BOLETÍN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
17 (4): 372 - 380 (2018)
© / ISSN 0717 7917 / www.blacpma.usach.cl

Artículo Original | Original Article

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)]

Andrés Quiroz[1,2], Leonardo Bardehle[1,2,3], Emilio Hormazabal[1,2], Fernando Ortega[4], Cristian Medina[2,5] & Ana Mutis[1,2]

[1]*Lab. de Química Ecológica, Departamento de Ciencias Químicas y Recursos Naturales, Universidad de La Frontera, Temuco, Chile*
[2]*Centro de Investigación Biotecnológica Aplicada al Medio Ambiente (CIBAMA), Universidad de La Frontera, Temuco, Chile*
[3]*Scientific and Technological Bioresource Nucleus, BIOREN-UFRO, Universidad de La Frontera, Temuco, Chile*
[4]*Instituto de Investigaciones Agropecuarias, Centro de Investigación Carillanca, Temuco, Chile*
[5]*Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera, Temuco, Chile*
Contactos | Contacts: **Andrés QUIROZ** - ***E-mail address:*** andres.quiroz@ufrontera.cl

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 Sabtoron 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 del 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 Sabtoron 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.

Recibido | Received: February 1, 2018
Aceptado | Accepted: May 5, 2018
Aceptado en versión corregida | Accepted in revised form: May 18, 2018.
Publicado en línea | Published online: July 30, 2018.
Declaración de intereses | Declaration of interests: This study was supported by FONDECYT (Project 1141245) belonging to Dr. Andrés Quiroz.
Este artículo puede ser citado como / This article must be cited as: A Quiroz, L Bardehle, E Hormazabal, F Ortega, C Medina, A Mutis. 2018. Differential formononetin content in cultivars and experimental lines of red clover (*Trifolium pratense* L.) plants affect the feeding behaviour of *Hylastinus obscurus* (Coleoptera: Curculionidae). **Bol Latinoam Caribe Plant Med Aromat** 17 (4): 372 – 380.

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 factor 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-INIA and Superqueli-INIA. 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 are there 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-INIA, Redqueli-INIA and Superqueli-INIA), 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 P_2O_5 ha^{-1} and 100 kg of K_2O 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 was 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 ($P \leq 0.05$), 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
Quantification (mg/g DM) by HPLC-UV of formononetin presents in the polar fractions obtained from the foliage of cultivars and experimental lines of 2-yr-old red clover plants (N = 12). Different letters indicate significant differences (P ≤ 0.05) based on ANOVA test followed by Fisher´s LSD test.

Cultivar/Experimental Line	Formononetin content (mg/g DM)
Sabtoron High	3.101 ± 0.540 a
Syn II Int 5	2.318 ± 0.324 b
Syn II Int 4	1.394 ± 0.435 c
Quiñequeli-INIA	1.400 ± 0.142 c
Superqueli-INIA	1.189 ± 0.187 c
Redqueli-INIA	1.097 ± 0.081 c

Tables No. 1 and No. 2 shows the formononetin content in foliage and roots of red clover plants respectively. Superqueli-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, Redqueli-INIA and Quiñequeli-INIA showed intermediate values, between 0.115 and 0.164 mg/g DM.

