EFFECT OF POTASSIUM NITRATE ON THE PRODUCTION OF RICININE BY *Ricinus communis* AND ITS INSECTICIDAL ACTIVITY AGAINST *Spodoptera frugiperda*

EFECTO DE NITRATO DE POTASIO EN LA PRODUCCIÓN DE RICININA POR *Ricinus communis* Y SU ACTIVIDAD INSECTICIDA CONTRA *Spodoptera frugiperda*

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SUMMARY

The effect of four nitrogen levels (KNO_3: 5, 10, 15 and 20 meq L^{-1}) on the production of ricinine was studied using a semi-hydroponic system. The insecticidal activity of methanolic extracts of *Ricinus communis* L. leaves against *Spodoptera frugiperda Smith* larvae was also tested. A dosage – response relationship showed strong positive correlation (R^2 = 0.92, P ≤ 0.05) between the nitrogen concentration in the hydroponic solution and ricinine percentage in leaves. A strong correlation (R^2 = 0.94, P ≤ 0.05) was also shown for nitrogen content in tissues and ricinine percentage. The use of nitrogen in the form of KNO_3 increased the production of ricinine, and it also affected mortality of *S. frugiperda* larvae. LC_{50} for ricine methanolic extracts of *R. communis* leaves on *S. frugiperda* were 13,469.12, 15,754.34, 16,046.11 and 18,155.75 mg mL^{-1} for nitrogen concentrations of 20, 15, 10 and 5 meq L^{-1} respectively. Increased nitrogen concentration in the hydroponic solution associated with increments in leaf area and ricinine concentration. This indicates that nitrogen concentration can be manipulated to improve production of this alkaloid, and the extracts used for crop protection.

Index words: *Ricinus communis, Spodoptera frugiperda,* hydroponic solution, nitrogen, ricinine.

RESUMEN

En este estudio se evaluó el efecto de cuatro niveles de nitrato de potasio (KNO_3: 5, 10, 15 y 20 meq L^{-1}) en la producción de ricinina mediante un sistema semi-hidropónico, y la actividad insecticida del extracto metanólico obtenido de hojas de *Ricinus communis* L contra larvas de *Spodoptera frugiperda* Smith. El extracto metanólico se usó para la obtención del alcaloide, y con él se evaluó su actividad biológica contra larvas de *S. frugiperda.* La relación dosis/respuesta demostró una alta correlación positiva (R^2 = 0.92, P ≤ 0.05) entre la concentración de nitrógeno en la solución hidropónica y el porcentaje de ricinina en las hojas. Lo mismo se observó (R^2 = 0.94, P ≤ 0.05) entre el contenido de nitrógeno foliar y el porcentaje de ricinina. El uso de KNO3 como fuente nitrogenada incrementó la producción de ricinina y también afectó la mortalidad larval de *S. frugiperda.* Las LC_{50} determinadas para *S. frugiperda* fueron de 13,469.12, 15,754.34, 16,046.11 y 18,155.75 mg mL^{-1} para los extractos metanólicos obtenidos de hojas de plantas crecidas en las concentraciones de nitrógeno de 20, 15, 10 and 5 meq L^{-1} respectivamente. El incremento en la concentración de ricinina al aumentar la concentración de nitrógeno en la solución hidropónica al indica que esta técnica puede utilizarse para mejorar la producción de este alcaloide y con ello elevar su capacidad de protección contra *S. frugiperda*.

Palabras clave: *Ricinus communis, Spodoptera frugiperda,* solución hidropónica, nitrógeno, ricinina.

INTRODUCTION

The composition of primary and secondary metabolites in plants depends on a combination of genetic factors and growth conditions. External factors such as nutrient levels, light, growth regulators and water stress can easily alter synthesis routes of secondary metabolites (Traw and Ackerly, 1995; Ramachandra and Ravishankar, 2002).

