Formononetin of Red Clover (*Trifolium pratense* L.) as Affected by Water Availability

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Key Words: Formononetin, isoflavones, water availability, plant survival

Abstract
Red clover is a valuable legume in the world. Among the biotic factors limiting persistence of the species in Chile is the root borer *Hylastinus obscurus* (Marsham). Our previous studies have shown a negative effect of formononetin content of the plant on the root borer fitness The purpose of this research was to assess the relationship between water availability and formononetin concentration. Two independent growth chamber experiments were established at Carillanca Research Center, INIA-Chile in order to study the effect of water availability on formononetin concentration in the aerial and root parts of the plants. In experiment I, three levels of water applied were studied: T1: non-stressed, maintained between 100 and 60% of the readily available soil water (RAW); T2: 60 and 30% of RAW; T3: <30% of RAW. Experiment II consisted of four water levels: T1: 100-80% RAW; T2: 80-60% RAW; T3: 60-40% of RAW; T4: <40% of RAW. In both experiments a complete randomized design was used. For sampling, plants were dug up from the pots and cut at the crown to separate the aerial part from the crown and roots. Formononetin concentration was evaluated by extracting with a methanol solution and relative quantifications based on HPLC–MS. Different water available levels were studied. Formononetin was much higher in the aerial part compared to roots. There were no significant effects of readily available soil water (RAW) over formononetin concentration.

Introduction

Red clover (*Trifolium pratense* L.) is a valuable legume around the world. The main limitation of the species is the lack of persistence related to the high mortality of plants due to a complex of biotic and abiotic factors (López-Olivari and Ortega-Klose 2020). There is a close relationship between plant population and forage yield potential (Ortega et al. 2014). Among the biotic factors associated with red clover mortality in Chile, the main one is the root borer *Hylastinus obscurus* (Marsham). Our previous studies of the relationship plant-borer mediated by semi-chemicals have shown that formonenotin is the main isoflavone of red clover eliciting an antifeedant response from *H. obscurus* (Quiroz et al. 2017; Quiroz et al. 2018). The purpose of this research was to study the relationship between soil water availability and formononetin concentration.

Methods and Study Site

Two independent experiments were established at Carillanca Research Center (38°41'SL and 72º25'WL), INIA-Chile, in a growth chamber maintained at 24/18°C day/night (14/10 hours) and 125 Photosynthetically Active Radiation (PAR) during the day. In both experiments, the diploid cultivar Superqueli INIA was sawn in greenhouse in seed trays and transplanted later to pots of 12.4 l each (22 cm deep; Andisol type soil with a loam texture). Fertilization was applied according to soil analysis, estimating the doses per pot to an equivalent of 40-113-329 kg/ha of N, K_2O and P_2O_5, respectively. In the experiment I, three levels of water applied were: T1: non-stressed, maintained between 100 and 60% of the readily available soil water (RAW); T2: 60 and 30% of RAW; T3: <30% of RAW. In this experiment I, 5 plants per pot were established with 36 pots in a complete randomized design with three levels of water applied, 2 evaluation dates and 6 pots per level and evaluation date. The experiment II consisted of four water levels: T1: 100-80% RAW; T2: 80-60% RAW; T3: 60-40% of RAW; T4: <40% of RAW. In this experiment II, 9 plants per pot were established with 32 pots in a complete randomized design with four levels of irrigation, 2 evaluation dates and 4 pots per water level and evaluation date. Water was replenishing based on the weight of each pot. Experiment I was cut and evaluated the first time after 16 days of establishing the water treatments and then evaluated again 33 days after the first cut. Experiment II was evaluated 36 days after establishing water treatments and again 48 days later. For sampling, plants were dug up from the pots and cut at the crown to separate the aerial part from the crown and roots; crown and roots were carefully washed. After sampling, the aerial and root tissues were stabilized immediately by immersion in liquid N2 for later lyophilization. Yield of the aerial and root sections was evaluated (mg / g DM). Formononetin concentration was evaluated by extracting with a methanol solution and relative quantifications based on HPLC–MS. HPLC analysis was performed using a Diode Array Detector and a C18 reversed-phase column according to the methodology reported by Ramos et al. (2008). Moisture availability per pot (W/W) was evaluated weekly by the weight of each pot. In the experiment II, before each sampling date, the stomatal conductance using a steady-state porometer (SC-1 leaf porometer, METER Group, Inc. USA) was measured. Also, the chlorophyll concentration in the leaf was measured once in the experiment II using a portable chlorophyll meter (SPAD-502, Minolta Camera Co. Ltd., Japan). Data was analyzed by ANOVA and means separated by Duncan (5%).