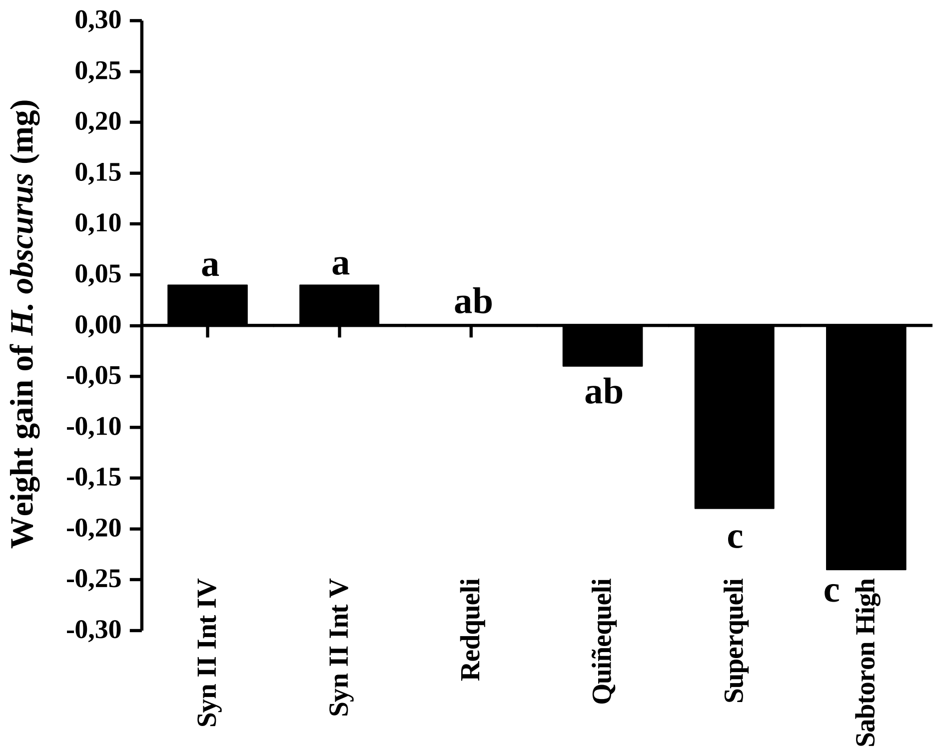
In relation to the foliage, the formononetin content was distributed in three groups: 1) Sabtoron

High with the highest amount of formononetin (3.101 ± 0.540 mg/g DM), 2) followed by Syn II Int 5 (2.138 ± 0324 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).

Table No. 2
Quantification (mg/g DM) by HPLC-UV of formononetin presents in the polar fractions obtained from the roots of cultivars and experimental lines of 2-yr-old red clover plants (N =12). Different letters indicate significant differences (P ≤ 0.05) based on ANOVA test followed by Fisher´s LSD test.

Cultivar/Experimental Line	Formononetin content (mg/g DM)
Superqueli-INIA	0.225 ± 0.030 a
Sabtoron High	0.204 ± 0.014 a
Redqueli-INIA	0.164 ± 0.006 b
Quiñequeli-INIA	0.115 ± 0.021 bc
Syn II Int 4	0.108 ± 0.042 bc
Syn II Int 5	0.095 ± 0.040 c

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 according 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 cultivars 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 "Quinqueli" –probably Quiñequeli-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 (Papodopoulus *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 (Quiñequeli-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 deterrency 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: Penthaleidae) 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, Quiñequeli-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.

ACKNOWLEDGEMENTS

This study was supported by FONDECYT (Project 1141245) belonging to Dr. Andrés Quiroz. To thank teachers and colleagues of the Laboratorio de Química Ecológica of Universidad de La Frontera, Temuco, Chile.

REFERENCES

Aguilera A, Cisternas E, Gerding M, Norambuena H. 1996. **Plagas de las praderas**. In Ruiz I: Praderas para Chile. INIA Carillanca, Temuco, Chile.

Andersen C, Weisbjerg MR, Hansen-Møller J, Sejrsen K. 2009. Effect of forage on the content of phytoestrogens in bovine milk. **Animal** 3: 617 - 622.

Anwar M. 1994. **Formononetin content in selected red clover strains and its effects on reproduction on ewes**. PhD Thesis, Massey University, Palmerston North, Nueva Zelanda.

Beck V, Rohr U, Jungbauer A. 2005. Phytoestrogens derived from red clover: An alternative to estrogen replacement therapy? **J Steroid Biochem Mol Biol** 94: 499 - 518.

