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RESEARCH ARTICLE

Antifeedant activity of red clover root isoflavonoids on *Hylastinus obscurus*

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Abstract

In the last decade, there has been increasing interest in investigating the impact of flavonoids on insects, specifically for pest control. In this study, we investigated the impact of isoflavonoids upon the feeding behavior of the clover root borer, *Hylastinus obscurus* Marsham (Coleoptera: Curculionidae), which is one of the most serious global pests associated with red clover, *Trifolium pratense* L. Four aglycones isoflavonoids: genistein (1), formononetin (2), daidzein (3) and biochanin A (4) were isolated and identified by HPLC, from roots of two Chilean red clover cultivars. The first two compounds, formononetin (2) and genistein (1), showed high feeding deterrent activity when they were evaluated in artificial diets. This antifeedant effect of isoflavones on feeding behavior of *H. obscurus* suggests that they are responsible for a decreasedin insect weight gain as compared with the control. This information could be useful respectively, to farmers and researcher to produce and create plants resistant to curculionid.

Keywords: *Trifolium pratense, Hylastinus obscurus*, isoflavonoids, antifeedant

1. Introduction

The red clover root borer, *Hylastinus obscurus* Marsham (Coleoptera: Curculionidae), is one of the most serious global pests associated with red clover, *Trifolium pratense* L. (Fabaceae) (Steiner and Alderman, 2003). Both larval and adult stages feed on the roots, causing weakening and subsequent death of plants (Aguilera *et al*., 1996). An average of 1.5 *H. obscurus* per plant can cause 5.5% reduction in forage yield in 2-3 year-old pastures and infestation levels can reach 70% to 100% (Aguilera *et al*., 1996).

To date, chemical control of H. obscurus has been unsuccessful (Aguilera *et al.*, 1996), and biological control has not been explored. Therefore, alternative strategies for controlling *H. obscurus* are a high priority for red clover producers. One such approach is the development of new pest-resistant red clover cultivars, and in Chile, new cultivars have been developed through polycross cycles and half-sib family evaluation, resulting in the creation of 10 synthetic lines, two of which have become registered varieties (Ortega *et al.*, 2014). Because this process is based on the use of individual red clover plants that survive *H. obscurus* attack for more than four seasons, it is proposed that the new lines could have developed resistance to *H. obscurus* through the production of secondary metabolites that are deleterious to the pest.

Several authors have studied plant secondary metabolites present in the roots of red clover and their effect on *H. obscurus* behavior. Results to date suggest that there is strong chemical communication between *T. pretense* and *H. obscurus*, ie, secondary metabolites present in the roots can elicit either attractant or repellent behaviour (Quiroz *et al*., 2005; Tapia *et al.*, 2005, 2007; Alarcón *et al*., 2010; Manosalva *et al*., 2011). Furthermore, plant-produced isoflavonoids act as phytoalexins and also as insect feeding deterrents (Sutherland *et al*., 1980) have been shown to play a role in *Trifolium* – pest interactions eg the isoflavones formononetin, genistein, and biochanin A, present in the leaves of *T. subterraneum* L. exhibit deterrent activity on the red-legged earth mite Halotydeus destructor (Tucker) (Acari: Penthaleidae) (Wang *et al.*, 1998). Specifically, the accumulation of the isoflavonoid formononetin in the roots of resistant white clover is believed to exhibit a defensive role on the stem nematode Ditylenchus dipsaci (Cook *et al*., 1995), whilst the presence of formononetin in red clover may act as a deterrent against adult weevils, Sitona lepidus (Gyllenhal) (Coleoptera: Curculionidae) Gerard *et al.*, (2005).

Following previous reports of the role of red clover secondary metabolites in mediating *H. obscurus* behavior, and the implicated role of isoflavonoids in modifying the feeding of pests on white and red clover, the aims of this study were; a) to identify and quantify the isoflavonoids present in red clover roots, b) to establish a qualitative and quantitative comparison of isoflavonoids content between two Chilean red clover cultivars with different levels of persistence and c) to evaluate the antifeedant responses of *H. obscurus* elicited by the identified isoflavonoids. Establishing a role for these secondary metabolites in the resistance of red clover cultivars to *H. obscurus* could provide the basis for the generation of new pest-resistant cultivars.

