RELATIONSHIP BETWEEN RADICAL INFESTATION OF *Hylastinus obscurus* (MARSHAM) AND THE YIELD OF CULTIVARS AND EXPERIMENTAL LINES OF RED CLOVER (*Trifolium pratense* L.)

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ABSTRACT

Red clover (*Trifolium pratense* L.) is a valuable forage legume grown alone or in mixture with grasses in temperate regions of the world. Red clover is adapted to a wide range of soil types, pH levels, environmental and management conditions. However, the main limitation of this species is the lack of persistence related to the high mortality of plants. In Chile the main biotic factor affecting survival of plants is the root borer *Hylastinus obscurus* (Marsham) (Coleoptera; Scolytidae). In 1989, a red clover (*T. pratense* L.) breeding program was started at Carillanca Research Center of the Institute of Agricultural Research (INIA), Chile, with the main objectives of improving the survival of plants, forage yield and persistence. In 2002 a research line to study the interaction between the root borer and the plant was started. This paper describes briefly the improvement obtained in survival of young plants after twenty years of breeding and the importance of root borer population regarding forage yield. Experiments were conducted at Carillanca Research Center under irrigated conditions, comparing under cutting the dry matter yield of new synthetic lines and Redqueli-INIA with Quiñequeli-INIA. The experimental lines Syn Int IV, Syn Pre III, Syn Int V and Syn Int VI were more productive than the cultivars Redqueli-INIA and Quiñequeli-INIA at the second season. The evaluation of the biological parameter allowed identifying a tolerant line, Syn Pre I, and three potential new varieties: Syn Pre III, Syn Int V and Syn Int VI. This is the first report showing an inverse relation between dry matter yield of red clover and root borer population ($P \leq 0.05$). Moreover, we report the first evidence that *H. obscurus* start the colonization of red clover plant of 6-month-old.

Keywords: *Hylastinus obscurus*, red clover, plant population, breeding, dry matter yield

INTRODUCTION

Red clover (*Trifolium pratense* L.) is an important forage legume grown on approximately 4 million hectares in temperate regions of the world and serves a multiple purpose in agricultural rotations (Riday, 2009). In Chile is a valuable resource for animal production and for the seed industry with exports in the last decade from 700 to 1700 tons year^{-1} and 100,000 ha cultivated, corresponding to 20% of the total sown pastures (Ortega, 2009). This is a legume that is used primarily in short-rotation pastures (Catrileo and Rojas, 1987), grazing and cutting (hay, silage and soiling). Although red clover is botanically considered a perennial plant, production levels decline dramatically two years after sowing (Cuevas and Balocchi, 1983; Leath, 1985; Steiner and Alderman, 2003), determined by the high mortality of plants (Ortega, 1996; Rhodes and Ortega, 1997).

Factors involved in mortality of red clover plants include root rot and infestations by the root borer *Hylastinus obscurus* Marsham (Coleoptera: Scolytidae) (Graham and Newton, 1959; Carrillo and Mundaca, 1974). This scolytid is the most important pests of red clover (*T. pratense*) throughout the world (Steiner and Alderman, 2003). Both larvae and adults bore and feed into the roots causing a significant reduction in production levels and persistence of red clover stands within two years after sowing (Cuevas and Balocchi, 1983; Steiner and Alderman, 2003). The root damage affects the carbohydrate reserves, the development of stems and leaves, resulting in a total destruction of the plant (Graham and Newton, 1959; Koehler *et al*., 1961; Carrillo and Mundaca, 1974; Matamala, 1976). The intensity of the attack can reach between 70 and 100% of plants affected the second and third year of clover established (Aguilera, 1995). In 1989, a red clover breeding program was launched at Carillanca Research Center of the Institute of Agricultural Research (INIA-Chile), with the main objectives of improving the survival of plants, forage yield and persistence compared to the Chilean diploid and double cut cultivar Quiñequeli-INIA released in 1962. The first new synthetic developed was Redqueli-INIA, cultivar released in 1997.

Previous studies have shown that H. obscurus respond to chemical stimulus from the plant (Quiroz *et al*., 2005; Tapia *et al*., 2005; Tapia *et al*., 2007), evidencing that this insect is able to recognize their host by detecting chemical compounds released from both aerial and root parts of red clover plant. These results suggested that the insect is strongly associated to the red clover roots because of the chemical compounds released from roots.