Results

Figure 1 shows the weight of pots during the second period of experiment II; the four water levels were maintained with three irrigations in this experimental period. There were clear responses in stomatal conductance and chlorophyll content. Stomatal conductance differences were significant and in accordance with water levels (Figure 2). Chlorophyll content was significantly higher with water deficit treatments (T1: 43,9 c; T2: 46,2 c; T3: 52,4 b; T4: 62,4 a). There was no significant response in dry matter yield of both aerial and root sections.

The highest content of formononetin was found in leaves and stems compared to roots. In the experiment I, there were non-significant tendencies to increase formononetin concentration in the aerial part with water stress; something similar occurred in experiment II (Figure 3).

Figure 1. Average weight of pots under four levels of irrigation in the Experiment II. FC: Field capacity; WP: Wilting point. T1: 100-80% of the readily available soil water (RAW); T2: 80-60% RAW; T3: 60-40% RAW; T4: <40% RAW.

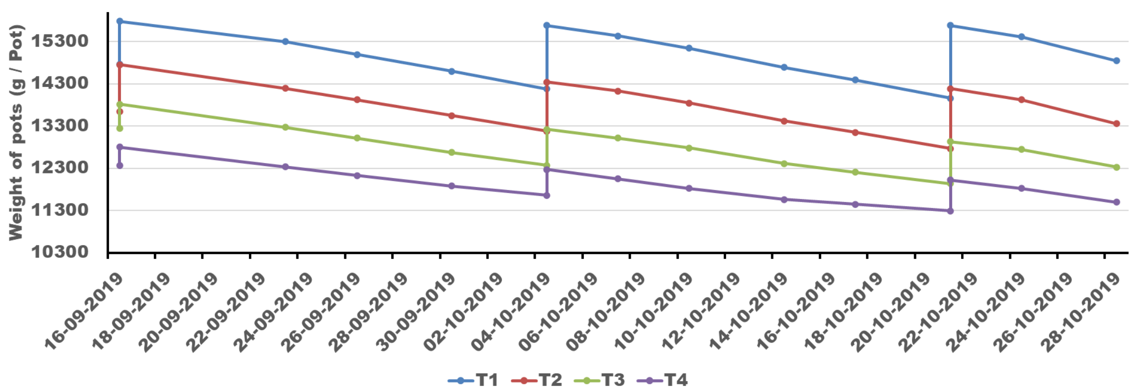


Figure 2. Stomatal conductance (mmol/m^2/s^1) of each treatment before two sampling dates of Experiment II. Different letters within dates indicate significant differences (Duncan's test, $p \leq 0.05$). T1: 100-80% of the readily available soil water (RAW); T2: 80-60% of RAW; T3: 60-40% of RAW; T4: <40% of RAW.