The effect of nutritional levels on the production of secondary metabolites has been previously studied, particularly nitrogen and its application, in producing cellular level of alkaloids (Abdolzadeh *et al.*, 2006; Hussain *et al.*, 2012; Ziba *et al.*, 2011). However, the effect of different hydroponic nitrogen levels on the induction to produce secondary metabolites in plant has not been reported (Gontier *et al.*, 2002).

Many secondary metabolites found in plants play a significant role in plant protection against insect pest and pathogens. These defensive roles act by influencing the behavior, growth, or survival of herbivores, and they may include feeding and oviposition deterrent effects, repellent and toxic effects. Several plant families have been reported to have insecticidal properties, such as Euphorbiaceae, Asteraceae, Lamiaceae, Fabaceae, Solanaceae and Meliaceae (García-Mateos *et al.*, 2004).

Castor bean (*Ricinus communis* L., Euphorbiaceae) has been investigated for its drying properties, medicinal and insecticidal effects (Rodríguez-Hernández, 2005). Ricinine (3-Cyano-4 methoxy-N-methyl-2-pyridone) is an alkaloid present in castor bean plants (Wang *et al.*, 2009), which

Recibido: 11 de Noviembre del 2014
Aceptado: 20 de Octubre del 2015

has insecticidal, acaricidal and nematicidal activities (Bigi *et al*., 2004; Bharadwaj and Sharma, 2007; Ramos-López *et al*., 2010; Bullangpoti *et al*., 2011), and whose production can be enhanced by nitrogen (Waller y Yang, 1965).

An important agricultural pest, the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae, is an insect species that displays a very wide host-plant range including 80 plant species across 23 families (Pashley, 1988). However, *S. frugiperda* has shown resistance to synthetic insecticides (Sayyed *et al*., 2008), then pointing out the need to develop new control methods, most particularly methods based on plant derived compounds with low impact on the environment and human health.

The aim of this study was to evaluate the effect of different nitrogen levels in a semi-hydroponic system on leaf growth, tissue nitrogen content and production of ricinine, and its subsequent role in crop protection. We tested the insecticidal effect of the methanolic extract of *R. communis* leaves containing different nitrogen contents against *Spodoptera frugiperda* larvae.

MATERIALS AND METHODS

This work was carried out at the Laboratory of Physiology and Technology of Crops and Center of Biological Research and Aquaculture Cuemanco (CIBAC) of Universidad Autónoma Metropolitana Unidad Xochimilco, and at the Insecticide Natural Compounds Laboratory of Universidad Autónoma de Querétaro, México, from January 2012 to June 2013.

Plant material

Seeds of *R. communis* were collected from plants located in the south of México City, (19º 18´ 54.6 N; 99º 05´ 59.9 W). The taxonomic authentication was performed by M. Sc. Patricia Zavaleta Beckler, and a voucher specimen was stored in the Herbarium of Universidad Autónoma Metropolitana Unidad Xochimilco (UAM-X 4626).

Plant growth

The seeds were germinated on peat moss 100 % as substrate, and the resulting seedlings were transplanted 25 d after emergency into polyethylene pots containing 15 L of substrate grounded red volcanic rock with particle size of 0.5 to 1.5 cm, previously disinfected with 1 % sodium hypochlorite solution. The plants were established under greenhouse conditions in semi-hydroponic production system containing four nitrogen concentrations (5, 10, 15 and 20 meq L^{-1}), as treatments. For this semi-hydroponic assay treatments were distributed in a completely randomized design with four replicates. The greenhouse conditions were monitored using a Hobo H21-001 climatologic station.

The nitrogen concentrations were formulated based on the nutrient solution according to Steiner (1961), and prepared with reagent grade salts, acidified with 1N H_2SO_4 to pH of 5.5. Nutrients provided included potassium nitrate, calcium sulfate, monopotassium phosphate, magnesium sulfate and potassium sulfate, and 1 L of the prepared solution contained iron sulfate (198 g), sulfate magnesium (81 g), zinc sulfate (44 g), copper sulfate (8 g) and borax (43 g). These solutions were applied using an automated drip irrigation system (2 L h^{-1}) which supplied 150 mL d^{-1} of solution per plant for 30 d, and thereafter 250 mL d^{-1} until harvest.