There are not studies about the population dynamic of *H. obscurus* and their potential influence on selecting new red clover cultivars. This research aims to relate *H. obscurus* population with conventional agronomical parameters evaluated in red clover breeding, such as dry matter yield, forage cover and plant density. Moreover, crown diameter of red clover plant was incorporated in these evaluation, because its relation with the oviposition behavior of *H. obscurus*. The research was performed studying two cultivars, Redqueli-INIA and Quiñequeli-INIA, and seven experimental lines proposed by INIA Carillanca.

MATERIALS AND METHODS

Location of the research and experimental design

A trial was established in spring of 2007 at INIA Carillanca Research Center, located in the Araucanía Region, Cautín Province, 38º 41' south latitude and 72º 25' west longitude and 200 m.s.n.m. Quiñequeli-INIA and Redqueli-INIA cultivars (Avendaño, 1965; Ortega *et al*., 2003) and 7 experimental lines under evaluation (Syn Pre I, Syn IV, Syn Pre II, Syn Int IV, Syn Pre III, Syn Int V and Syn Int VI) were distributed in an experimental design of randomized complete blocks with three replicates. Plot size was 1.8 x 7 m each.

Agroclimatic characteristics of the research site

The climate is characterized by annual average temperatures of 10°C, with a maximum mean monthly value of 21.5ºC January and a minimum of 2.3ºC in July. Average annual precipitation is close to 1,400 mm. There is an annual sum of 2,346 hours of cold and thermal summation base temperature of 5°C of 1.394 day. The frost-free period is between January and February.

The soil is an Andisol, belonging to the series Vilcún, Temuco family, classified as Medial, Music, Entice, Dystrandept. Agriculture has depth of 80 cm and a flat topography with a slope of 0-2%.

Trial establishment and management

Cultivars and experimental lines were sown in previously prepared soil on September 12, using a Planet Junior manual seeder. Seedlings were in rows with a separation of 20 cm each other. The seed dosage was 15 kg ha^{-1}, using rhizobium inoculated seed.

Fertilizers were applied annually according to soil analysis; fertilization at sowing consisted of 140 kg P_2O_5 ha^{-1}, 151 kg K_2O ha^{-1} , 5 kg S ha^{-1}; 1 kg Mg ha^{-1}; 65 kg Na ha^{-1}; 153 kg CaO ha^{-1}; maintenance fertilization the second season was 140 kg P_2O_5 ha^{-1} and 200 kg K_2O ha^{-1}.

The trial was irrigated five times in the first season (from January to march of 2008) and eight times during the second season (from November of 2008 to March of 2009. More prominent weeds were removed by hand but no herbicide was applied.

Evaluation of *Hylastinus obscurus* (Marsham) in red clover roots

Insect were sampled monthly from November 2007 to May 2009. An average of 12 plants of each cultivar and experimental line were randomly chosen from the three replicates. Plants were extracted with both aerial and radical parts, and they were put into a paper bag and transferred to the Chemical Ecology Laboratory of the Universidad de La Frontera. The radical part was separated from the aerial part and it was carefully cut with a scalpel for evaluating the presence of adult and larvae of *H. obscurus* inside red clover roots because these stages cause weakening and death of red clover plants (Aguilera *et al*., 1996). These surveys were started when the plants were 4-month-old.

Crown diameter and length of the main root

Crown diameter and length of the main root was evaluated in the same plants used for *H. obscurus* evaluation. Diameter was measured with a Vernier caliper and root length with a metallic rule. Evaluations were performed with the total number of plants collected in the field.

Evaluation of agronomic parameters

Seven cuts were carried out for determining dry matter (DM) yield. Three cuts were performed in the first growing season (2007-2008), and four in the second season (2008-2009). The aged of the plants in the first season were 5 (January 2 2008), 6 (February 20 2008) and 7 (March 31 2008) months, while for the second season the aged of the plants were 14 (October 16 2008), 16 (December 11 2008), 18 (February 9 2009) and 21 (May 5 2009) months.

