INIA and Quiñequeili-INIA showed intermediate values, between 0.115 and 0.164 mg/g DM.

In relation to the foliage, the formononetin content was distributed in three groups: 1) Sabtoron
High with the highest amount of formononetin (3.101 ± 0.540 mg/g DM), 2) followed by Syn II Int 5 (2.138 ± 0.324 mg/g DM) and 3) Syn II Int 4, Quiñequeli-NIA, Superqueli-INIA and Redqueli-INIA with the lowest formononetin amount value (1.097 ± 0.081 to 1.394 ± 0.435 mg/g DM).

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<td>Redqueli-INIA</td>
<td>0.164 ± 0.006 b</td>
</tr>
<tr>
<td>Quiñequeli-INIA</td>
<td>0.115 ± 0.021 bc</td>
</tr>
<tr>
<td>Syn II Int 4</td>
<td>0.108 ± 0.042 bc</td>
</tr>
<tr>
<td>Syn II Int 5</td>
<td>0.095 ± 0.040 c</td>
</tr>
</tbody>
</table>

**Figure No. 1**

*Hylastinus obscurus* (Coleoptera: Curculionidae) weight gain (mg) in feeding bioassays using roots from different cultivars and experimental lines of red clover. Different letters indicate significant differences (P ≤ 0.05) based on ANOVA test followed by Fisher’s LSD test.
When adults individuals of *H. obscurus* were allow to feed on the same root material, a differential behavior was observed. Figure No. 1 shows that Superqueli-INIA and Sabtoron High elicited a weight decreasing of the root borer. On the contrary, the rest of the tested materials did not elicited any response from the insects.

**DISCUSSION**

Both the estrogenic characteristics (Beck et al., 2005; Luther et al., 2014) and the protection effect against herbivores (Gerard et al., 2005; Quiroz et al., 2017) showed by isoflavonoid has already been studied in red clover. The content of isoflavonoids in the foliage is crucial if this species is used as source of phytoestrogens. However, the amount of these secondary metabolites depend on the cultivar, age, time of the year, locality, etc. (Wong, 1963; Rossiter, 1970; Kelly et al., 1979; McMurray et al., 1986; Anwar, 1994; Booth et al., 2006).

Moreover, it is important to point out that under certain circumstances, these substances could cause disorders in grazing animals, such as depression of fertility in females. Formononetin, an isoflavone, present in red clover is the substance implicated in these reproductive problems. This compound is not oestrogenic itself, but it is metabolized to the oestrogenic compound equol in the sheep rumen. Hence, breeding and use of low formononetin cultivars must be considered to minimize these problems. Our results showed that formononetin content ranged from 0.11 to 0.31% in the foliage of the different red clover genotypes studied. Marshall (1973) reported that formononetin content below 0.3% to be unlikely to have detrimental effect on ewe fertility. Schubiger and Lehmann (1994) compared formononetin content in leaves of 32 red clover varieties in Switzerland, reporting a decreasing at the second cut (0.49% in August) in comparison to the first cut (0.77% in May, both at starting flowering). Interestingly, Sivesind & Seguin (2005) reported that red clover variety had the most effect on isoflavone contents followed by the effect of sites. In accordance to that reports, McMurray et al. (1986) informed a decreasing in formononetin content from the first cut (0.56%) harvested early May to the second cut (0.35%) occurring at mid-June under the conditions of North Ireland.