At 60 d after the transplant all the leaves were harvested and leaf area quantified with a leaf area meter (Li-Cor Li -3100; Lincoln, NE, USA), and the N concentration was quantified by the Micro-Kjeldahl method (Guebel *et al*., 1991).

The average temperature during the experiment was 21.2 ºC; the highest temperature was 49.0 ºC and the minimum temperature was 8.2 ºC. The relative average humidity was 74.1%; with maximum humidity observed at sunset (after 18:00 h) and sunrise (0600-0800 h), while the minimum humidity occurred close to 14:00 h when the maximum average solar radiation (830.6 Wm^{-2}) also occurred.

Methanol extract of ricinine from aerial parts of *R. communis*

Dried aerial parts of *R. communis* (300 g) were grounded to a powder, and 50 g were placed in an Erlenmeyer flask and soaked in 150 mL of methanol with intermittent shaking for 24 h at room temperature. The extract was filtered through a Buchner funnel with Whatman number 1 filter paper. The resulting solution was concentrated by reduced pressure in a rotary evaporator at 35 ºC and stored at 4 ºC in a refrigerator until use. The obtained extracts were analyzed by HPLC to quantified extract ricinine.

HPLC analysis

Samples of ricinine standards and methanolic extracts were analyzed in triplicate by High Performance Liquid Chromatography (HPLC) (Varian StarPro335), equipped with a C18 column in reverse phase (250 × 4.6 mm and 5 µm particle size) (Grace). The water and the methanol used were HPLC grade (Caledon) and reference compound ricinine (Latoxan, France) was used. The equipment was adjusted to a flow of 0.6 mL min^{-1}, UV absorbance detector

was set at 250 nm, ratio methanol: water was 75:25 %, and the running time per sample was 20 min.

The calibration curve was done by triplicate in HPLC, using a series of concentrations (2.0, 0.2, 0.02, 0.002 and 0.0002 mg mL^{-1}) of standard ricinine and the corresponding area under the curve. The ricinine content in each methanolic extract (*RME*) was calculated with the formulae:

$$RME = AUCME \times CSR / AUCSR$$

Where: *AUCME* = area under the curve from methanolic extract at 2 mg mL^{-1}; *CSR* = concentration of standard ricinine (2 mg mL^{-1}); *AUCSR* = area under the curve of standard ricinine at 2 mg mL^{-1}.

Bioassay

The methanolic extracts from leaves of *R. communis* were evaluated for their insecticidal activity on first instar larvae of *S. frugiperda* growing on the artificial diet proposed by Bergvinson and Kumar (1997).

A preliminary test was performed to evaluate five concentrations varying between 24×10^3 and 0.16×10^3 mg mL^{-1}, each added to the diet before culture medium solidification. Larval mortality was assessed 15 days after the insects were put into the treatments, according to a modified method used by Rodríguez-Hernández and Vendramim (1996). This experiment allowed to determine the minimum and maximum concentrations affecting larval mortality.

The subsequent testing concentrations (24×10^3, 16×10^3, 9.6×10^3, 4.0×10^3, and 1.6×10^3 mg mL^{-1}) looking for a strong correlation between concentration and mortality. One mL of each mixed treatment diet was poured into 28.3 g acrylic vessels (Bio-Serv No. 9051) with 24 replicates per treatment, prior to the medium solidification at room temperature for 24 h. Thereafter, a first instar larva of *S. frugiperda* was placed into each compartment and covered with a white cardboard adjustable lid (Bio-Serv No. 9049). The vessels were then randomly placed n a growth chamber set at 27± 2 ºC, 70 % ± 5 % RH, and 14/10 h light/dark cycles, according the methodology reported by Pérez-Gutiérrez *et al.* (2011).