Because red clover is a perennial, temperate climate species, the beginning of the second growing season cycle was considered as spring 2008. Cuts were made when the clover was found with 5 to 10% of flowering or when it reached an altitude of 40 to 50 cm. Aerial growth was mowed leaving a residue of approximately 5 cm from the soil. A central section of each (1.2 m²) with two quadrants of 0.6 m² each, was used by determining the agronomical parameters: a) plant population was determined as number of plants per m^2; b) the foliage cover was evaluated determining the percentage of empty spaces of at least 10 cm between plants found in 1.2 m^2; c) dry matter (DM) was evaluated from a subsample of 150 to 200 grams dried in an oven at 65ºC for 48 hours, for obtaining the yield in t DM ha^{-1}.

Statistical Analysis

The normality of the data was determined by Shapiro Wilk test. As the number of insects did not correspond to normal data, it was performed the non parametric Friedman test followed of Conover ($P \leq 0.05$) test. Accumulative number of larvae and adult of *H. obscurus* were used for statistical analysis. As agronomical parameters correspond to normal data, ANOVA ($P \leq 0.05$) analysis was carried out and the separation of the groups was determined by LSD ($P \leq 0.05$). Correlation between parameters was tested with Kendall´s rank and Spearman´s rank tests ($P \leq 0.05$).

RESULTS AND DISCUSSION

Total dry matter yield of red clover in two seasons is shown in Figure 1. The results showed that there were not significant differences among cultivars and experimental lines in the first season. However, the experimental lines Syn Int IV, Syn Pre III, Syn Int V and Syn Int VI were more productive than the cultivars Redqueli-INIA and Quiñequeli-INIA at the second season. Ortega *et al*. (2003) reported 17.40 and 15.00 t DM ha^{-1} for Redqueli-INIA and Quiñequeli-INIA respectively for the total yield of the first two seasons. In this study, both cultivars were roughly less productive, 15.25 and 14.00 t DM ha^{-1}, respectively. However, DM yield from the experimental line Syn Int VI (20.32 t DM ha^{-1}) at second season was comparable to the forage yield of Redqueli (21.10 t DM ha^{-1}) reported by Ortega *et al*. (2003) after three seasons.

There were not significant differences of forage yield among cultivars and experimental lines in the three cuts at the first season (Figure 2), and plants did not show infestation by *H. obscurus* at the first cut performed when plants were 5-month-old. However, the beginning of *H. obscurus* colonization started in all cultivars and experimental lines when plants were six months old. This differs from report of González (1989), indicating that this scolitid does not attack red clover during the first year of establishment. Our results indicated that the insects were found at the end of February, after the fly period and when the insect deposit the eggs in the base of the crown of red clover plants (Matamala, 1976; Aguilera, 1989).

Considering the presence of *H. obscurus*, there were not significant differences among cultivar and experimental lines at the second and third cuts, which could be due to percentage of damaged roots represented on average only the 26% of the roots sampled in each cultivar and experimental line evaluated. Nevertheless, a trend for bearing a higher amount of *H. obscurus* per plant was observed on the Redqueli-INIA cultivar at second and third cuts, with 1.78 and 1.18 insects per plant respectively.

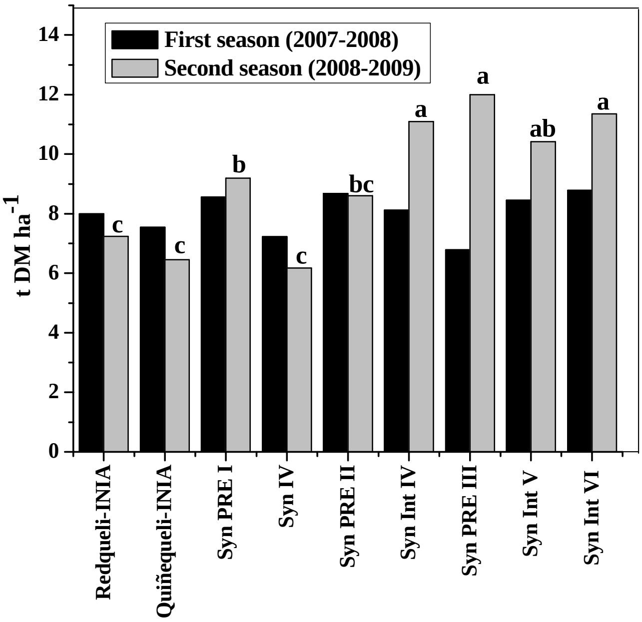

Figure 1. Forage yield (t DM ha^{-1}) of red clover cultivars and experimental lines during two seasons at INIA-Carillanca Experimental Station. Different letters indicates significant differences based on ANOVA analysis and LSD test ($P \leq 0.05$). Absence of letters indicates no significant differences.

