Could formononetin of Red Clover (*Trifolium pratense* L.) be enhanced by phosphorus and arbuscular mycorrhizal fungi management?

Ortega-Klose, F. *; Quiroz, A. †; Bardehle, L. †; Aguilera, P. †
* Instituto de Investigaciones Agropecuarias, INIA Carillanca, Chile.
† Universidad de La Frontera, Chile.

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Abstract. Red clover is a forage legume of importance in the world with limited persistency; in Chile this is due mainly to the root borer (*Hylastinus obscurus* Marsham) infestation. Our previous studies have shown that there is a strong relationship between the root borer and the formononetin content in roots of the plants; therefore, studying factors that enhance the concentration of formononetin in the plant could help to decrease the negative effect of the root borer. The purpose of this research was to assess the relationship between phosphorus availability (P) in the soil interacting with arbuscular mycorrhizal fungi (AMF) over the concentration of formononetin in shoots and roots of red clover. One trial was carried out in a growth chamber at Carillanca Research Center, INIA-Chile, using 6.000 cc pots filled with an Andisol soil. Three levels of soil available phosphorus (10 ppm Olsen-P; 17 ppm Olsen-P; 24 ppm Olsen-P) and two levels of arbuscular mycorrhizal fungi (inoculated and non-inoculated with a commercial mixture) were implemented in a factorial arrangement in a completely randomized design. Soil water was maintained between 50 and 100% of the readily available soil water (RAW) by weighting each pot. Formononetin concentration of shoots and roots was evaluated in two sampling dates by extracting with a methanol solution and relative quantifications based on HPLC. Shoot and root biomass were affected significantly by P and not by AMF, being higher with increased P; however, formononetin concentration was higher with reduced P. On the other hand, there was a significant increase of formononetin concentration both in shoots and roots in the treatments inoculated with AMF. The medium level of P (17 ppm) with AMF inoculation shows a good compromise between biomass production and 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). It has been demonstrated that 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 general objective of our project is to enhance the concentration of formononetin by managing biotic and abiotic factors. The purpose of this particular research was to study the relationship between phosphorus availability in the soil (P) interacting with arbuscular mycorrhizal fungi (AMF) over formononetin concentration of shoots and roots.

Methods

The experiment was established at Carillanca Research Center in a growth chamber maintained at 24/18°C day/night (14/10 hours) and 400 Photosynthetically Active Radiation (PAR) at canopy level during the day. Three levels of P (P10: 10 ppm Olsen-P; P17= 17 ppm Olsen-P; P24= 24 ppm Olsen-P) and two levels of AMF (AMF+: inoculated with a commercial mixture; AMF-: non-inoculated) were implemented in a completely randomized design with a factorial arrangement (3 x 2 with 5 replicates, two sampling dates, 60 pots in total) in pots of 6.000 cc each (20 cm deep) with 16 equidistant plants each of the diploid cultivar Superqueli-INIA. Soil readily available water (RAW) was maintained between 50 and 100% based on the weight of each pot. A destructive sampling was performed twice; the first time 21 days after moving pots to the growth chamber. The remaining pots were cut the same date without evaluation and sampled 22 days after. For sampling, plants were dug up from the pots and cut at the crown level 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. Colonization of roots with AMF (%) was evaluated in each sampling using the methodology described by Giovannetti and Mosse (1980). Plant height (cm) and root length (cm) were measured with a ruler in each cut. Yield of shoots and

roots was evaluated (DM g / plant) by weighting the total fresh tissue per plot, stabilizing the fresh weight in liquid nitrogen and later lyophilization for estimating weights in DM and formononetin extraction. Chlorophyll content in the leaves was evaluated five times during the experiment using a portable chlorophyll meter (SPAD-502, Minolta Camera Co. Ltd., Japan). Formononetin concentration (mg/g) 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). Data was analyzed by ANOVA and means separated by Duncan (5%).

Results and Discussion

There was no significant effect of AMF or P on colonization of roots by AMF. Chlorophyl contend (SPAD Units) was significantly affected by P in four of the five dates evaluated, being higher as the phosphorus increased. By contrary, AMF did not affect significantly the chlorophyl content.

Biomass produced was higher in shoots than in roots (Figure 1). Both in shoots and roots, this was significantly increased by phosphorus level in the first and second sampling dates and not by AMF.

In both sampling dates the highest concentration of formononetin was found in shoots compared to roots. In the first sampling date there were significant effects of P and AMF on the concentration of formononetin in shoots and roots, with no interaction between them. In shoots, concentration (mg/g) increased as P decreased (Figure 2A). The tendency was similar in the roots, concentration (Figure 2B) was higher with lower P. Regarding mycorrhiza, both in shoots and roots the formononetin concentration (Figure 3) was higher in AMF+ compared to AMF-.