Booth NL, Overk CR, Yao, P, Totura, S, Deng Y, Hedayat AS, Bolton JL, Pauli GF, Farnsworth NR. 2006. Seasonal variation of red clover (*Trifolium pratense* L., Fabaceae) isoflavones and estrogenic activity. **J Agric Food Chem** 54: 1277 - 1282.

Carrillo R, Mundaca N. 1974. Biología de *Hylastinus obscurus* (Marsham) (Coleoptera: Scolytidae). **Agric Tec** 24: 29 - 35.

Cook R, Tiller SA, Mizen KA, Edwards R. 1995. Isoflavonoid metabolism in resistant and susceptible cultivars of white clover infected with the stem nematode *Ditylenchus dipsaci*. **J Plant Physiol** 146: 348 - 354.

Chang C, Suzuki A, Kumai S, Tamura S. 1969. Chemical studies on “clover sickness” II. Biological functions of isoflavonoids and their related compounds. **Agric Biol Biochem** 33: 398 - 408.

Cuevas E, Balocchi O. 1983. **Producción de forraje.** Instituto de Producción Animal. Universidad Austral de Chile, Valdivia.

Daems F. 2016. **Développement et validation de méthodes analytiques pour la quantification des isoflavones et de l’équol dans des matrices biologiques**. Thèse de doctorat, Université de Liège, Gembloux Agro-Bio Tech, Gembloux, Belgique.

Debnam R, Smith IM. 1976. Changes in the isoflavones and pterocarpans of red clover on infection with *Sclerotinia trifoliorum* and *Botrytis cinerea*. **Physiol Plant Pathol** 9: 9 - 23.

Faccoli M, Schlyter F. 2007. Conifer phenolic resistance markers are bark beetle antifeedant semiochemicals. **Agric Forest Entomol** 9: 237 - 245.

Gerard PJ, Crush JR, Hackell DL. 2005. Interaction between *Sitona lepidus* and red clover lines selected for formononetin content. **Ann Appl Biol** 147: 173 - 181.

Iwashima T. 2003. Flavonoid function and activity to plants and other organisms. **Biol Sci Space**