During the second season, significant differences were observed on both forage yield and insect population (Figure 3). In the second (December 2008) and third (February 2009) cuts of the second season, Syn Int VI, Syn Int V and Syn Pre III were more productive than the cultivars Requeli-INIA and Quiñequeli-INIA. The low forage yield shown by all the cultivars and experimental lines at the fourth cut, 1.8 t DM ha^{-1}, can be explained because this evaluation was carried out during the autumn (May 2009).

The decrease in dry matter production is common during the winter according to data reported by Demanet (2008), indicating that Quiñequeli-INIA and Redqueli-INIA produce only between 9 to 11% of the total annual production of dry matter, and the maximum production is obtained during the summer. Significant differences in the population of *H. obscurus* between cultivars and experimental lines were only observed in the second season (Figure 3).

The negative association between *H. obscurus* population and DM yield is shown in Figure 4. The experimental lines Syn Pre III, Syn Int V and Syn Int VI showed significantly higher forage yields and the lowest *H. obscurus* populations. The same effect was observed when the insect population was related to plant density (Figure 5). A correlation analysis of both parameters showed that *H. obscurus* population is negatively associated to plant density (Kendal´s rank test, $P \leq 0.05$; Spearman´s rank test, $P \leq 0.05$).

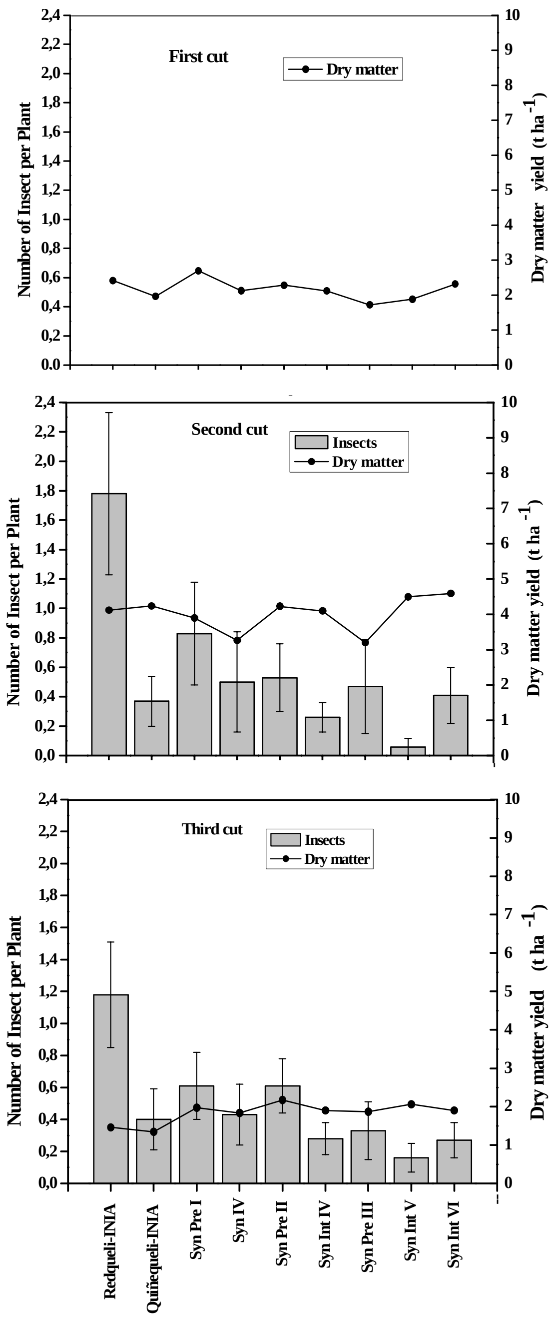

Figure 2. *H. obscurus* population and forage yield of different cultivars and experimental lines of red clover at the first season (2007-2008). Forage yield was analyzed by ANOVA ($P \leq 0.05$) and number of insect were analyzed with Friedman test followed by Conover-Inman test ($P \leq 0.05$). Absence of letters indicates no significant differences.