Statistical data analysis

The collected data were tested for normality (Shapiro-Wilk W test) and homocedasticity (Bartlett test). The Kruskal-Wallis non-parametric analysis of variance was used when data violated the normality assumption and could not be corrected using a transformation. One way ANOVA analysis and Tukey test ($P < 0.05$) were also performed for mortality data at different concentrations of each extract. Linear regression analysis was used for quantifying the relationship between tissue nitrogen-concentration and N concentration in nutrient solution, and between tnitrogen concentration and ricinine content in leaf tissue. The LC_{50} (concentration causing 50 % mortality compared to the control) was calculated for each treatment by the probit analysis, based on the percent of mortality obtained at each concentration of the samples, using the Systat 8.0 statistical analysis program (SPSS Inc., 1998).

RESULTS

Nitrogen content in leaf tissue

The nitrogen content in leaf tissue showed a gradual increase with the increasing concentration of nitrogen in the hydroponic solution ($R^2 = 0.97$, $P \leq 0.05$), demonstrating a significant difference between treatments 5 ($F = 0.049$) and 10 meq L^{-1} ($F = 0.79$) and treatments 15 and 20 meq L^{-1} (Figure 1). There was also a significant increase in leaf area with increasing nitrogen accumulation in tissue, as demonstrated by the slope of the regression curve ($R^2 = 0.71$; $P \leq 0.05$) (Figure 2).

Correlation between nitrogen content in leaf tissue and ricinine production

The chromatograms of standard ricinine curve exhibited a linearity and high correlation of nitrogen ($R^2 = 0.992$; $P \leq 0.05$), since ricinine concentrations were 0.0894, 0.101, 0.103 and 0.1042 mg mL^{-1} for the 5, 10, 15 and 20 meq L^{-1} potassium nitrate solutions, respectively. The ricinine synthesized in the foliage of *R. communis* grown at 5 meq L^{-1} of N solution, was significantly less as compared with higher N concentrations.

The results also show a significant increase in leaf area in response to an increasing amount of nitrogen in the hydroponic solution. There is also a positive correlation ($R^2 = 0.94$; $P \leq 0.05$) between the content of ricinine in leaf tissue and the nitrogen content in the hydroponic solution (Figure 3). A positive correlation ($R^2 = 0.92$, $P \leq 0.05$) was also observed in the content of ricinine associated with increase in nitrogen leaf tissue (Figure 4).

Mortality assay

Mortalities differed among different concentrations (24×10^3, 16×10^3, 9.6×10^3, 4.0×10^3 and 1.6×10^3 mg mL^{-1}) of the methanolic extract of *R. communis* (Table 1) at varying nitrogen content. The lowest LC_{50} (13.469×10^3 mg mL^{-1}) was observed with the extract containing the