In the second sampling date, there was a significant effect of P on the formononetin concentration of shoots; the tendency was similar to the first sampling date, being higher the concentration with the lowest P. Regarding the roots, there was an interaction of P and AMF; the highest concentration was achieved with AMF+ and the lowest P. These results show that, even though there was no significant effect of AMF on colonization, there was effect of AMF on the concentration of formononetin both in shoots and roots; AMF- was probably colonized by native mycorrhiza, which was less effective in terms of enhancing formononetin concentration compared to AMF+.

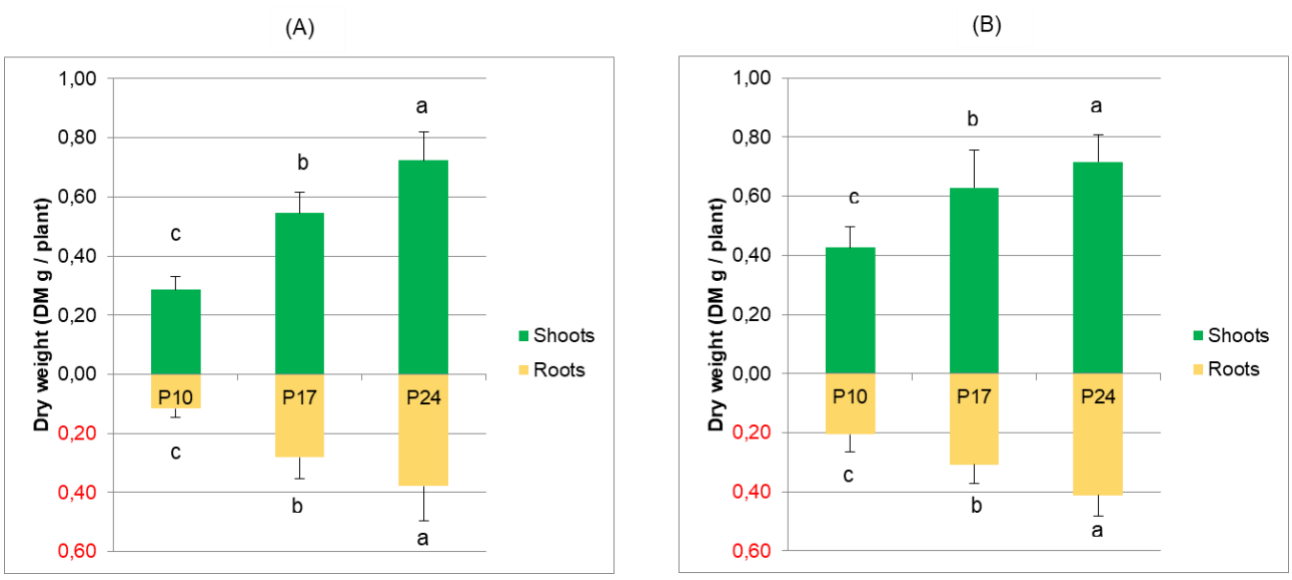

Figure 1. Shoot and root dry weight (DM g/plant) according to soil phosphorus level in the first (A) and second sampling dates (B). The values are provided as mean + standard deviation; diferent letters indicate Duncan significant differences (P<0,05).