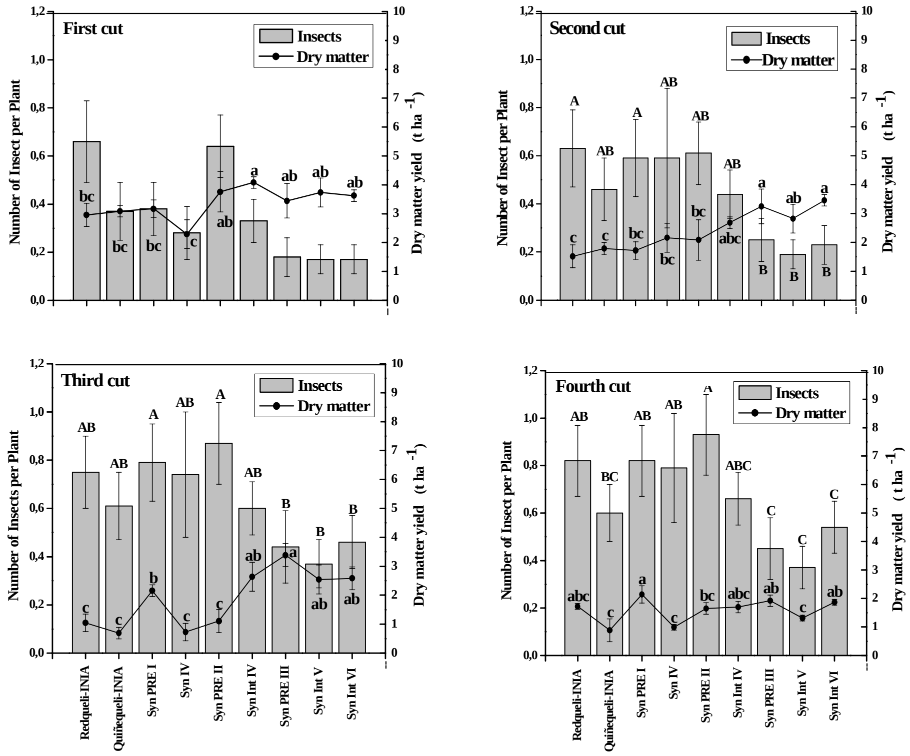

Figure 3. *H. obscurus* population and forage yield of different cultivars and experimental lines of red clover at the second season (2008-2009). Different letters indicate significant differences based on ANOVA ($P \leq 0.05$) for the forage yield. Bars with different letters are significantly based on the non-parametric statistic of Friedman test followed by Conover-Inman test ($P \leq 0.05$).

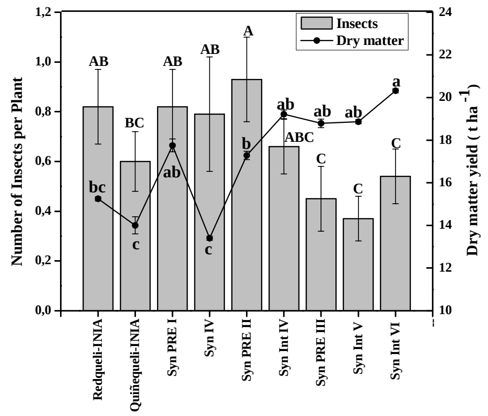

Figure 4. Total forage yield of red clover from two season (2007-2008 and 2008-2009) and number of individuals of *H. obscurus* per plant. Different letters in dry matter indicate significant differences based on ANOVA analysis and LSD test ($P \leq 0.05$). Bars with different letters are significantly based on the non-parametric statistic of Friedman test followed by Conover-Inman test ($P \leq 0.05$).