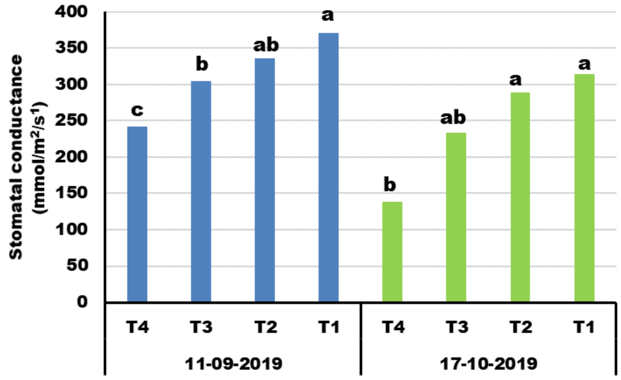
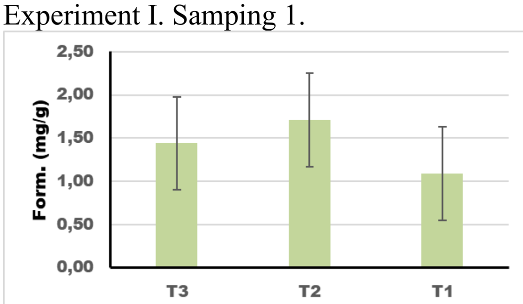
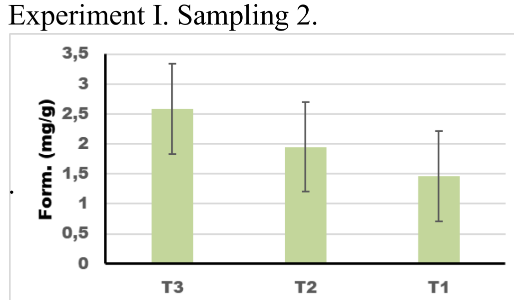


Figure 3. Formononetin content (mg / g) of red clover aereal part (shoots and leaves) in two experiments and two sampling dates each. Experiment I: T1: non stressed, maintained between 100 and 60% of the readily available soil water (RAW); T2: 60 and 30% of RAW; T3: <30% of RAW. Experiment II: T1: 100-80% of RAW; T2: 80-60% of RAW; T3: 60-40% of RAW; T4: <40% of RAW. Bars indicate the standar error of the means.

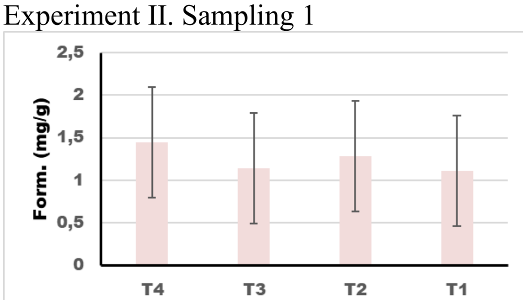
Experiment I. Samping 1.

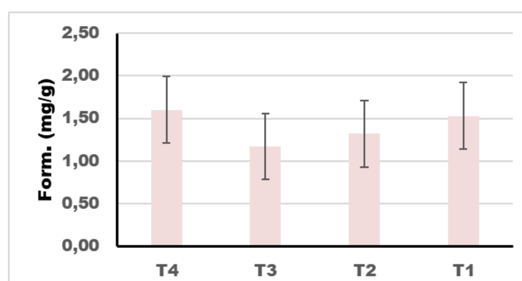


Experiment I. Sampling 2.



Experiment II. Sampling 1





Discussion

times after a period of 3 weeks treatment (De Rijke et al., 2005). By contrary, our results did not show an increase of formononetin content in treatments with water stress; moreover, there was no significant effect of available soil water on formononetin concentration in the aerial part and roots of red clover. Considering the repellent effect elicited by formononetin on *H. obscurus*, the non-effect shown by water availability can be considered a good result since the genetic expression of formononetin content in selected red clover genotypes will be independent of the soil available water level.

Conclusions

There was a higher concentration of formononetin in the aerial parts of red clover compared to roots. There was no significant effect of soil water availability on formononetin content; this is a positive finding if a red clover genetic line with a higher formononetin content is selected since the expression of the trait will be independent of the water soil available level. It is necessary to study other abiotic soil factor (phosphorus level as example) and confirm this findings in field conditions.

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