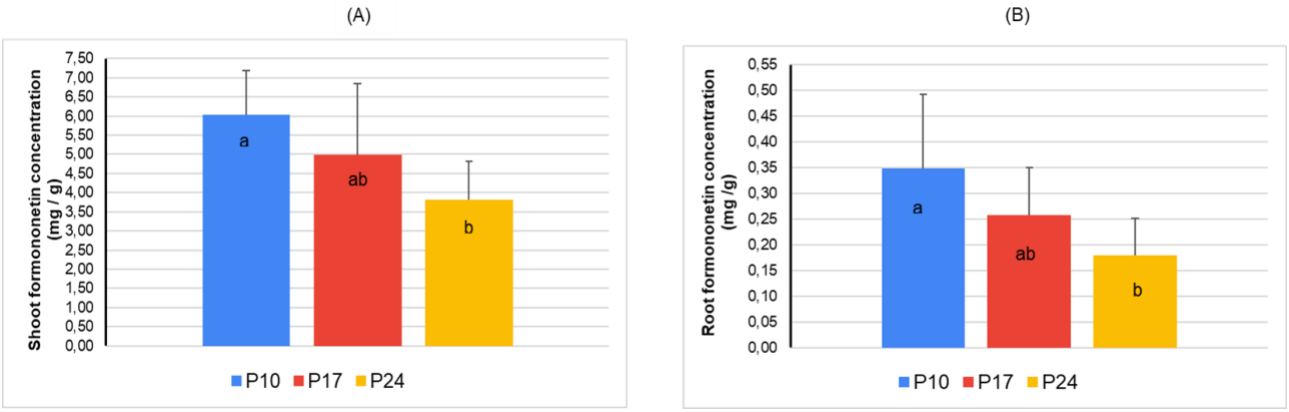

Figure 2. Formononetin concentration (mg/g) (A shoots; B roots) according to soil phosphorus level in the first sampling date. The values are provided as mean + standard deviation; diferent letters for each tissue and graph indicate Duncan significant differences (P<0,05%).

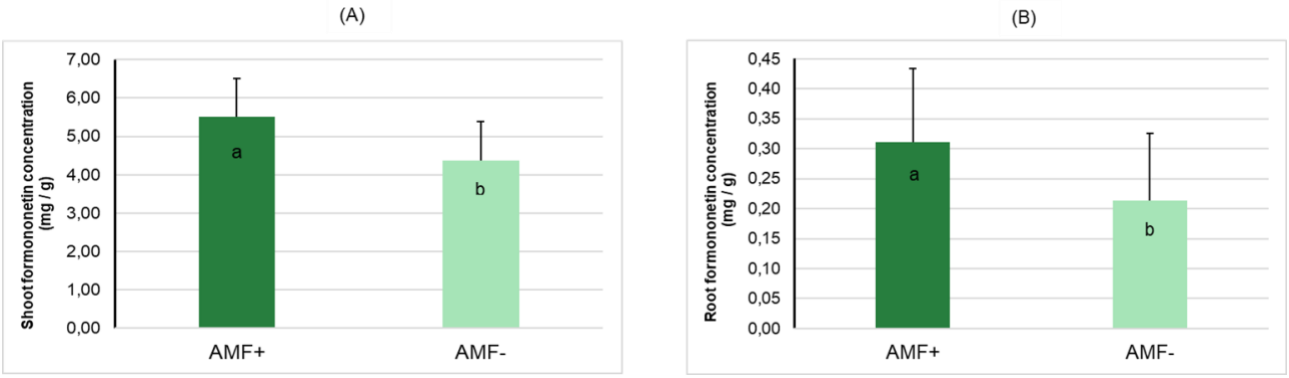

Figure 3. Formononetin concentration (mg/g) in shoots (A) and roots (B) according **to** arbuscular mycorrhizal fungi (AMF+: inoculated; AMF-: non-inoculated) in the first sampling date. The values are provided as mean + standard deviation; diferent letters within tissue indicate Duncan significant differences (P<0,05%).

Conclusions

P and AMF did affect the formononetin concentration and biomass production of red clover. The higher concentration of formononetin was obtained with reduced P and AMF inoculation; by contrary, the biomass production increased with P and also with AMF inoculation. The medium level of P (17 ppm) with AMF inoculation shows a good compromise between biomass production and formononetin concentration. Future research will consider the validation of the results in field trials.

Acknowledgements

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References

Giovannetti, M., & Mosse, B. 1980. An Evaluation of Techniques for Measuring Vesicular Arbuscular Mycorrhizal Infection in Roots. *New Phytologist*, 84, 489-500. http://dx.doi.org/10.1111/j.1469-8137.1980.tb04556.x

López-Olivari, R. and Ortega-Klose, F. 2020. Response of red clover to deficit irrigation: dry matter yield, populations, and irrigation water use efficiency in southern Chile. *Irrig Sci*, https://doi.org/10.1007/s00271-020-00693-0.

Fernando Ortega, Leonardo Parra and Andrés Quiroz. 2014. Breeding red clover for improved persistence in Chile: a review.*Crop and Pasture Science*, CSIRO publishing, 65(11):1138-1146.

Quiroz, A., L. Méndez, A. Mutis, E. Hormazábal, F. Ortega, M.A. Birkett & L. Parra. 2017. Antifeedant activity of red clover root isoflavonoids on *Hylastinus obscurus*. *Journal of Soil Science* 17(1): 231-239.

Quiroz, A., L. Bardehle, E. Hormazábal, F. Ortega & 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). *Blacpma* 17(4): 372-380. Boletín Latinoamericano y del caribe de plantas medicinales y aromáticas. ISSSN 0717 7917.

Ramos, G.P., Dias, P.M.B., Morais, C.B., Froehlich, P.E., Dall'Agnol, M., & Zuanazzi J.A.S. 2008. LC Determination of four isoflavone aglycones in red clover (*Trifolium pratense* L.). Chromatographia 67: 125-129.