Evaluation of pyrethroids toxicity in a laboratory strain and a field population of *Rachiplusia nu* (Lepidoptera: Noctuidae) using two bioassay techniques

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Evaluación de la toxicidad de piretroides en una cepa de laboratorio y una población de campo de *Rachiplusia nu* (Lepidoptera: Noctuidae) usando dos métodos de bioensayo

■ **RESUMEN.** La soja es el cultivo más importante en la República Argentina y *Rachiplusia nu* (Gueneé) (Lepidoptera: Noctuidae), una de sus principales plagas. En este estudio se evaluó la toxicidad de cuatro cianopiretroides y un no-cianopiretroide, aplicados en forma tópica o por exposición a filmes sobre papeles de filtro, en ninfas del tercer estadio de una cepa de laboratorio y una población de campo de *R. nu.* Todos los cianopiretroides mostraron el mismo orden de toxicidad creciente, independientemente de la forma de aplicación y del origen de las larvas: cipermetrina < λ-cihalotrina < deltametrina < β-ciflutrina. Los valores de Dosis de Volteo para el 50% (DV50) y de Tiempo de Volteo para el 50% (TV50) aumentaron en función de la solubilidad de los cianopiretroides en agua. El no-cianopiretroide permetrina mostró un comportamiento diferente: fue el insecticida más tóxico para la cepa de laboratorio cuando se hizo una aplicación tópica, pero el menos tóxico cuando las larvas fueron expuestas a filmes sobre papeles de filtro. En general, todos los piretroides fueron más tóxicos para las larvas criadas en laboratorio que para las provenientes del campo. La estimación de los valores de Factor de Resistencia (FR) permitió establecer que la población de campo presentaba resistencia moderada o baja a permetrina, cipermetrina y λ-cihalotrina, cuando los insecticidas fueron aplicados en forma tópica. Sin embargo, no se detectó resistencia cuando se realizó la exposición en papeles de filtro tratados. No hubo correlación entre los valores de FR obtenidos por ambos métodos. Estos resultados sugieren que la población de campo de *R. nu* estudiada, presentaba resistencia moderada a algunos piretroides, y que la aplicación tópica es un método más apropiado que la exposición a filmes sobre papeles de filtro para cuantificar la resistencia.

PALABRAS CLAVE. Oruga medidora. Plagas de la soja. Resistencia a insecticidas.

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■ **ABSTRACT.** Soybean is the most important crop in Argentina and *Rachiplusia nu* (Gueneé) (Lepidoptera: Noctuidae) is one of its main pests. In this study, the toxicity of five pyrethroids applied topically and by exposure to films on filter paper was evaluated on third instar larvae from a laboratory strain and a field population of *R. nu.* Four cyanopyrethroids and one non-cyanopyrethroid (permethrin) were tested. All cyanopyrethroids showed the same order of increasing toxicity, regardless of the form of application and origin of the larvae: cypermethrin < λ-cyhalothrin < deltamethrin < β-cyfluthrin. Knock down Dose 50% and Knock down Time 50% values increased as a function of the solubility of cyanopyrethroids in water. Permethrin showed a different behavior: it was the most toxic insecticide for the laboratory strain when applied topically, but the least toxic when larvae were exposed to filter papers. In general, the pyrethroids were more toxic for laboratory larvae than for the field ones. After calculating Resistance Factor (RF) values, low-moderate resistance to permethrin, cypermethrin and λ-cyhalothrin was observed in the experiments with topical application. However, exposure to films on filter papers failed to detect resistance. There was not correlation between the RF values obtained by both methods. These results suggest that the population of *R. nu* studied here has low-moderate resistance to some pyrethroids, and that topical application is a more appropriate method for quantifying resistance than exposure to insecticide films on filter paper.

KEY WORDS. Soybean looper. Soybean pests. Insecticide resistance

INTRODUCTION

Soybean was a minor crop in Argentina until the 1990's, when intensive techniques associated to the cultivation of transgenic seeds resistant to glyphosate were adapted. The soybean production rose from 3.5 in the end of 1970's to 35 millon tons in the mid 2000's (Pengue, 2005). This important increase in production made Argentina the third largest soybean producer in the world.