2. Materials and Methods

2.1. Plant material

Two red clover (*Trifolium pratense* L.) cultivars, Quiñequeli INIA and Superqueli INIA with low and high persistence respectively, were collected at

INIA-Carillanca (La Araucanía, Chile). These plants were established in September 2014 under irrigated conditions at a seeding rate of 15 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 manually and no herbicides were applied. The whole plants were sampled in October 2015 with a sufficient amount of soil to avoid root damage.

2.2. Insects

The methodology used for the insect collection was based on the method described by Manosalva *et al.*, (2011). Adult *H. obscurus* were collected daily from 3-year-old red clover plants (cv. Quiñequeli INIA) located at INIA-Carillanca. The insects were gently removed from the roots and were maintained in Petri dishes (4ºC) with pieces of red clover roots (cv. Quiñequeli INIA). The insects were deprived of food 24 h before each feeding assay. Prior to each test, insects that were able to walk were selected and used for the feeding assay.

2.3. Chemicals

Isoflavones: genistein (1), formononetin (2), daidzein (3) and biochanin A (4) were purchased from Sigma-Aldrich, Chile and solvents from Merck, Chile.

2.4. Isoflavonoid extraction and analysis by high performance liquid chromatography (HPLC)

Red clover root tissue (20 mg) was lyophilized, milled and extracted with 80% MeOH (2.0 mL) for 16 h using an orbital shaker in dark at room temperature to obtain a polar fraction of isoflavonoids. The extract was spun down by centrifugation at 3,000 rpm for 1 h and the supernatant evaporated under stream of nitrogen to dryness. The dried residue was suspended in 300 µL of 45% methanol (Farag *et al*., 2007). The identification and quantification of isoflavonoids in root extracts was performed using a HPLC method, reported by Franke et al., (1994), with modifications. Samples (20 µL) were injected into a Shimadzu HPLC (LC-20A Prominence, Kyoto, Japan) equipped with a C-18 column (300 x 4.6 mm I.D.; particle size 5 µm) was eluted with a mobile phase composed of acetic acid: water (1:9 v/v) as solvent A and acetonitrile as solvent B at a flow rate of 1 mL min. The following gradient, which is expressed as the percentage of solvent B, was used: 0 min, 23% B; 0-8 min, 70% B; and 8-16 min, 23% B. The detection was performed at the preferred wavelength of 260 nm. The standard solutions for the calibration curve were prepared using a mixture of all isoflavonoids (Table 1) ranging in concentration from 0.5 to 15 ppm.

2.5. No-choice feeding (antifeedant) assays

Antifeedant experiments (no-choice feeding assays) with adult *H. obscurus* were carried out using an artificial diet according to Faccoli and Schlyter (2007). Approximately 500 µL of the diet, composed of 87.6% water, 2% cellulose, 2.6% glucose, 4.3% starch and 3.5% agar, was placed into transparent Eppendorf tubes (10 mm diameter x 35 mm length). Then, 10 µL of ethanolic solutions of the identified isoflavonoid aglycones, at different concentration, maximum, minimum and two intermediates (Table 1), were added separately, ensuring homogeneous distribution of the solution in the whole diet along the tube. The Eppendorf tube was kept open in a vertical position for 30 min at 21°C for ventilation, thus allowing the solvent to completely evaporate. Subsequently, a pre-weighed *H. obscurus* (iw) was introduced into each tube, which was then closed

with a plastic cap of Eppendorf tube. The insect was allowed to feed on the diet in the vertical tube for 5 d under darkness at room temperature. After this period, the insects were removed from the tubes and weighed again (fw). Each dose was tested individually on 20 adults. Moreover, 20 adults feeding in tubes containing only artificial diet were used as a control and each insect was used only once. The feeding performance was evaluated by the weight increase (%), where: weight increase (%) = ((fw-iw)/fw) × 100 (Toledo *et al.*, 2014).

Table 1. Quantification (mg g^{-1} dry matter) by HPLC-UV of the isoflavonoids presents in the polar fractions obtained from the roots of Quiñequeli INIA and Superqueli INIA.