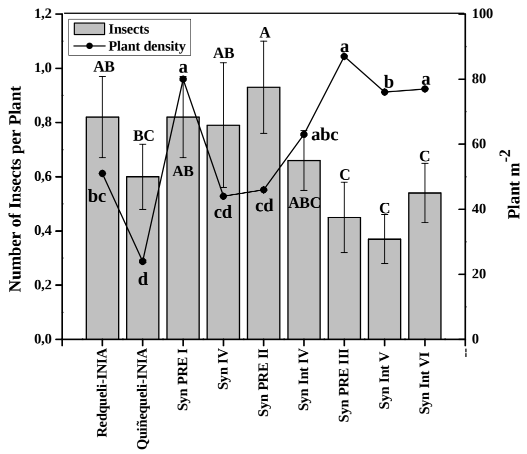

Figure 5. Plant density of cultivars and experimental lines of red clover determined after two seasons of evaluation and *H. obscurus* population. Lines with different letters indicate significant differences based on ANOVA analysis and LSD test ($P \leq 0.05$). Bars with different letters are significantly based on the non-parametric statistic of Friedman test followed by Conover-Inman test ($P \leq 0.05$).

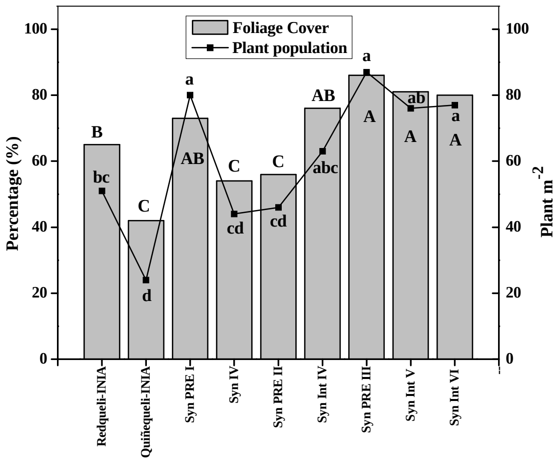

Figure 6. Foliage cover and plant population of cultivars and experimental lines of red clover determined after two seasons of evaluation. Different letters indicate significant differences based on ANOVA analysis and LSD test ($P \leq 0.05$).

Because of this association, these experimental lines could be considered with a higher partial resistance to the root borer attack. On the other hand, Syn Pre I showed forage yield similar to the synthetic lines mentioned above, but with a significantly higher load of insects (Figure 4). Moreover, Syn Pre I showed a similar plant density, foliage cover and plant population to resistant lines Syn Pre III, Syn Int V and Syn Int VI (Figures 5 and 6). These data suggest that Syn Pre I could be considered as tolerant to *H. obscurus*, according to the definition reported by Rosenthal and Agrawal (1994) and Strauss and Agrawal (1999),