The soybean looper *Rachiplusia nu* (Guenée) (Lepidoptera: Noctuidae) is one of the main defoliating Lepidoptera of soybean crops in South America (Sánchez & Pereira 1995). Its distribution area includes Bolivia, Chile, Uruguay, Argentina and southern Brazil (Pastrana, 2004). In Argentina, it occupies a vast territory that extends from the provinces of Chaco, Tucumán and Misiones to the provinces of Mendoza, Río Negro and south of the province of Buenos Aires. In Argentina, crop infestation begins in the middle of December and reaches its peak during January and February (Aragón *et al.*, 1998). It is particularly abundant in hot, dry summers, and chemical control is the main tool for controlling it.

Pyrethroids are a family of neurotoxic insecticides whose site of action is the voltage-dependant sodium channels (Sternersen, 2004). Their insecticide activity covers a wide range of species and they have been used for controlling all sorts of agricultural plagues since the mid-1970's (Perry *et al.*, 1998). The Cámara de Sanidad y Fertilizantes from Argentina (CASAFE) recommends twelve pyrethroids to control *R. nu* (CASAFE, 2009)

Cases of insecticide resistance have been reported for diverse species of the Noctuidae family: *Helicoverpa zea* (Boddie) (Abd-Elghafar *et al.*, 1993), *Heliothis virescens* (Fabricius) (Bagwell, 1992; Campanhola & Plapp, 1989a, b; Elzen *et al.*, 1992), *Plutella xylostella* (L.) (Shelton et al., 1993; Zhao and Grafius, 1993), and *Pseudoplusia includens* (Walker) (Felland *et al.*, 1990; Leonard *et al.*, 1990; Mink & Boethel, 1993; Plapp *et al.*, 1990; Thomas & Boethel, 1993). To our knowledge, there is only one scientific report indicating resistance in *R. nu* populations

(Araya *et al.*, 2003). The authors of this study found low levels of resistance to endosulfan and methamidophos in several populations from Chile.

The object of the present study was to compare the toxicity of five pyrethroids applied by two methods on a laboratory strain and a field population of *Rachiplusia nu* from the province of Santa Fe (Argentina). Topical application and exposure to insecticide films on filter papers were the methods used. The following pyrethroids were evaluated: permethrin, β-cyfluthrin, deltamethrin, λ-cyhalothrin and cypermethrin. Cypermethrin and λ-cyhalothrin had been used on the crop where the field population was sampled (J.C. Gamundi, personal communication). Permethrin, β-cyfluthrin and deltamethrin had not been used on that crop, although they are recommended for controlling *R. nu* and other soybean plagues and are regularly used in Argentina (CASAFE, 2009).

MATERIAL AND METHODS

Biological material

Following the Entomological Society of America recommendation for other Lepidoptera (Anonymous, 1970), all bioassays were carried out on *R. nu* third instar. Laboratory individuals were from a colony at Estación Experimental Oliveros (Instituto Nacional de Tecnología Agropecuaria, provincia de Santa Fe, Argentina). This colony has been reared for several generations without exposure to insecticides, at 28°C and under a 12:12 L:D photoperiod. Eggs were collected on paper napkins and placed in plastic trays with tops containing the artificial rearing medium described by Greene et al. (1976). Adults were fed on a mixture of: distilled water, 1 litre; sacarose 99.5% (Ledesma, Libertador General San Martín, Argentina), 60 g; honey (Cristina Brunel, Chapuy, Argentina), 10 g; ascorbic acid 99.0% (Laboratorio Cicarelli, San Lorenzo, Argentina), 1 g; and methyl-paraben 100% (Novalquim, Rosario, Argentina).

Adult individuals from the field population were collected from a soybean crop at the Oliveros Experimental Station (32° 33′ 48.82″ S, 60° 51′ 38.58″ W) using a light trap. They were transferred to the laboratory and reared as described above. Their progeny (F_1-F_3) were maintained on the artificial rearing diet until ready for bioassays.

Chemicals

The following insecticides were used (all technical grade): permethrin and cypermethrin (Chemotecnica, Carlos Spegazzini, Argentina); β-cyfluthrin (Bayer, Martínez, Argentina); deltamethrin (Roussel-Uclaf, Lyon, France); and λ-cyhalothrin (Syngenta, Buenos Aires, Argentina). Pro-analysis acetone (Merck, Buenos Aires, Argentina) was used as solvent. Water solubility values at 20°C were obtained from Kidd & James (1991).