Retention time (min)	Compound	Quiñequeli INIA	Superqueli INIA
8.50	Daidzein	1.043 ± 0.22	1.025 ± 0.27
11.01	Genistein	0.494 ± 0.11	0.651 ± 0.08
12.75	Formononetin	0.098 ± 0.01	0.195 ± 0.01 *
20.6	Biochanin A	1.575 ± 0.24	2.668 ± 0.23 *

Asterisk indicates significant differences in the concentration of flavonoid between cultivars by the non-parametric Mann-Whitney test ($p < 0.05$).

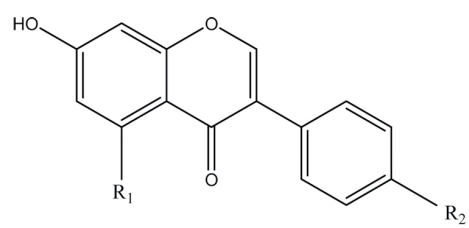
2.6. Statistical analysis

Non-parametric tests were used because the data did not conform to the statistical requirements of normality and homoscedasticity. The total isoflavonoid data in both cultivars were analyzed using the Wilcoxon signed rank test for paired samples ($p < 0.05$). Differences among isoflavonoids by cultivar were analyzed by Mann-Whitney test ($p < 0.05$). Results from feeding bioassays were analyzed by Friedman and Kruskall-Wallis test ($p < 0.05$) followed by the Conover-Inman test (Conover, 1999).

3. Results

3.1. Identification and quantification of isoflavonoids

The analysis of isoflavonoids from *T. pratense* revealed the presence of four aglycones in the polar extracts of both Quiñequeli INIA and Superqueli INIA. Aglycones identified were diadzein, genistein, formononentin and biochanine A (Table 1, Figure 1).



(1) R_1=OH, R_2=OH = Genistein

(2) R_1=H, R_2=OCH3 = Formoninetin

(3) R_1=H, R_2=OH = Daidzein

(4) R_1=OH, R_2=OCH_3 = Biochanin A

Figure 1. Structures of isoflavonoid from *Trifolium pratense*: (1) genistein, (2) formononetin and (3) daidzein. (4) biochanin A.

Levels (mg g^{-1} of dry matter) of genistein (0.494 ± 0.11 vs 0.651 ± 0.08) and daidzein (1.043 ± 0.22 vs 1.025 ± 0.27) were not significantly different between cultivars ($p > 0.05$). Levels of formononetin were significantly higher in Superqueli INIA (0.195 ± 0.01) than Quiñequeli INIA (0.098 ± 0.01) ($p < 0.05$). Significant differences in the level of biochanin A were detected between the cultivars (1.575 ± 0.24 vs 2.668 ± 0.23) ($p < 0.05$). Moreover, there were significant differences when the averages of the four isoflavonoids was compared between both cultivars evaluated, with the level being greater in Superqueli INIA (1.135 ± 0.54) than Quiñequeli INIA (0.802 ± 0.32) (p = 0.0261).

3.2. Feeding bioassays

Antifeedant bioassays using an artificial diet indicated that the addition of formononetin and genistein to the diet clearly affected the feeding behavior of *H. obscurus*, compared to the diet controls (Figure 2). Weight increase data demonstrated that whereas control weights steadily increased (3.45 ± 1.14 %), individuals with doses of 0.098 to 0.195 mg/g of formononetin had lower weight gains (Figure 2A). The aglycone genistein elicited a similar antifeedant behavior at all doses, with a decreased in insect weight gain from 2.56 ± 0.95 % (control) to -3.71 ± 1.53 % (0.651 mg/g) (p = 0.0013, Figure 2B). By contrast, daidzein revealed a significant feeding-preference activity (p = 0.0205) from 1.9 ± 4.28 % (control) to a maximum of 10.24 ± 3.43 % in all doses evaluated (1.025, 1.031, 1.037 and 1.043 mg/g) (Figure 2C) and biochanin A showed similar results (Figure 2D).

Only the intermediate concentration of 2.3 mg/g was statistically significant to the control and all other doses tested, reaching a significant weight increase of 10.58 ± 2.69 %. The rest of the doses and the control did not differ significantly ($p > 0.05$).