Isoflavones are distributed unevenly within the aerial parts of red clover. Tsao et al. (2006) studied the isoflavone profiles of 13 red clovers cultivars and their distribution in different parts at different growing stages, finding that the isoflavone compositions were similar and the individual concentrations differed significantly. Specifically, formononetin content ranged from 0.6 to 1.3% in leaves, 0.6 to 1.5% in stems and 0.05 to 0.11% in flowers. With the aim of studying the broad-sense heritability of red clover isoflavones, Papadooulos et al. (2006) analyzed twelve-week-old plants of 13 cultivars determining that formononetin ranged from 0.66 to 1.00% showing a high heritability degree (74%). Differential amount of formononetin were found depending on plant stage, flower colour, plant part and cultivar (Saviranta et al., 2008). Using 6-weeks-old plant, the authors reported that leaves were rich in formononetin (0.56 to 0.91%), and the content was dependently of the maturity stage; young leaves contained more amount of formononetin than big leaves (Saviranta et al., 2008). Moreover, bright flowers showed higher amount of this isoflavonoids than light or brown flowers (0.35% in comparison to 0.75%). Results reported by Andersen et al. (2009) showed higher formononetin content in red clover than white clover, and lucerne, 1.4, 0.041, and 0.016% respectively. Formononetin content in 77 accessions from the USDA core collection and a Brazilian line were reported by Ramos et al. (2012). The mean content of this isoflavonoid in leaves of 12-month-old red clover plants was 11.4 mg/g DM. In this work two Chilean accessions were mentioned, a cultivar (PI 304824) and a “Landrace” genotype (PI 449326), containing 1.8 and 1.1% of formononetin respectively. As previously mentioned, the content of this isoflavonoid is dependent on the season of the year, this is how the highest content is observed during the winter (1.32% in average). Lemežienė et al. (2015) reported that formononetin was in higher amount in leaves than stems and flowers in 11 genotypes collected from Lithuania, Russia and Latvia. In leaves at flowering stage ranged between 0.40 and 0.72% DM (0.58% DM in average); in stems at flowering stage ranged between 0.15 and 0.23% DM (0.18% DM); in flowers at flowering stage ranged between 0.036–0.084% DM (0.052% DM). Low formononetin (0.19% DM) content in leaves of three-year-old plants of red clover was reported by Daems et al. (2016), the author applied a laborious enzymatic methodology for determining of this isoflavonoids with several steps included.

Phytoestrogen concentrations, including formononetin, were measured on leaves of 17

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culturavsb and 47 accessions of 2-year-old red clover plants from Australia and overseas (Little et al., 2017). Formononetin content ranged from 0.06 to 0.68% DM. This study included two Chilean accessions, PAC 19 and “Quinquel” – probably Quínequeli-INIA- reporting 0.59 and 0.62% DM of formononetin.

The fact that different cultivars had different isoflavone levels (Table No. 1) is suggestive of the genetic impact on the biosynthesis of isoflavones; thus, isoflavone concentrations can be potentially elevated to higher levels through breeding, which in turn favors the extraction and processing of isoflavones for the development of nutraceuticals and food supplements (Tsao et al., 2006). Our results and the literature would suggest that selecting individual plant phenotypes for high formononetin would be highly effective for cultivar development (Papadopoulus et al., 2006).

The differences found among our results and those reported in the literature can be explained by the sensible of the system to several factors, such as season harvesting, UV radiation, growing stage, plant part and genotype among the main abiotic and biotic factors (Booth et al., 2006; Saviranta et al., 2010). It has been reported that formononetin content below 0.3% to be unlikely to have detrimental effect on mammals fertility. This recommendation would indicate that pure swards of Sabtoron High (0.31% of formononetin, Table No. 1) should never be grazed with breeding ewes or ewe lambs by long term. This is not the case of the experimental lines (Syn II Int 4 and Synt II Int 5) and the Chilean cultivars (Quínequeli-INIA, Redqueli-INIA and Superqueli-INIA) where the formononetin content ranged from 0.11 to 0.23% (Table No. 1).

The information about formononetin root content is scarce. Saviranta et al. (2008) reported that root formononetin content depended on the cultivar; 6-weeks-old plant of Tepa, Bjursele, Acendure, Venla and Varte contained 0.49, 0.43, 0.38, 0.35 and 0.29% of formononetin under greenhouse conditions, increasing between 0.5 and 0.6% under field conditions for the case of Bjursele cultivar. The results proved Andersen et al. (2009), who determined 11.4, 0.41, and 0.16 mg/g dry matter (DM) of formononetin in red clover, white clover, and Lucerne, respectively. Later, Saviranta et al. (2010) found lower formononetin content in roots of the Bjursele cultivar in samples collected under field conditions (around 0.05%). However, the content of the respective malonate glycoside was significantly higher in the same samples (0.39 - 0.43%). In this work glycosides were not analyzed.