Bioassays

Topical application

Concentrated solutions of 1 mg ml^{-1} were prepared in acetone for each insecticide, and then diluted serially to obtain different concentrations (ranging between 0.3 and 500 µg ml^{-1}) according to the results of preliminary assays. The solutions were applied using a Hamilton 50 μl microsyringe with repeating dispenser (Hamilton, Reno, NV). According to the Entomological Society of America recommendation, each larvae received 1 μl of solution (Anonymous, 1970). Five doses between 0.3 and 500 ng per insect were used, and each dose was applied to 10 larvae. A control group treated only with acetone was included in each assay.

Treated larvae were placed in plastic trays of 128 wells (C-D International, Pitman, NJ). Each well contained a cube of artificial diet of approximately 0.4 mg. To avoid cannibalism, only one larva was placed in each well, and all wells were covered with adhesive plastic tops with breathing holes. Trays were kept in a breading chamber under constant temperature throughout each experiment. Insecticide effect was registered 24 h after treatment. Larvae that remained still after prodding them gently with the end of soft

entomological tweezers were considered knocked down. Each assay was repeated independently three times.

Exposure to pyrethroid films on filter paper

Solutions of 1 mg ml^{-1} were prepared for each insecticide in acetone. Whatman # 1 filter paper circles (Whatman International, Maidstone, UK), 7 cm in diameter, were treated with the solutions. Each circle was treated with 0.5 ml solution (5 mg $(cm^2)^{-1}$). A glass ring (10 cm in diameter, 5 cm high) was placed on each filter paper and 20 larvae were positioned in the perimeter delimited by each ring. Each assay included a control group that was exposed to a circle of filter paper treated with acetone alone. Knock down was recorded every 20 minutes during 8 h using the same criterion described for topical application. Each assay was repeated independently three times.

Statistical analysis

Data from each set of three replicates obtained in the topical application bioassays were pooled for estimation of Knockdown Dose 50% (KD_{50}) values and their respective 95% Confidence Intervals (CI 95%) using the PoloPlus 2.0 programme (LeOra software, 2002). Data from each set of three replicates obtained in the exposure to films on filter papers bioassays were pooled for estimation of Knockdown Time 50% (KT_{50}) values and their respective CI 95% using the software for correlated data developed by Throne *et al.* (1995). Differences between values were considered significant ($P < 0.05$) if the respective CI 95% did not overlap.

Resistance Factor (RF) values were calculated as the quotient between the KD_{50} (or KT_{50}) of each insecticide evaluated on field individuals and the KD_{50} (or KT_{50}) of the same insecticide on the laboratory strain. RF CI 95% values were calculated as described by Robertson & Preisler (1992). Insecticide resistance level was classified by using RF values as following (Torres-Vila et al. 2002): susceptibility (RF = 1), low resistance (RF = 2-10), moderate resistance (RF = 11-30), high resistance (RF = 31-100) and very high resistance (RF > 100).

RESULTS

In the first experimental series, pyrethroids were applied topically on *R. nu* larvae and KD_{50} values were estimated (Table I). The pyrethroids presented the following increasing order of toxicity on the laboratory strain (the respective values of KD_{50} expressed in ng $insect^{-1}$ are indicated in brackets): cypermethrin (8.1) < λ-cyhalothrin (5.2) < deltamethrin (2.5) < β-cyfluthrin (1.9) < permethrin (1.5). A similar pattern of increasing toxicity was observed in the field population but in this case, the toxicity of permethrin was lower and came second in the sequence: cypermethrin (25.9) < permethrin (19.3) < λ-cyhalothrin (13.2) < deltamethrin (3.7) < β-cyfluthrin (2.6).

In the second series of experiments, larvae were exposed to insecticide films on filter paper and values of KT_{50} were estimated (Table II). The pyrethroids presented the following increasing order of toxicity on the laboratory strain (the respective values of KT_{50} expressed in min are indicated in brackets): permethrin (136.1) < cypermethrin (114.7) < λ-cyhalothrin (90.0) < deltamethrin (55.8) < β-cyfluthrin (21.8). The increasing order of toxicity for the field population was the same: permethrin (251.4) < cypermethrin (217.5) < λ-cyhalothrin (133.4) < deltamethrin (54.7) < β-cyfluthrin (29.6)

Regression analysis showed a statistically not significant relationship between the KD_{50} (or KT_{50}) values for the five pyrethroids tested and their solubility in water ($P > 0.05$). However, when KD_{50} (or KT_{50}) values for permethrin were excluded from the analysis, the KD_{50} (or KT_{50}) values for the remaining four cyanopyrethroids and their solubility in water fitted very well to a linear regression (Figs. 1-2). The following equations were obtained: for the laboratory strain, KD50: $y = 0.9 + 0.7 x$, ($R^2 = 0,973$) and KT50: $y = 24.9 + 9.6 x$ ($R^2 = 0,804$); for the field population, KD50: $y = -2.2 + 2.9 x$ ($R^2 = 0,993$), and KT50: $y = 4.0 + 22.1 x$, ($R^2 = 0,968$).