17: 24 - 44.
Johnson SN, Gregory PJ, Greenham JR, Zhang X, Murray PJ. 2005. Attractive properties of an isoflavonoid found in white clover root nodules on the clover root weevil. **J Chem Ecol** 31: 2223 - 2229.
Johnson SN, Gregory PJ. 2006. Chemically-mediated host-plant location and selection by root-feeding insects. **Physiol Entomol** 31: 1 - 13.
Kelly RW, Hay RJM, Shackell GH. 1979. Formononetin content of “Grasslands Pawera” red clover and its oestrogenic activity to sheep. **New Zealand J Exp Agric** 7: 131 - 134.
Lane GA, Sutherland ORW, Skipp RA. 1987. Isoflavonoids as insect feeding deterrents and antifungal components from root of *Lupinus angustifolius*. **J Chem Ecol** 13: 771 - 783.
Lemežienė N, Padarauskas A, Butkutė B, Cesevičienė J, Taujenis L, Norkevičienė E, Mikaliūnienė J. 2015. The concentration of isoflavones in red clover (*Trifolium pratense* L.) at flowering stage. **Zemdirbyste** 102: 443 - 448.
Lin LZ, He XG, Lindenmaier M, Yang J, Cleary M, Qiu SX, Cordell GA. 2000. LC-ESI-MS study of the flavonnoid glycoside malonates of red clover (*Trifolium pratense* L.). **J Agric Food Chem** 48: 354 - 365.
Little V, Reed KFM, Smith KF. 2017. Variation for Concentrations of various phytoestrogens and agronomic traits among a broad range of red clover (*Trifolium pratense*) cultivars and accessions. **Agronomy** 34: 1 - 11.
Lutter S, Schmalbach K, Esch HL, Lehmann L. 2014. The isoflavone irilone contributes to the estrogenic potential of dietary supplements containing red clover. **Arch of Toxicol** 88: 309 - 321.
Manosalva L, Pardo F, Perich F, Mutis A, Parra L, Ortega F, Isaacs R, Quiroz A. 2011. Behavioral responses of clover root borer to long-chain fatty acids from young red clover (*Trifolium pratense*) roots. **Environ Entomol** 40: 399 - 404.
Marshall T. 1973. Clover disease- what we know and what we can do. **J Departm Agric** 14: 198 - 206.
McMurray CH., Laidlaw AS, McElroy M. 1986. The effect of plant development and environment on formononetin concentration in red clover (*Trifolium pratense* L.). **J Sci Food Agric** 37: 333 - 340.
Ortega F, Levío J. 2011. **SuperQueli INIA, nuevo cultivar chileno de trébol rosado (*Trifolium pratense* L.). I- Origen y descripción morfológica.** XXXVI Congreso Anual de la Sociedad Chilena de Producción Animal (SOCHIPA). Punta Arenas, Chile.
Ortega KF. 1996. **Variation in mortality, yield and persistence of red clover (*Trifolium pratense* L.).** Doctoral Thesis, University of Wales, Aberystwyth, UK.
Ortega F., Galdames R, Soto P, Teuber N, Torres, A, Levío J. 2012a. **Avances en mejoramiento genético de trébol rosado (*Trifolium pratense* L.) en Chile.** 63° Congreso Agronómico, Contribuyendo a la sustentabilidad alimentaria.
Ortega F, Quiroz A, Parra L, Levío J. 2012b. **Relaciones entre población de plantas, rendimiento de forraje y persistencia productiva en trébol rosado (*Trifolium pratense* L.).** 63° Congreso Agronómico, Contribuyendo a la sustentabilidad alimentaria.
Ortega F, Parra L, Quiroz L. 2014. Breeding red clover for improved persistence in Chile: a review. **Crops Pasture Sci** 65: 1138 - 1146.
Ortega F, Inostroza L, Moscoso C, Parra L, Quiroz A. 2018. **Temperate grassland legumes for sustainable animal production**. In Marshall A, Collins R. Improving grassland and pasture management in temperate agriculture Burleigh Dodds Science Publishing Limited, Cambridge, UK.
Palma R, Mutis A, Manosalva L, Ceballos R, Quiroz A. 2012. Behavioral and electrophysiological responses of *Hylastinus obscurus* to volatiles released from the roots of *Trifolium pratense* L. **J Soil Sci Plant Nutr** 12: 183 - 193.
Papadopoulos YA, Tsao R, McRae KB, Mellish AE, Fillmore SA. 2006. Genetic variability of principal isoflavones in red clover. **Can J Plant Sci** 86: 1345 - 1347.
Parra L, Mutis A, Ortega F, Quiroz A 2013. Field response of *Hylastinus obscurus* Marsham (Coleopetra: Curculionidae) to *E*-2-hexenal and limonene, two host derived semiochemicals. **Cienc Inv Agric** 40: 637 - 642.
Quiroz A, Ortega F, Ramírez C, Wadhams L, Pinilla

K. 2005. Response of the beetle *Hylastinus obscurus* Marsham (Coleoptera: Scolytidae) to red clover (*Trifolium pratense* L.) volatiles in a laboratory. **Environ Entomol** 34: 690 - 695.

Quiroz A, Méndez L, Mutis A, Hormazábal A, Ortega F, Birkett MA, Parra L. 2017. Antifeedant activity of red clover root isoflavonoids on *Hylastinus obscurus*. **J Soil Sci Plant Nutr** 17: 231 - 239.

Ramos GP, Dias PMB, Morais CB, Dall´Agnol M, Zuanazzi JAS. 2012. Genetic variability of isoflavones in the USDA red clover core collection. **Braz J Pharmacognosy** 22: 1241 - 1252.