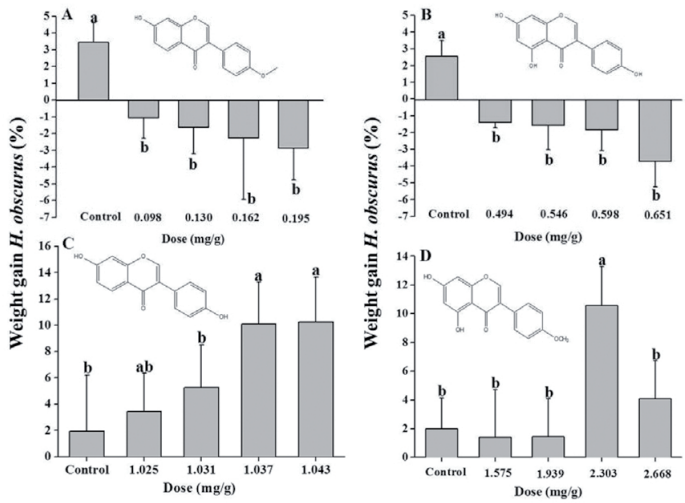


Figure 2. Weight gain (%) of *Hylastinus obscurus* feeding bioassays using artificial diet. Treatment: A) formononetin (0.098, 0.130, 0.1626 and 0.195 mg/g), B) genistein (0.494, 0.546, 0.598 and 0.651 mg/g), C) daidzein (1.025, 1.031, 1.037 and 1.043 mg/g) and D) biochanin A (1.575, 1.939, 2.303 and 2.668 mg/g). Control = artificial diet. Different letters indicates significant differences between treatment and control by the non-parametric Kruskal-Wallis test followed by Conover-Inman test.

4. Discussion

In general, forage legumes such as *T. pratense* produce phytoestrogens mainly belonging to the isoflavone group. Isoflavonoids are known to mediate plant-insect interactions, either deterring or stimulating insect feeding behaviour (Simmonds, 2003). For red clover, Saviranta *et al.*, (2010) previously reported that the most abundant phenolic compounds in field-grown roots were the maleate glycoside of formononetin (3.90–4.27 mg g^{-1}), maackiain (2.35–3.02 mg g^{-1}) and pseudobaptigenin (1.80–2.58 mg g^{-1}), and the concentration of formononetin-aglycone was approx. 0.5 mg g^{-1}. Similar to that study, our work identified here 4 flavonoid aglycones (Table 1, Figure 1) in the polar extract of red clover roots. The level of formononetin found in Superqueli INIA (0.195 ± 0.1 mg g^{-1}) cultivar was similar to those reported by Saviranta *et al*., (2010), and formononetin (2) levels differed considerably between Quiñequeli INIA (0.098 ± 0.01 mg g^{-1}) and Superqueli INIA. However, the highest content of formononetin were found in roots of red clover (cv. Bjursele) samples ranging from 5.0 to 6.0 mg g^{-1} of dry matter (Saviranta *et al*., 2008). The differences could be explained by different factors, such as seasonality, environmental and organ or part of plant used for the analysis (Saviranta *et al*., 2010). On the other hand, Papadopoulus *et al*., (2006) indicated that there is significant genetic variability for formononetin (2) content among red clover cultivars, and concluded that the selection of individual plant phenotypes for high formononetin (2) could be effective for cultivar improvement. Other authors have reported that variations in the levels of flavonoids, especially formononetin (2) is because this compound is found mostly in aerial part of the plant (leaves, flowers and stems) (Vetter, 1995). In this regard, several studies reported that different breeding programs have been successful in the selection of cultivars and experimental lines with high and low formononetin (2) content (Rumball *et al*., 1997). In relation to the bioactivity against insects of flavonoids, Iwashina (2000) reported that a wide variety of flavonoids have been used as feeding deterrents towards harmful insect by some plants. During the last few decades many flavonoids with deterrent or antifeedant effects against the grass grub Costelytra zealandica (White) (Coleoptera: Scarabaeidae), causing significant impacts on the persistence and productivity of improved pastures in New Zealand have been reported (Zydenbos *et al*., 2011). In total, 23 isoflavonoids isolated from roots of Fabaceae species *Lupinus angustifolius* and *Lotus pedunculatus* were evaluated, being genistein one of the responsible for the deterrent activity (Lane *et al*., 1987). In this sense, Gerard *et al*., (2005) informed that formononetin (2) ranging from 0.62 to 1.53 % in dry matter in three cultivars of red clover, GF131, G27 and Pawera, acted as chemical defenses against the curculionid *Sitona lepidus,* suggesting that their distribution in forage legumes can be manipulated by plant breeding to improve root health. The isoflavones formononetin (2), genistein (1), and biochanin A (4) present in the leaves of *Trifolium subterraneum* L. have been reported to exhibit a greater deterrent activity on the red-legged earth mite *Halotydeus destructor* (Tucker) (Acari: Penthaleidae) than the respective glycosylated isoflavones (Wang *et al.*, 1998). In relation to this study, formononetin has been reported by Cook *et al*., (1995) as responsible for conferring defence to white clover roots against the stem nematode *Ditylenchus dipsaci* because its accumulation in the meristems. Furthermore, some flavonoid aglycones may affect other parameters in insects such as reduced growth rate, to extend the duration of both larvae instar and progeny. Lahtinen *et al*., (2004) reported that larvae of *Epirrita autumnata* (Lepidoptera: Geometridae) reduced the growth rate and prolonged the duration of their first instars because to the high content of the aglycone 5-hydroxy-