In the topical application bioassays, the KD50 values for permethrin, λ-cyhalothrin and cypermethrin where significantly higher in the laboratory strain than in the field

Table I. Values of Knockdown Dose 50% and Resistance Factor for five pyrethroids applied topically to a laboratory strain and a field population of *R. nu.*

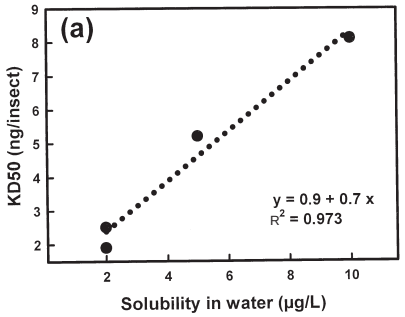
	Laboratory strain		Field population		
Insecticide	Slope (SE)	KD50, ng per insect[a] (95%CI)	Slope (SE)	KD50, ng per insect[a] (95% CI)	RF[b] (95% CI)
Permethrin	1.8 (0.3)	1.5aA[c] (0.9-2.1)	2.5 (0.3)	19.3a,bB (13.5-27.6)	13.3 (8.4-21.2)
β-Cyfluthrin	2.2 (0.4)	1.9aA (1.3-2.5)	2.5 (0.2)	2.6cA (1.0-4.8)	1.4 (0.7-2.9)
Deltamethrin	1.3 (0.3)	2.5a,bA (0.6-4.8)	1.3 (0.2)	3.7cA (2.1-5.8)	1.4 (0.8-2.7)
λ-Cyhalothrin	2.4 (0.4)	5.2bA (3.8-6.9)	1.8 (0.2)	13.2aB (9.9-17.3)	2.5* (1.7-3.8)
Cypermethrin	0.8 (0.2)	8.1bA (3.0-17.6)	1.5 (0.2)	25.9bB (17.9-37.7)	3.2* (1.4-7.6)

[a] KD50, Knockdown Dose 50%; 95% CI, 95% Confidence Interval. In these columns, values followed by different lower case letters are significantly different ($P < 0.05$).
[b] Resistance Factor = KD50 for the field population / KD50 for the laboratory strain. In this colum, means followed by an asterisk are significantly different from unity ($P < 0.05$).
[c] In each row, values of KD50 followed by different capital letters are significantly different ($P < 0.05$).

Table II. Values of Knockdown Time 50% and Resistance Factor for five pyrethroids applied to a laboratory strain and a field population of *R. nu* using insecticide films on filter papers.

	Laboratory strain		Field population		
Insecticide	Slope (SE)	KT50, ng per insect[a] (95% CI)	Slope (SE)	KT50, ng per insect[a] (95% CI)	RF[b] (95% CI)
Permethrin	3.4 (0.3)	136.1aA[c] (99.3-186.1)	2.6 (0.3)	251.4aA (151.4-443.7)	1.9* (1.4-2.4)
β-Cyfluthrin	3.4 (0.4)	21.8bA (18.2-25,4)	3.2 (0.4)	29.6bA (24.0-43.0)	1.3 (1.0-1.7)
Deltamethrin	2.8 (0.2)	55.8cA (47.3-65.1)	1.7 (0.2)	54.7b,cA (27.5-102.6)	1.0 (0.7-1.4)
λ-Cyhalothrin	3.4 (0.3)	90.0a,cA (64.9-124.3)	2.5 (0.2)	133.4a,cA (92.7-192.0)	1.5 (0.5-4.3)
Cypermethrin	4.6 (0,4)	114.7aA (103.5-167.8)	2.0 (0.2)	217.5aA (115.8-457.8)	1.9* (1.5-2.5)

[a] KT50, Knockdown Time 50%; 95% CI, 95% Confidence Interval. In these columns, values followed by different lower case letters are significantly different ($P < 0.05$).
[b] Resistance Factor = KT50 for the field population / KT50 for the laboratory strain. In this colum, means followed by an asterisk are significantly different from unity ($P < 0.05$).
[c] In each row, values of KT50 followed by the same capital letter are not significantly different ($P > 0.05$).



