

1 **Title:**

2 Monitoring resistance of *Helicoverpa armigera* to different insecticides used in cotton
3 in Spain.

4

5 **Authors:** Carlos Avilla, José E. González-Zamora

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7 **Affiliations:** Department of Ciencias Agroforestales, University of Seville. Carretera
8 de Utrera, km 1. E-41012-Seville (Spain)

9

10 **Corresponding author:** José E. González-Zamora

11 zamora@us.es

12 Phone: +34-954 486 459

13 Fax: +34-954 46 436

14 **Published in: Crop Protection 29 (2010) 100–103**

15 **doi:10.1016/j.cropro.2009.09.007**

1 **Abstract**

2 *Helicoverpa armigera* is the key pest of cotton in Spain, resulting in many
3 insecticide treatments against it. The resistance status of *Helicoverpa armigera* to
4 different insecticides currently used in cotton was evaluated in Spain in two different
5 seasons, 1999 and 2004. Four populations were tested in total, two in each season.
6 Toxicological bioassays were conducted in the laboratory, and performed on third
7 instar larvae by topical application of the insecticides. LD₅₀'s were estimated by
8 probit analysis and resistance factors (RF) were calculated at the LD₅₀ level. Four
9 insecticides were evaluated, but only endosulfan reached a moderate resistance level
10 (RF=11.4), and the others (methomyl, chlorpyrifos and lambda-cyhalothrin) showed
11 low resistance (RF between 1.9 and 6.0). Such results indicate the generally low
12 resistance of *H. armigera* to most of the insecticides used against this pest in cotton
13 in Spain. Possible explanations for this situation are discussed.

14
15 **Key words:** insecticide resistance, cotton, *Helicoverpa armigera*, endosulfan,
16 methomyl, chlorpyrifos, lambda-cyhalothrin.

1 **1. Introduction**

2 Cotton is an important crop in the Guadalquivir river valley (Andalucía, southern
3 Spain), especially in the provinces of Seville, Córdoba and Cádiz. One of its main
4 economic costs comes from insecticide treatments against pests like *Helicoverpa*
5 *armigera* Hübner (Lepidoptera: Noctuidae), the key pest of cotton in southern Spain
6 (Durán, 1999). *H. armigera* can severely reduce cotton yields, mainly because it
7 feeds on flower buds, flowers and on the boll. Its control is based mainly on an IPM
8 schedule, which involves estimating populations of the pest and its natural enemies:
9 predatory bugs (Heteroptera) and lacewings (Neuroptera), and recommending
10 insecticides only when *H. armigera* populations surpass a threshold and natural
11 enemies are scarce (Durán, 1999; 2003).

12
13 The control of this pest in Spanish cotton may require several applications
14 through the season (the IPM cotton is sprayed with insecticides between 2 and 9
15 times, range obtained from 23 seasons, Durán (1999; 2003)), mainly of endosulfan
16 and methomyl (2-6 treatments), complemented with other products including
17 pyrethroids (e.g. lambda-cyhalothrin), organophosphates (e.g. chlorpyrifos) or other
18 carbamates (e.g. thiodicarb) (Durán, 2003). The continuous use of these products
19 can lead to the development of resistant populations of *H. armigera*, and in the 2003
20 season important control failures of *H. armigera* were observed in cotton fields. The
21 failure reports involved mainly the Seville province and the two products that were
22 most used against this caterpillar, endosulfan and methomyl. There are many reports
23 of the resistance of *H. armigera* (and other Heliothinae) to insecticides around the
24 world, especially to pyrethroids, but also to other groups of insecticides, like

1 cyclodienes (such as endosulfan), carbamates (such as methomyl and thiodicarb)
2 and organophosphates (such as clorpyrifos) (McCaffery, 1999; Bues et al., 2005;
3 Ahmad et al., 2007; Pietrantonio et al., 2007; Ugurlu and Gurkan, 2007; Saleem et
4 al., 2008).

5
6 Cotton is a good crop in which to study the presence of resistance to insecticides
7 due to the high number of treatments that may be applied (Deguine et al., 2008),
8 but until now no specific study has been done in this crop in Spain, even though,
9 with Greece and Bulgaria, it is one of the few countries in the European Union where
10 cotton is cultivated. On the other hand, our study complements others carried out in
11 Spain to analyze the status of resistance in *H. armigera* in different crops (Torres-Vila
12 et al., 2002a,b). This study covered two seasons: the 1999 season, in which the
13 baseline resistance to the insecticides that are most frequently used against *H.*
14 *armigera* in cotton was established with a laboratory strain, and compared with a
15 field population, and the 2004 season, in which the evolution of susceptibility to the
16 same insecticides over the period of 5 years, and control failures of *H. armigera* in
17 cotton reported in the previous 2003 season, were analysed.