4´,7-dimethoxyflavanone (10 mg/g leaf dry weight). The progeny produced by Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) was significantly decreased at 30 days after the application of 50 µg of biochanin A (4) and genistein (29.9 and 18.6%, respectively) (Boué and Raina, 2003). Genistein also has been demonstrated as detrimental on the feeding behavior of the pea aphid *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) at higher concentrations (≥ 1,000 µg cm-3) stopped completely the salivation and passive ingestion (Goławska and Łukasik, 2012).

Similar to the reports by Simmonds and Stevenson (2001) and Yu *et al*., (2003), our results demonstrated that independent of the doses of individual flavonoids, in our research, all the concentrations of formononetin (0.098, 0.13, 0.162 and 0.195 mg/g) and genistein (0.494, 0.546, 0.598 and 0.651 mg/g) were detrimental on feeding behavior of *H. obscurus* (Figure 2). Similarly, feeding studies have suggested that formononetin and biochanin A elicit a deter behavior from both clover root weevil (*S. lepidus*) larvae which feed on the roots, and adults which feed on the red clover leaves (Gerard *et al.*, 2005). These results could be related to both higher persistence and better agronomic yields observed in Superqueli INIA than Quiñequeli INIA (Ortega *et al*., 2014).

Moreover, the isoflavonoids genistein (1) and daidzein, when exuded by the roots, are important signals to *Rhizobium* sp. bacteria for the establishment of root nodules where the bacteria reside and fix atmospheric nitrogen in a symbiotic relationship with the plant (Subramanian *et al*., 2006).

5. Conclusions

We evaluated 4 isoflavonoid aglycones: genistein (1), formononetin (2), daidzein (3) and biochanin A (4) in feeding bioassays with artificial diet. The first two aglycones elicited an antifeedant effect on H. obscurus while daidzein (3) and biochanin A (4) elicited phagostimulant behaviour. In summary, this work establishes detrimental effect of the flavonoids formononetin and genistein on the clover root borer, H. obscurus. Our results suggest that these isoflavonoids present in the roots of red clover are responsible for the decreased gain in *H. obscurus*, suggesting that the higher formononetin content in Superqueli-INIA than Quiñequeli-INIA would explain the high persistence of Superqueli-INIA. This research demonstrates the usefulness of isoflavonoids present in *T. pratense* as natural controllers of *H. obscurus*. Our results cannot be extrapolated to field conditions, but constitute an important basis to carry out future experiments that evaluate the resistance of these plants with high contents of formononetin (2), and present a new opportunity for plant breeders.

This information, associated with cultural practices, could help farmers and breeders to reduce *H. obscurus* populations in grassland, where this curculionid constitute an important pest.

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