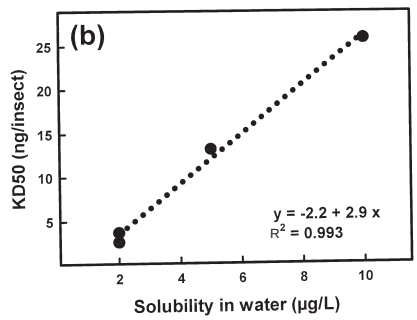
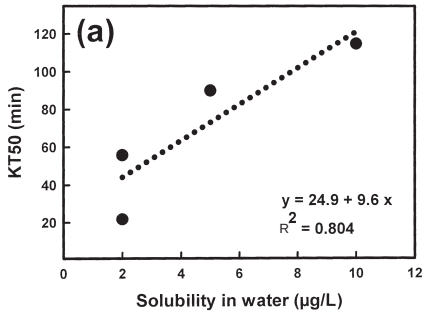


Fig. 1. Variation of Knockdown Dose 50% (KD50) values for cyanopyrethroids as a function of their solubility in water on (a) a laboratory strain, and (b) a field population of *R. nu*.



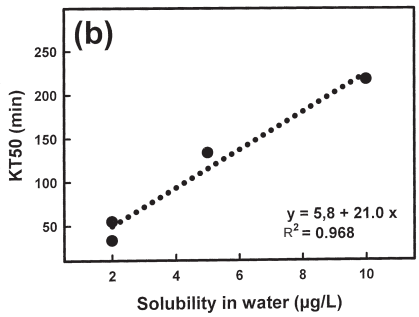


Fig. 2. Variation of Knockdown Time 50% (KT50) values for cyanopyrethroids as a function of their solubility in water on (a) a laboratory strain, and (b) a field population of *R. nu*.

population ($P < 0.05$). The latter showed susceptibility to β-cyfluthrin and deltamethrin (RF = 1.4 in both cases), low resistance to λ-cyhalothrin (RF = 2.5) and cypermethrin (RF = 3.2), and moderate resistance to permethrin (RF = 13.3).

In the insecticide films on filter paper bioassays, no significant differences were observed between KT_{50} values for the laboratory strain and the field population ($P > 0.05$). The values of RF varied between 1.0 (deltamethrin) and 1.9 (cypermethrin and permethrin), showing susceptibility in all cases. Regression analysis showed a statistically not significant relationship between the RF values for topical application and for exposure to films on filter paper ($P > 0.05$).

DISCUSSION

The toxicity of five pyrethroids on *R. nu* third instar larvae was studied using different methods of application. Pyrethroids are highly lipophilic substances with very low vapour pressure that dissolve in non-polar or low-polar solvents (Perry *et al.*, 1998). In the present work, acetone was used as solvent, because it has an intermediate polarity and is commonly used in this type of bioassays (Lietti *et al.*, 2005; Sfara *et al.*, 2006; Tarelli *et al.*, 2009).

In this work, two methods of application were used: topical application and exposure to insecticide films on filter papers. When a lipophilic insecticide is applied topically with an organic solvent as vehicle, the latter dissolves the cuticle and the insecticide comes into direct contact with the exocuticle that is relatively more polar (Olson & O'Brien, 1963). In the method with exposure to filter papers, once the solvent evaporates, the insecticide remains on the surface of the paper in the form of crystals or as an oily film (depending on its physico-chemical characteristics), and the insects come into contact with it when they move.

The main difference between both methods of application is the way the product enters the organism. By topical application, the solvent vehicle modifies the epicuticle and each insect instantly receives a known quantity of insecticide. When insects are exposed to a treated surface, the epicuticle is intact when the insects contact the insecticide, exposure is constant throughout the duration of the experiment, and the amount of insecticide that each insect receives is unknown.

Here, the toxicity of four cyanopyrethroids and one non-cyanopyrethroid was tested on larvae of *R. nu*. The four cyanopyrethroids had the same pattern of toxicity regardless of the form of application and origin of the larvae. Furthermore, the values of KD_{50} and KT_{50} for these cyanopyrethroids increased linearly as a function of their solubility in water (which varied between 2 and 10 $\mu g\ l^{-1}$). As the polarity of a molecule is directly proportional to its solubility in water, the values of KD_{50} and KT_{50} of the cyanopyrethroids increased in direct proportion to their polarity.