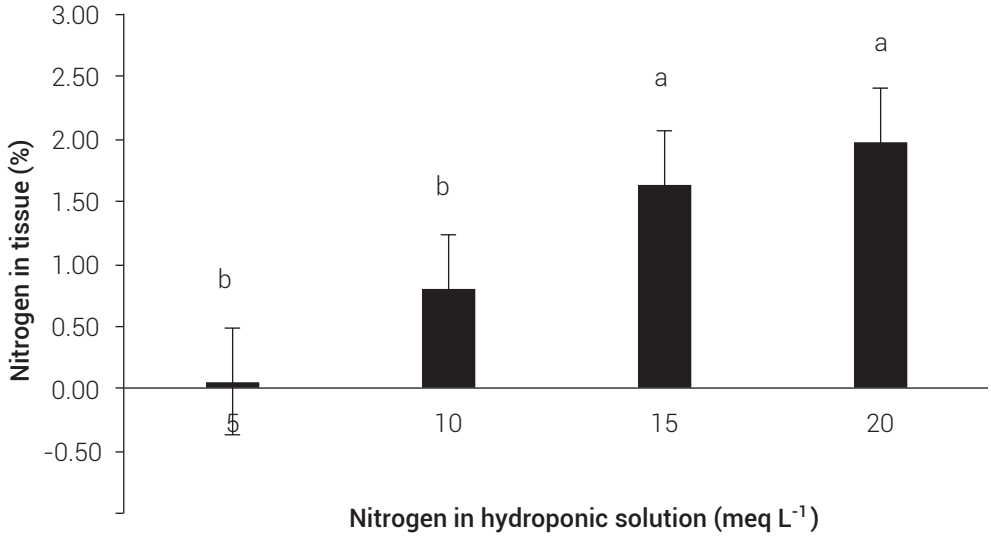

Figure 1. Mean values for nitrogen content in leaf tissue for plants growing at different nitrogen concentrations in hydroponic solution. Different letters indicate significantly different means (Tukey, 0.05; n = 4).

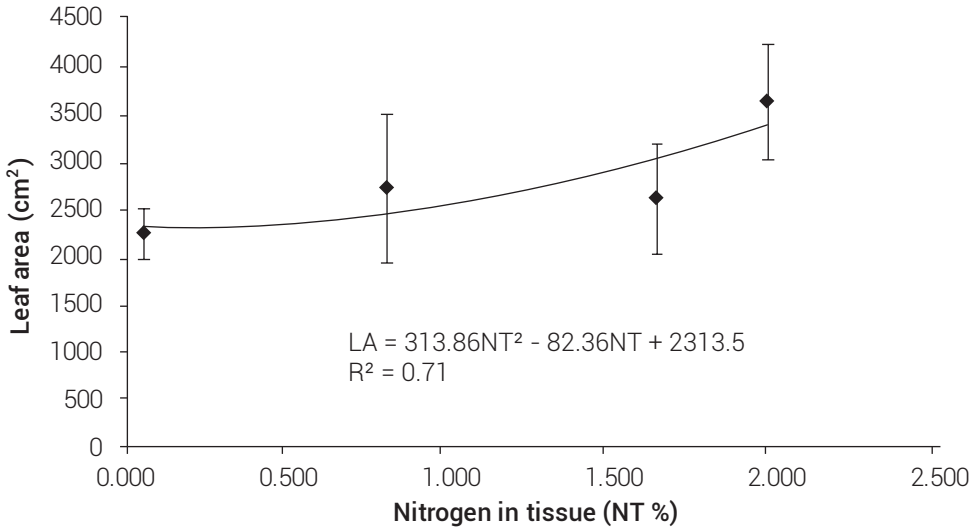

Figure 2. Relationship between leaf area (LA) and nitrogen content in leaf tissue (n = 4; P ≤ 0.05).

highest nitrogen content (20 meq L^{-1}), followed by the extracts containing 15 meq L^{-1} (LC_{50} = 15.754 × 10^3), 10 meq L^{-1} (LC_{50} = 16.046 × 10^3) and 5 meq L^{-1} (LC_{50} = 18.156 × 10^3 mg mL^{-1}) for nitrogen content.

DISCUSSION

The results of nitrogen content in leaf tissue showed in this study are consistent with those reported by Gontier *et al.* (2011) whom demonstrating gains in leaf area, specific leaf weight and chlorophyll content when increasing nitrogen applications. Cramer *et al.* (2000), who studied the response of tree canopy to nitrogen applications, also found a significant increase in leaf N concentration in response to higher doses of N in the nutrient solution, for most of the species studied.