Phytophagous insects use flavonoids for host selection (Simmonds, 2001), and, in general, the effects of isoflavonoids, including those sequestered by insects, depend on the species of plant and insect (Simmonds, 2003). Iwashima (2003) reported a number of flavonoids that elicit feeding deterrenry toward harmful insects. Johnson & Gregory (2006) showed that 50% of the reported deterrent compounds are isoflavonoids. For example, the resistant characteristic of Lupinus angustifolius L. to the scarabs Costelytra zealandica (White) (Coleoptera: Scarabaeidae) has been found to be related to the content of isoflavones (Lane et al., 1987). The “ecological” role of these phytoestrogens in clover plants is not clear yet. In the case of red clover, it is suggested that it would slow down germination of the seeds of the same species as well as those of white clover (T. repens) and it could also have some antifungal activity (Chang et al., 1969; Debnam & Smith, 1976; Saviranta et al., 2008). Specifically, the isoflavones formononetin, genistein, and biochanin A present in the leaves of T. subterraneum L. have been reported to exhibit a greater deterrent activity on the red-legged earth mite Halotydeus destructor (Tucker) (Acari: Pentaleidae) than the glycosylated isoflavones (Wang et al., 1998). In relation to this study, accumulation of the flavonoid formononetin in the meristems of resistant white clover roots exhibits a defensive role on the stem nematode Ditylenchus dipsaci (Cook et al., 1995). Gerard et al. (2005) suggested that the presence of formononetin in red clover may act as a deterrent against adult Sitona lepidus (Gyllenhal) (Coleoptera: Curculionidae). However, Johnson et al. (2005) reported that formononetin is found in high concentrations in nitrogen-fixing rhizobial nodules of white clover roots, where the clover root weevil, S. lepidus, feeds. In this work, 500 mg of each root material was applied in the feeding bioassay, corresponding to 0.113, 0.102, 0.082, 0.058, 0.054 and 0.0475 formononetin content in Superqueli-INIA, Sabtoron-High, Redqueli-INIA, Quínequeli-INIA, Syn Int 4 and Syn Int 5 respectively. Superqueli-INIA and Sabtoron-High elicited an antifeedant behaviour from H. obscurus. This result is agree with the previous report from Quiroz et al. (2017) who showed that doses higher than 0.098 mg of formononetin elicited...
this behaviour. In this work, the content of formononetin in Superqueli-INIA and Sabtoron-High were higher than 0.098 mg (Table No. 1 and Figure No. 1). These results are in agreement with literature data indicating that Superqueli-INIA is more persistent under field experimental conditions (Ortega et al., 2014). It is important to point out that in this research red clover plants of 2-year-old were used for all the experiments; however, in the most of the reviewed literature the authors did not specify the age of the vegetal material, and mostly the hydrolysis by hydrochloric acid was used for isoflavonoids determinations. In this work formononetin determination was carried applying a significantly non-laborious analytical technique in a short time (1.5 h) without using a hydrolysis acid catalysed. Our approach was to determine the free aglycones. Nonetheless, our results are in accord of the reported in the literature.

Finally, the main contribution of this work lies in the fact that through a rapid analytical methodology and an efficient bioassay, experimental lines of red clover with a higher degree of resistance to *H. obscurus* can be selected for their use in breeding programs.

In conclusion, the relative low content of formononetin in leaves of Superqueli-INIA (0.12% DM) and the high formononetin content in roots (0.023% DM) suggest to this cultivar as a germplasm base for improving the content of this isoflavonoid in red clover roots without a detriment effect on mammals due to the low formononetin content in the aerial part.

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