18

19 **2. Material and methods**

20 The *H. armigera* populations used in this study were obtained from cotton fields
21 in Seville and Córdoba (Andalucía, southern Spain) from larvae collected in different
22 plots. In 1999, the laboratory strain was created from a number of pupae from a
23 colony that had been maintained in the University of Córdoba by Professor E. Vargas-
24 Osuna since 1996, with periodical reintroductions of individuals from cotton fields,

1 and with no contact with any kind of insecticide. The 1999 field strain was obtained
2 in June by collecting larvae from different cotton fields from the province of Seville.
3 In the 2004 season, two populations were collected, the first in June (prior to
4 application of insecticides against *H. armigera* in that season) from larvae from
5 different cotton fields in the province of Seville in which control failures of *H.*
6 *armigera* had been observed in the previous 2003 season, and the other in
7 September, from the same or nearby cotton plots.

8
9 Larvae were brought to the laboratory and reared following the methodology of
10 Poitout and Bues (1974), at 26 ± 1 °C and 55 ± 10 %RH with a 16:8(L:D) photoperiod.
11 Adults were reared in vertical cylindrical tubes of 11 cm in diameter and 26 cm in
12 height, constructed from laboratory drying paper and covered with paper. Three to
13 five pairs of adults were placed in each tube for mating and oviposition in the
14 conditions described above, and were fed with a 10% honey solution. Eggs were
15 collected in cotton bundles and placed in containers. After hatching, larvae were fed
16 with a semi-synthetic diet (Poitout and Bues, 1974) and reared in the conditions
17 described above. Third instar larvae were transferred to individual cages of 4 cm in
18 diameter and 2 cm in height, with a portion of diet, for insecticide bioassays.

19
20 Laboratory testing of the insecticides was carried out with the first or second
21 generation of larvae reared in the laboratory. Toxicological bioassays were performed
22 on third instar larvae using the standard topical application procedure as
23 recommended by the Entomological Society of America (Anonymous, 1970) and
24 following the guidelines of Robertson and Preisler (1992). Four products were used:

1 endosulfan (Thiodan-35[®], 350 g l⁻¹ a.i. from Bayer CropScience), methomyl (Bonsul[®],
2 200 g l⁻¹ a.i. from Bayer CropScience), chlorpyrifos (Cuspide-48[®], 480 g l⁻¹ a.i. from
3 Comercial Química Massó), and lambda-cyhalothrin (Karate King[®], 25 g l⁻¹ a.i. from
4 Syngenta). For each insecticide, serial dilutions in acetone [or 0.075% humectant
5 (Agridexa, from Bayer CropScience) in water for lambda-cyhalothrin] were prepared
6 so that each was one-half of the previous dose. A drop of solution (2 µl) was applied
7 with a 0.5–10 µl micropipette to the thoracic dorsum of each third instar larvae.
8 Controls for each replicate were treated with acetone (or water with Agridexa) alone.
9 The cages were closed with a lid ventilated by a hole covered with mesh, and left in
10 the same rearing conditions described above. For each strain, at least 240 larvae
11 were treated per insecticide, usually with 4 replicates of 10 larvae at each of 5 or
12 more insecticide concentrations. After 48 hours mortality was assessed: larvae were
13 considered dead if they were desiccated, or unable to move when disturbed with a
14 brush.

15
16 Dose-mortality regressions, LD₅₀'s and their fiducial limits were estimated by
17 probit analysis (Finney, 1971) using the POLO Plus 1.0 software (LeOra Software,
18 Berkeley, CA, USA). Resistance factors (RF) were estimated at the LD₅₀ level as RF=
19 LD₅₀ collected strain/ LD₅₀ reference strain (either the 1999 laboratory strain or the
20 strains collected in the field in 1999 or June 2004). The insecticide resistance level
21 was classified according to the criteria reported in Torres-Vila et al. (2002a,b):
22 susceptibility (RF=1), low resistance (RF=2–10), moderate resistance (RF=11–30),
23 high resistance (RF=31–100), and very high resistance (RF=>100).

24

1 **3. Results and Discussion**

2 The resistance in *Helicoverpa armigera* to different insecticides that are usually
3 applied in Spain on cotton was tested in two seasons (Table 1). In all cases, the t -
4 ratio of the slope was significant ($P < 0.05$), so that a significant dose-response line
5 was obtained.