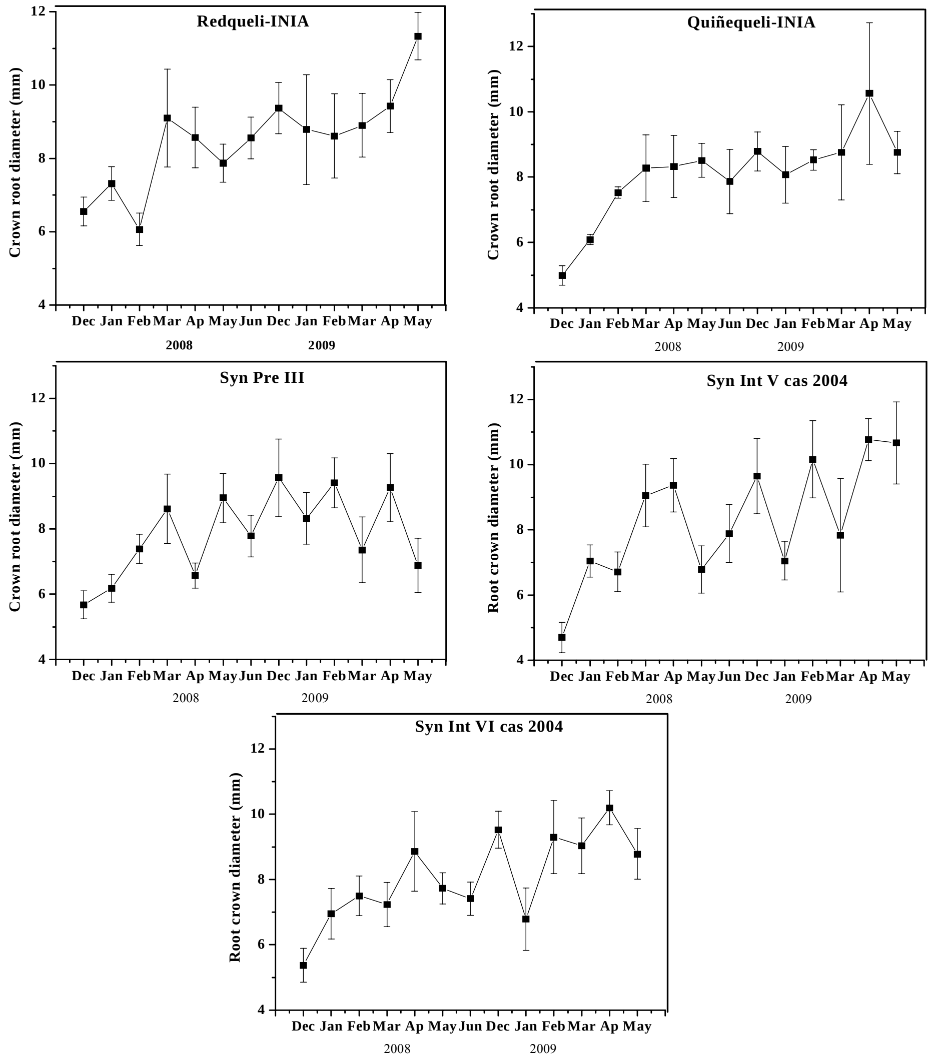

Figure 7. Root crown diameter of cultivars and experimental lines of red clover during two seasons (2007-2008 and 2008-2009). Bars indicate standard error.

and can be evaluated under both artificial and natural environments (Tiffin and Inouye, 2000).

The results shown in Figure 4 allow distinguishing two groups: a) one formed by the partially resistant lines Syn Pre III, Syn Int V and Syn Int VI, and b) Redqueli-INIA and Quiñequeli-INIA and the rest of experimental lines, including the tolerant line, Syn Pre I. The second group is significantly more attacked by *H. obscurus* than the first group. Redqueli-INIA and Quiñequeli-INIA showed an increase growing pattern of the root crown diameter, in comparison to the resistant lines (Figure 7).

This finding is in accordance to the existence of a positive correlation coefficient between the crown diameter of red clover plants and the number of individuals of *H. obscurus* founded inside the roots (Matamala, 1976).

CONCLUSIONS

Our study reports the first evidence that *H. obscurus* start the colonization of red clover plant of 6-month-old. Moreover, we report a complementary new biological tool for selecting new varieties of red clover, such as the evaluation of *H. obscurus* population. The results showed an inverse relation between forage yield and *H. obscurus* population.

The evaluation of the biological parameter allowed identifying a tolerant line, Syn Pre I, and three potential new varieties: Syn Pre III, Syn Int V and Syn Int VI. These experimental lines showed the lowest population of *H. obscurus* and the highest forage yield of red clover under the experimental conditions.

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REFERENCES

Aguilera, A. 1989. Ficha entomológica de la IX Región de La Araucanía, *Hylastinus obscurus* (Marsham) (Coleoptera: Scolytidae). IPA-Carillanca 8, 2.

Aguilera, A. 1995. Control selectivo de plagas en frutales de la zona sur. In: A. Aguilera, O. Andrade, J. Díaz, N. Espinoza, R. Galdames, H. Norambuena (eds). Seminario de Protección Vegetal. INIA Carillanca, Temuco, pp: 141-180.

Aguilera, A., Cisternas, E., Gerding, M., Norambuena, H. 1996. Plagas de las praderas. In: I. Ruiz (ed.). Praderas para Chile. Ministerio de Agricultura, Chile, pp: 309-339.

Avendaño, R. 1965. La variedad Quiñequeli y su evaluación con respecto a algunos tréboles rosados corrientes. Agric. Téc. 25,167-171

Becerra, L., Soto, P. 1982. Calendario de utilización de trébol rosado. Instituto de Investigaciones Agropecuarias, Investigación y Progreso Agrícola (IPA), Estación Experimental Quilamapu, Chillán, Chile.