To explain these results it must be taken into account that the insect cuticle's external surface (epicuticle) is highly lipophilic; however, cuticle's lipophilicity decreases towards the inner part of the body (Hepburn, 1985). Therefore, the polarity of an insecticide greatly influences the rate it enters the organism when the cuticle is the route of entrance. Insecticides with very high or very low polarity have poor insecticidal activity, whereas the polarity of more active substances is nearer an optimal value (Briggs *et al.*, 1976). Within a certain range, as the polarity of an insecticide increases, the rate it enters an organism will be lower. This characteristic could explain the positive regression observed between the values of KD_{50} and KT_{50} for the four cyanopyrethroids and their solubility in water.

Permethrin showed a different behaviour to the rest of pyrethroids: it was the most toxic when applied topically but the least toxic when larvae were exposed to insecticide films on filter papers. Permethrin is a non-cyanopyrethroid with solubility in water of 200 $\mu g\ l^{-1}$, in other words one order of magnitude greater than the polarity of the four cyanopyrethroids studied in the present work. Due to this high polarity, the capacity of permethrin to cross the lipophilic cuticle is much lower, which could explain its reduced toxicity in the assays with exposure to films on filter papers. When applied topically, the acetone modifies the epicuticle facilitating the entrance of permethrin to the more polar exocuticle.

The values of KD_{50} and KL_{50} were higher for all pyrethroids when field larvae were used instead of laboratory reared larvae. The values of RF ranged between 1.4 and 13.3 in the topical application assays, and between 1.0 and 1.9 in assays using insecticide films on filter papers. When the insects were exposed to pyrethroids films on filter paper, the RF values for permethrin and cypermethrin were significantly different from unity. However, the 95% CI of KT_{50} for laboratory and field larvae overlapped for both insecticides. So the exposure to treated filter papers method failed to detect resistance to pyrethroids. These results are consistent with previous studies on other insects. Topical application allowed detecting crossed resistance to several pyrethroids in a DDT resistant population of *Blattella germanica* (L.); however, no resistance was observed when cockroaches were exposed to surfaces treated with the same insecticides (Scott *et al.*, 1990). In other study with ten populations of *B. germanica*, the values of RR for deltamethrin varied between 434.0 and 4,234.6 when applied topically, but only between 2.6 and 22.0 when using the method of exposure to a treated surface (Wi Choo *et al.*, 2000). Similar tendencies were observed on the human lice, *Pediculus humanus capitis* (De Geer); the lepidopteran *Plutella xylostella* (L.), and other populations of *B. germanica* (Scott *et al.*, 1990; Zhao & Grafius, 1993; Vassena *et al.*, 2003).

When an insect is placed on a treated surface, the amount of insecticide it enters into contact with depends on its movement: the more it moves, the greater the amount of insecticide it is exposed to. It has been suggested that insects exposed to a treated surface could incorporate so much insecticide

that the mortality rate among resistant individuals would increase and produce lower values of RF than those obtained by topical application (Zhai & Robinson, 1996). In the case of pyrethroids, it should be taken into account that these insecticides increase the locomotion in insects.

The first visible sign of intoxication in insects treated with pyrethroids is an increase in their locomotive activity (hyperactivity) (Gammon, 1978; Miller & Adams 1982; Benoit *et al*., 1985; Alzogaray *et al*., 1997). This could explain the results reported here, as hyperactivation could have increased the amount of insecticide of larvae exposed to films on filter papers, producing greater toxicity and hence reducing the values of RF compared to the method of topical application.

The results suggest the presence of low-moderate resistance to pyrethroids in the population of *R. nu* from Santa Fe. Judging by the lower values of RF, it is improbable that this condition produces control failures. Resistance to permethrin could be a crossed resistance phenomenon as the crops from which the insects were obtained were not exposed to any treatments with this pyrethroid. However, other explanations cannot be discarded such as the exposure to permethrin by applications carried out on neighbouring crops, or the migration of permethrin resistant insects from other cultures in which this pyrethroid is used.

Exposure to insecticide films on filter papers allows to obtain results quickly, but is more expensive than topical application because it requires greater quantity of solvent and insecticides. Furthermore, it is less precise because the exact amount of insecticide each insect receives is unknown. In this study, topical application was a more sensitive method for detecting pyrethroid resistance than exposure to 5 mg $(cm^2)^{-1}$ of pyrethroids. This shows that it is necessary to test different methods of application when assessing insecticide resistance in order to choose the most appropriate.

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