It has been demonstrated that the secondary metabolites of hydroponic grown plants of *Datura* and *Taxus* can be 'milked' using hydroponic conditions and permeabilization (Gontier *et al.*, 2002). In plants of *Acmella oleraceae* L (Asteraceae) growing in hydroponics, Abeysinghe *et al.* (2014)

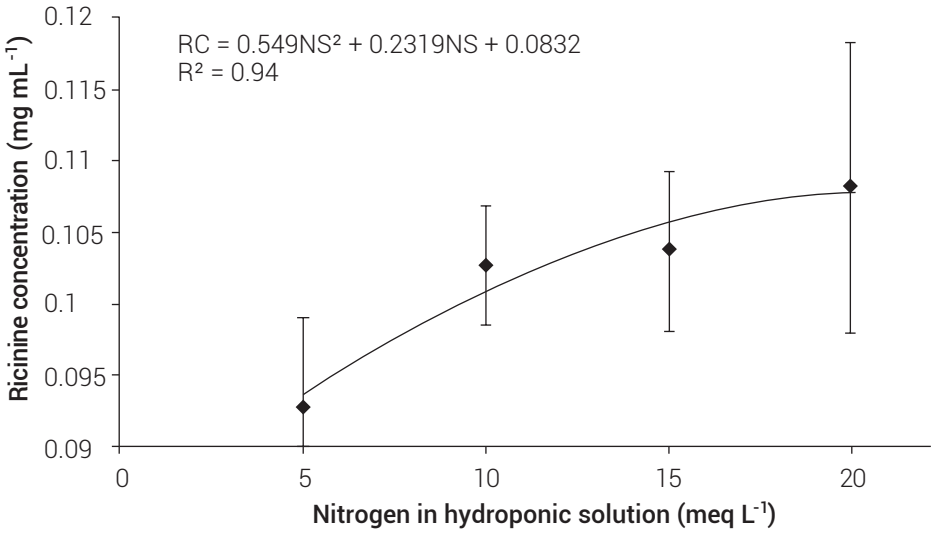

Figure 3. Relationship between ricinine concentration in the leaf and nitrogen content in the hydroponic solution (n = 4; P ≤ 0.05).

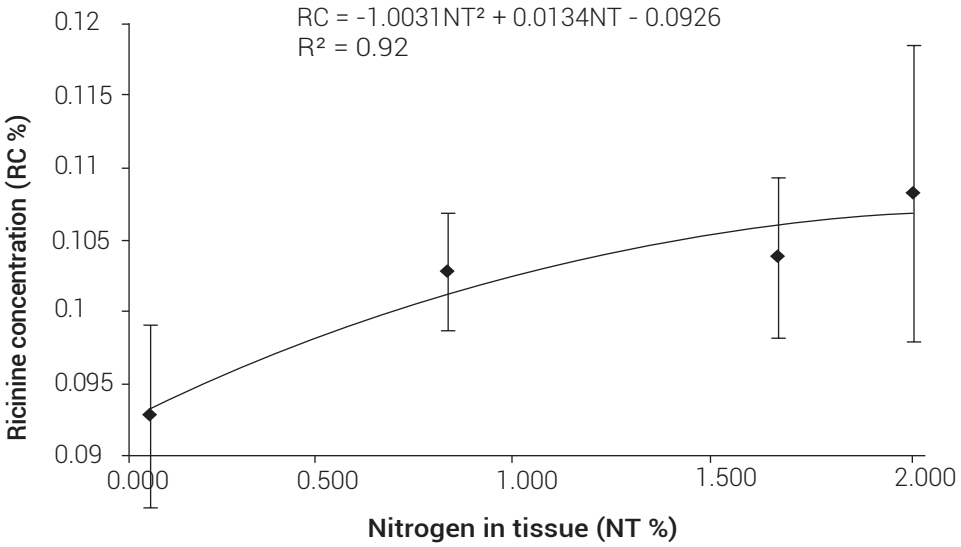

Figure 4. Relationship between ricinine concentration and nitrogen content, both in the leaf tissue (n = 4; P ≤ 0.05).

were able to increase the total phenolics and flavonoids contents by supplying more N to the nutrient solution.