Carrillo, R., Mundaca, N. 1974. Biología de *Hylastinus obscurus* (Marsham) Col., Scolytidae. Agric. Téc. 34, 28-35.

Catrileo, A., Rojas, C. 1987. Ganado y cultivos: posibilidades para enfrentar mejor las variaciones del mercado. IPA Carillanca. 6, 9-11.

Cuevas, E., Balocchi, O. 1983. Producción de forraje. Instituto de Producción Animal. Universidad Austral de Chile. Serie B7, Valdivia, 210 p.

Demanet, R. 2008. Manual de especies forrajeras y manejo pastoreo. Plan Desarrollo Lechero Watt´s. Imprenta Americana, Valdivia. 200 p.

González, R. H. 1989. Insectos y Ácaros de importancia Agrícola y Cuarentenaria en Chile. Editora Ograma S.A. 310 p.

Graham, J., Newton, R. 1959. Relationship between root feeding insects and incidence of crown and root rot in red clover. Plant Dis. Res. 43, 1114-1116.

Koehler, C. S, Gyrisco, G. G, Newsom, L. D., Schwardt, H. H. 1961. Biology and control of the clover root borer, *Hylastinus obscurus* (Marsham). Mem. Cornell Agriculture Experiment Station, New York. 36 p.

Leath, K. 1985. General diseases. In: N.L. Taylor (ed). Clover Science and Technology, American Society of Agronomy, Madison, WI, pp: 205-233.

Matamala, J. 1976. Biología, niveles de infestación, daño y combate químico de *Hylastinus obscurus* (Marsham). Tesis Agronomía, Universidad Austral de Chile, Chile, 51 p.

Quiroz, A., F. Ortega, C. Ramírez, L.J. Whadams, K. Pinilla. 2005. Response of the beetle *Hylastinus obscurus* Marsham (Coleoptera: Scolytidae) to red clover (*Trifolium pratense* L.) volatiles in a laboratory olfactometer. Environ. Entomol. 34, 690-695.

Ortega, F. 1996. Variation in mortality, yield and persistence of red clover (*Trifolium pratense* L.). Ph.D. Thesis, University of Wales, Aberystwyth, United Kingdom, 255 p.

Ortega, F., Galdames, F., Aguilera A., Romero, O., Ruiz, I., Soto, P., Torres, A. 2003. Redqueli-INIA, nuevo cultivar sintético de trébol rosado. Agric. Téc. 63, 207-211.

Ortega, F. 2009. Fitomejoramiento de trébol Rosado: Variedades para el mercado nacional y de exportación. Tierra Adentro 85, 21.

Rhodes, I., Ortega, F. 1997. Plant breeding achievements and prospects, Forage legumes. In: J.R. Weddell (ed.). Seeds of Progress. British Grassland Society, Symposium #31, Nottingham, pp: 15-27.

Riday, H. 2009. Correlations between visual biomass scores and forage yield in space planted red clover (*Trifolium pratense* L.) breeding nurseries. Euphytica 170, 339-345.

Rosenthal, J.P., Agrawal, P.M. 1994. Terrestrial plant tolerance to herbivory. Trends Ecol. Evol. 9, 145-148.

Steiner, J., Alderman, S. 2003. Red clover seed production: VI. Effect and Economics of soil pH Adjusted by lime application. Crop. Sci. 43, 624-630.

Strauss, S.Y., Agrawal, A.A. 1999. The ecology and evolution of plant tolerance to herbivory. Trends Ecol. Evol. 14, 179-185.

Tapia, S., Pardo, F., Perich, F., Quiroz, A. 2005. Clover root borer *Hylastinus obscurus* (Marsham) (Coleoptera: Scolytidae) has no preference for volatiles from root extract of disease infected red clover. Acta Agri. Scand. Sect. B Soil Plant Sci. 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.

Tiffin, P., Inouye, B.D. 2000. Measuring tolerance to herbivory: accuracy and precision of estimates made using natural versus imposed damage. Evolution 54, 1024-1029.

Torres, A., Sierra, C. 1991. Descripción, adaptación y establecimiento. In: Torres and Dumont (Ed.). Producción y Utilización de Trébol Rosado. Serie Remehue N° 13. Instituto de Investigaciones Agropecuarias, Estación Experimental Remehue, Osorno, Chile, pp: 13-25.