Rhodes I, Ortega KF. 1996. **Progress in forage legume breeding**, In Younie D: Legumes in sustainable farming systems. British Grassland Society, Occasional symposium #30, Aberdeen, Scotland.

Rhodes I, Ortega KF. 1997. **Plant breeding achievements and prospects**, In Weddell JR: Forage Legumes. Seeds of Progress. British Grassland Society, Occasional symposium #31, Nottingham, England.

Rossiter RC. 1970. Factors affecting the oestrogen content of subterranean clover pastures. **Austr Vet J** 46: 141 - 144.

Saviranta NMM, Anttonen MJ, von Wright A, Karjalainen RO. 2008. Red clover (*Trifolium pratense* L.) isoflavones: determination of concentrations by plant stage, flower colour, plant part and cultivar. **J Sci Food Agric** 88: 125 - 132.

Saviranta NMM, Julkunen-Tiito R, Oksanen E, Karjalainen RO. 2010. Red clover (*Trifolium pratense* L.) isoflavones: root phenolic compounds affected by biotic and abiotic factors. **J Sci Food Agric** 90: 418 - 423.

Schubiger FX, Lehmann J. 1994. Stoffe mit östrogener Wirkung in Rotkleesorten. **Agrarforschung Schweiz** 1: 361 - 363.

Simmonds M. 2003. Flavonoids-insect interactions: recent advances in our knowledge. **Phytochem** 64: 21 - 30.

Sivesind E, Seguin P. 2005. Effects of the environment, cultivar, maturity, and preservation method on red clover isoflavone concentration. **J Agric Food Chem** 53: 6397 - 6402.

Singh N, Johnson DT. 2013. Baseline responses of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) to insect growth regulators. **J Agric Urban Entomol** 29: 35 - 54.

Steiner JJ, Alderman SC. 2003. Red clover seed production: IV. Effect and economics of soil pH adjusted by lime application. **Crop Sci** 43: 624 - 630.

Tapia S, Pardo F, Perich F, Quiroz A. 2005. Clover root borer *Hylastinus obscurus* (Marsham) (Coleoptera: Scolytidae) has no preference for volatiles from root extracts of disease infected red clover. **Acta Agric Scand B** 55: 158 - 160.

Tapia T, Perich F, Pardo F, Palma G, Quiroz A. 2007. Identification of volatiles from differently aged red clover (*Trifolium pratense*) root extracts and behavioural responses of clover root borer (*Hylastinus obscurus*) (Marsham) (Coleoptera: Scolytidae) to them. **Biochem Syst Ecol** 35: 61 - 67.

Taylor NL, Quesenberry KH. 1996. **Biosytematics and interspecific hybridization**, In Taylor NL, Quesenberry KH: Red clover science. Current Plant Science and Biotechnology in Agriculture. Boston, USA.

Toledo D, Parra L, Mutis A, Ortega F, Hormazábal E, Quiroz A. 2014. Influence of long-chain fatty acids on weight gain of *Hylastinus obscurus* (Coleoptera: Curculionidae). **Cienc Inv Agric** 41: 357 -364.

Tsao R, Papadopoulos Y, Yang R, Young JC, McRaf K. 2006. Isoflavone profiles of red clovers and their distribution in different parts harvested at different growing stages. **J Agric Food Chem** 54: 5797 - 5805.

Wang S, Ridsdill-Smith T, Ghisalberti E. 1998. Role of isoflavonoids in resistance of subterranean clover trifoliates to the redlegged earth mite *Halotydeus destructor*. **J Chem Ecol** 24: 2089 - 2100.

Wong E. 1963. Isoflavone content of red and subterranean clovers. **J Sci Food Agric** 14: 376 - 379.

Wu QL, Wang MF, Simon JE. 2003. Determination of isoflavones in red clover and related species by high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection. **J Chromatography A** 1016: 195 - 209.