Waller and Henderson (1961) and Waller and Yang (1965), who studied the biosynthetic pathway of ricinine from organic and inorganic compounds labeled with N^{15}, found that the relative efficiency of incorporation of labeled compounds followed a gradient: formamide glutamine aspartate > > > NH_4NO_3 nicotinamide (Amida-N) > > nicotinitrile KNO_3 (nitrile-N) 1-methyinicotinamida > (Amide-N) 1-methyilnicotinonitrilo > (nitrile N). These results indicate that the soluble nitrogenous fertilizer (KNO_3) of the nutrient solution is an efficient source of precursor in the production of ricinine. However, nitrogen supplied in the form NH_4NO_3 reaches higher incorporation efficiency than the KNO_3 used in this experiment; there could be also a higher induction of ricinine production.

On the other hand, it has been previously shown that the ammonium/nitrate-nitrogen ratio and overall levels of total nitrogen markedly affect the production of secondary plant products (Ramachandra and Ravishankar, 2002; Sae-Lee *et al.*, 2014). In our research noted that the ricinine content in leaf tended to increase as the level of nitrogen leaf concentration raised.

Table 1. Average (± se) larval mortality of *S. frugiperda* caused by methanolic leaf extracts of *R. communis* from plants growing at different nitrogen concentrations in the hydroponic solution.

N concentration (mg mL^{-1})	20 meq L^{-1}	15 meq L^{-1}	10 meq L^{-1}	5 meq L^{-1}
24,000	87.5 ± 2.9*	79.2 ± 2.9*	79.2 ± 2.9*	75 ± 3.1*
16,000	54.2 ± 4.0*	41.7 ± 4.6*	41.7 ± 4.6*	33.3 ± 3.7*
9600	37.5 ± 4.0*	37.5 ± 2.9*	33.3 ± 3.7*	25 ± 3.1*
4000	25 ± 4.6*	20.8 ± 2.9*	16.7 ± 3.7*	8.3 ± 3.1
1600	8.3 ± 3.1	4.2 ± 2.9	8.3 ± 3.1	4.2 ± 2.9
0	4.2 ± 2.9	4.2 ± 2.9	4.2 ± 2.9	4.2 ± 2.9
LC_{50}	13.46912 (11.12145-16.44106) × 10^3 mg mL^{-1}	15.75434 (13.08424-19.47266) × 10^3 mg mL^{-1}	16.04611 (13.33269-19.87345) × 10^3 mg mL^{-1}	18.15575 (15.36153-22.25480) × 10^3 mg mL^{-1}

* Significant differences respect to control (n = 24; P ≤ 0.05).

According to Ramos-López *et al.* (2010) the mortality of *S. frugiperda* larvae caused by methanolic leaf extracts from *R. communis* estimated by the Viability Larvae Concentration 50 (LC_{50}) was 5.07×10^3 mg mL^{-1} for the leaf extract and 0.38×10^3 mg mL^{-1} for the ricinine standard. With these results they demonstrated for the first time that ricinine is an active ingredient of *R. communis* that acts against *S. frugiperda*.

In our research the obtained VLC_{50} values were higher for the same extract but when the ricinine concentration was highest the larval mortality was highest too, corroborating that this alkaloid is the principal insecticide active ingredient of this plant. This alkaloid has also shown activity against other insects as *Atta sexdens rubropilosa Forel* (Hymenoptera: Formicidae) (Bigi *et al.*, 2004) and *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) (Bullangpoti *et al.*, 2011).

CONCLUSIONS

By increasing the N concentration in hydroponic solution significant gains in leaf area of *R. communis* can be obtained, which would be directly proportional to the amount of ricinine in the leaf methanol.

There is also a positive and significant correlation between the concentration of ricinine in leaf extracts of *R. communis* and their insecticidal activity against *S. frugiperda* larvae.

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