6
7 Both inter-season and intra-season comparisons can be made with the data. In
8 the inter-season comparison, the results showed that the LD_{50} of all products tested
9 had increased at the end of the 2004 season (in September), when compared
10 against the LD_{50} of the 1999 laboratory strain, with the fiducial limits not
11 overlapping, except in chlorpyrifos. This increment was most obvious with
12 endosulfan, which reached the category of moderate resistance ($RF = 11.4$), whereas
13 the other products were placed in the category of low resistance (RF range between
14 1.9 and 6.0). Comparison of the data from September 2004 with the field population
15 of 1999 produced RF s that ranged from 1.1 to 3.1, i.e., from susceptibility to low
16 resistance, but only endosulfan had fiducial limits that did not overlap, indicating an
17 increment in its LD_{50} in this period.

18
19 The second kind of comparison is intra-season. The only comparison that is
20 meaningful is for the year 2004, when field populations were collected at the
21 beginning and at the end of the season. The September population showed
22 susceptibility/low resistance (RF between 0.6 and 1.7) when compared with the June
23 population, but no increment of the LD_{50} was detected, with the fiducial limits
24 overlapping for all the insecticides.

1

2 The results obtained in this work are similar to those obtained in a study by
3 Torres-Vila et al. (2002a,b) during 1995 to 1999, which included the same
4 insecticides tested here, but in different crops (excluding cotton). They found the
5 LD₅₀ of endosulfan to be between 0.93 and 6.70 µg/larva, which includes our result.
6 In terms of the RF, the value obtained in this study was higher (RF=11.4, included in
7 the moderate resistance category), but this is probably because it was compared
8 with a more susceptible laboratory strain. If the less susceptible strain of Torres-Vila
9 et al. (2002a) had been used instead, then the RF would be 3 (included in the low
10 resistance category).

11

12 The other insecticides tested in this study (i.e., methomyl, chlorpyrifos and
13 lambda-cyhalothrin) followed the same pattern as endosulfan: their LD₅₀ is included
14 in the range of values reported in Torres-Vila et al. (2002a,b), and with RF values
15 that are always within the susceptibility to low resistance category.

16

17 A clear increment of the LD₅₀ has been detected in most of the insecticides tested
18 between 1999 and 2004, compared with the laboratory strain. Only a small change
19 was observed in the LD₅₀ in the period from 1999 to 2004 in the field strains (only
20 significative in the case of endosulfan), and no significant increment was detected in
21 the 2004 season. Overall, our results showed little or no difference from the results
22 obtained in the general study carried out from 1995 to 1999 by Torres-Vila et al.
23 (2002a,b) in Spain, even though the study presented here included two seasons,
24 1999 and 2004, that were very separate in time. Such results indicate a general

1 scenario of low resistance of *H. armigera* to almost all the products tested in Spain,
2 except for some pyrethroids (cypermethrin, lambda-cyhalothrin and deltamethrin),
3 for which Torres-Vila et al. (2002b) found high or very high levels of resistance,
4 although only in 4 out of 111 insecticide–strain combinations tested, involving 2 out
5 of 35 *H. armigera* strains.

6
7 The level of resistance in *H. armigera* to endosulfan in our study can be
8 considered as low to moderate. This is similar to the situation in other cotton-
9 growing regions such as those in Turkey (Ugurlu and Gurkan, 2007) and India
10 (Kranthi et al., 2002; Suryawanshi et al., 2008). In terms of other insecticides, like
11 methomyl, several organophosphates, and especially pyrethroids (such as
12 cypermethrin, deltamethrin, lambda-cyhalothrin, and others), there are many studies
13 from different countries reporting high levels of resistance in this species (Kranthi et
14 al., 2002; Pietrantonio et al., 2007; Ugurlu and Gurkan, 2007; Suryawanshi et al.,
15 2008). Studies of insecticide resistance in *Helicoverpa/Heliothis* in Europe have not
16 been limited to Spain, and in France there is also evidence of significant levels of
17 resistance to pyrethroids (deltamethrin), and moderate resistance to methomyl in *H.*
18 *armigera*, but no resistance has been detected against endosulfan (Bues et al.,
19 2005).

20
21 The development of insecticide resistance is primarily the result of the selection
22 pressure exerted on sprayed populations, which increases the frequency of resistant
23 individuals. However, the cotton crop in Spain is sprayed with insecticides against *H.*
24 *armigera* from 2 to 6 times per season (Durán, 2003), which does not help to explain

