World Academy of Science, Engineering and Technology
International Journal of Agricultural and Biosystems Engineering
Vol:10, No:08, 2016

# Enzymatic Determination of Limonene in Red Clover Genotypes

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**Abstract :** Red clover (Trifolium pratense L.) is an important forage species in temperate regions of the world. The main limitation of this species worldwide is a lack of persistence related to the high mortality of plants due to a complex of biotic and abiotic factors, determining a life span of two or three seasons. Because of the importance of red clover in Chile, a red clover breeding program was started at INIA Carillanca Research Center in 1989, with the main objective of improving the survival of plants, forage yield, and persistence. The main selection criteria for selecting new varieties have been based on agronomical parameters and biotic factors. The main biotic factor associated with red clover mortality in Chile is Hylastinus obscurus (Coleoptera: Curculionidae). Both larval and adults feed on the roots, causing weakening and subsequent death of clover plants. Pesticides have not been successful for controlling infestations of this root borer. Therefore, alternative strategies for controlling this pest are a high priority for red clover producers. Currently, the role of semiochemical in the interaction between H. obscurus and red clover plants has been widely studied for our group. Specifically, from the red clover foliage has been identified limonene is eliciting repellency from the root borer. Limonene is generated in the plant from two independent biosynthetic pathways, the mevalonic acid, and deoxyxylulose pathway. Mevalonate pathway enzymes are localized in the cytosol, whereas the deoxyxylulose phosphate pathway enzymes are found in plastids. In summary, limonene can be determinated by enzymatic bioassay using GPP as substrate and by limonene synthase expression. Therefore, the main objective of this work was to study genetic variation of limonene in material provided by INIA´s Red Clover breeding program. Protein extraction was carried out homogenizing 250 mg of leave tissue and suspended in 6 mL of extraction buffer (PEG 1500, PVP-30, 20 mM MgCl2 and antioxidants) and stirred on ice for 20 min. After centrifugation, aliquots of 2.5 mL were desalted on PD-10 columns, resulting in a final volume of 3.5 mL. Protein determination was performed according to Bradford with BSA as a standard. Monoterpene synthase assays were performed with 50 µL of protein extracts transferred into gas-tight 2 mL crimp seal vials after addition of 4 µL $MgCl_2$ and 41 µL assay buffer. The assay was started by adding 5 µL of a GPP solution. The mixture was incubated for 30 min at 40 °C. Biosynthesized limonene was quantified in a GC equipped with a chiral column and using synthetic R and S-limonene standards. The enzymatic the production of R and S-limonene from different Superqueli-Carillanca genotypes is shown in this work. Preliminary results showed significant differences in limonene content among the genotypes analyzed. These results constitute an important base for selecting genotypes with a high content of this repellent monoterpene towards H. obscurus.

**Keywords :** head space, limonene enzymatic determination, red clover, Hylastinus obscurus
**Conference Title :** ICABBBE 2016 : International Conference on Agricultural, Biotechnology, Biological and Biosystems Engineering
**Conference Location :** Amsterdam, Netherlands
**Conference Dates :** August 04-05, 2016