1 the low level of resistance observed in this study. Other mechanisms (such as the
2 behaviour and physiology of target individuals) can modulate insecticide resistance,
3 and agroecological factors, like the presence of refugia that harbour susceptible or at
4 least less resistant individuals, may dilute resistant gene frequencies, allowing them
5 to remain at an acceptable level for successful control. Furthermore, there is a fitness
6 cost associated with resistance genes (Sayyed et al., 2008). All these factors (and
7 others) have been considered in an attempt to understand the low level of resistance
8 to insecticides in Spain (Torres-Vila et al., 2002a,b), but the same authors considered
9 the most important factor in the low level of resistance to be the gene flow that
10 happens during the seasonal migrations of *H. armigera*. The capacity for migration of
11 *H. armigera* is well known (Feng et al., 2005; Feng et al., 2009), and it may be of
12 great importance for IPM programmes, given its potential effects on insecticide
13 resistance dynamics. In Australia it is considered a key factor in the variation in
14 resistance frequencies from year to year (Forrester et al., 1993; Scott et al., 2005),
15 and in India, the migratory movements of resistant individuals is considered to be the
16 origin of the high pyrethroid resistance that is prevalent in the subcontinent (Armes
17 et al., 1996), as well as in Central Africa (Brevault et al., 2008). In the case of Spain,
18 Northern Africa is the origin of *H. armigera* immigrants, and these populations may
19 act as refugia that dilute insecticide resistance in northern countries and help to
20 explain the general low level of resistance found (Torres-Vila et al., 2002a,b). Equally,
21 the recently developed resistance in *H. armigera* to some pyrethroids in southern
22 France could be explained by the migration of resistant populations from Spain (Bues
23 et al., 2005).

24

1 The study presented here focused on the insecticides used in cotton. The general
2 conclusion is that there was no substantial increase in resistance to the insecticides
3 tested in the two years of the study (1999 and 2004), and only when both seasons
4 were compared was there an indication of an increase in resistance to endosulfan,
5 and a slight increase in resistance to methomyl and lambda-cyhalothrin. For that
6 reason, it is unlikely that the failure to control *H. armigera* that was observed in
7 cotton fields was due to resistance. Regarding endosulfan and methomyl, there is
8 evidence of a significant positive correlation between the LD₅₀ values of both
9 insecticides, which could indicate cross-resistance ($r_s=0.383$, $P<0.05$, Torres-Vila et
10 al., 2002a; $r=0.516$; $P\leq 0.1$, Ahmad et al., 2007). As methomyl and endosulfan are
11 the most frequently used insecticides against *H. armigera* in Spanish cotton, more
12 attention should be given to evaluating possible control failures in field conditions,
13 complemented by periodical evaluations of the susceptibility of *H. armigera* to both
14 (and other) insecticides.

15

16 **Acknowledgments**

17 We thank Antonio Jiménez, Bruno Ferreras and Saúl Herrera, for their help in the
18 experiments. This work was possible thanks to the financial support and interest
19 from the Federación Andaluza de Empresas Cooperativas Agrarias. We also thank the
20 Laboratorio de Sanidad Vegetal from the Consejería de Agricultura y Pesca of the
21 Junta de Andalucía (Montequinto, Seville), in particular José Manuel Durán, for their
22 help in contacting cotton growers and finding the plots from which the caterpillars
23 were collected.

24

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- 7

Final Version

1 Table 1. Toxicity of topically applied insecticides, currently used in cotton in
 2 Spain, to different strains of *Helicoverpa armigera* larvae (F1 and F2, third instar), in
 3 two years.

Insecticide	Year–Strain	Intercept	Slope±S.E.	LD ₅₀ (µg/larva)	95% fiducial limits	RF ^a
Endosulfan	1999–Laboratory	6.31	2.19±0.34	0.25	0.18–0.32	--
	1999–Field	5.05	1.81±0.26	0.93	0.72–1.28	3.7
	2004–June	4.54	2.11±0.27	1.64	1.30–2.05	6.6
	2004–September	4.10	1.97±0.33	2.86	1.44–5.03	11.4
Methomyl	1999–Laboratory	6.57	1.43±0.28	0.08	0.05–0.12	--
	1999–Field	6.14	1.92±0.27	0.26	0.19–0.33	3.3
	2004–June	5.46	1.01±0.16	0.35	0.22–0.50	4.4
	2004–September	5.47	1.48±0.22	0.48	0.19–0.90	6.0
Chlorpyrifos	1999–Laboratory	7.83	3.06±0.40	0.12	0.09–0.14	--
	1999–Field	7.16	3.21±0.46	0.21	0.17–0.25	1.8
	2004–June	5.88	2.21±0.28	0.40	0.32–0.50	3.3
	2004–September	6.41	2.22±0.20	0.23	0.11–0.41	1.9
Lambda-Cyhalothrin	1999–Laboratory	7.88	1.63±0.28	0.02	0.00–0.03	--
	1999–Field	7.12	1.40±0.25	0.03	0.02–0.04	1.5
	2004–June	6.87	1.64±0.19	0.07	0.06–0.09	3.5
	2004–September	7.08	1.85±0.27	0.08	0.05–0.12	4.0

4 In all strain–insecticide combinations the probit dose-response lines were significant (*t*-ratio
 5 test, *P*<0.05).

6 ^a Resistance factor (RF) estimated as RF= LD₅₀ specific strain/LD₅₀ 1999 laboratory strain.

7