Bulletin of Insectology ***58*** *(1): 57-64, 2005*
ISSN 1721-8861

Interfacial forces and permeation of the codling moth cocoon silk

Teodoro Stadler[1], Adriana Fornés[2], Micaela Buteler[3]
[1]*National ResearchCouncil (CONICET) UNC, Neuquen, Argentina*
[2]*Facltad de Ingenieria, Universidad Nac. Cuyo, Mendoza, Argentina*
[3]*Department of Entomology, Montana State University, Bozeman, MT, USA*

Abstract

The silk from *Cydia pomonella* (L.) (Lepidoptera Tortricidae) cocoon was considered as a key factor affecting the effectiveness of control practices on over-wintering codling moth populations. The interaction energy between the silk cocoon surface and different products -Diphenilamine, sprayoils (winter and summer) and vegetable oils (castor-, soy-, jojoba- and peanut oil) - was determined. Dynamic viscosity, surface tension, and silk permeation (L^2) of these products were also determined. To establish whether the silk cocoon may act as a barrier to chemical treatments, the effectiveness of these products when applied to fifth instar codling moth larvae in contact bioassays was compared to their toxicity when applied to cocooned fifth instar larvae.

The highest codling moth silk permeation activity was shown by summer sprayoils, followed by winter mineral sprayoils and vegetable oils. No strong correlation was found between measured silk permeation and oil viscosity (R = -0.06685) or oil surface tension (R = -0.51074). Hence, the behaviour of a substance on the silk surface cannot be predicted by analyzing individual physical characteristics. However, the calculated permeation (L^2) is shown as a useful tool to recognize silk wettability because measured permeation correlates with L^2 (R = 0.97354).

Some products presented a reduced effectiveness when they were applied to cocooned larvae, even though they caused high mortality to codling moth larvae when exposing them directly. Interestingly there was no difference in mortality between contact application to larvae and spray application to cocooned larvae for products with L^2 values between 13 and 22 cm.

These results suggest that the lack of efficacy of certain products is linked to the presence of the silk cocoon and that calculated L^2 values can provide valuable information to identify a product that penetrates actively the codling moth silk cocoon.

Key words: *Cydia pomonella*, overwintering larvae, cocoon silk, permeation, pest management.

Introduction

The codling moth (CM), *Cydia pomonella* (Lepidoptera Tortricidae) is the most serious pest of apples and pears in Patagonia - Argentina, in an approx. 60.000ha fruit producing area. This species is not indigenous to America, but was accidentally introduced to Canada and the USA in the 1800's (Higbee *et al.*, 2001) and in Argentina in the early 1930's. It first appeared in orchards in Patagonia, and by 1932, *C. pomonella* was officially declared a pest (Oscos and Gianotti, 1960).

Diapause is the mechanism that synchronises the life cycle of the codling moth with agroecological conditions and its occurrence and maintenance depend upon environmental and genetic factors. The ecological features of codling moth life cycle reflect adaptation to latitude and climate (Riedl, 1983). The codling moth has a facultative type of diapause but a gradual increase in obligatory diapause occurs from warmer to cooler climates. Obligatory diapause in cooler climates is advantageous to the species because the growing season is short and the food supply and weather tend to be more variable. On the contrary, facultative diapause is advantageous to the populations from mild climates, where the growing season is longer and the food supply and weather are more stable (Riedl and Croft, 1978). In Patagonia, codling moth has successfully adapted to the local mild agro-ecological conditions, producing three generations a year and a partial fourth generation (Cichón, 2004).

Because the codling moth is not native to Argentina, there are only a few unspecific enthomophagous species present in Patagonia, that could contribute to reduce the population densities of this pest, e.g. *Trichogramma minutum* Riley, *Chrysoperla externa* (Hagen) (= *Chr. lanata*) and *Eriopis connexa* (Germar) (Coscaron and Gianotti, 1960). Moreover, after having been devastated by pesticides during decades, these beneficial organisms are inefficient at regulating pest populations. Therefore, at present, the major codling moth control effort in this region relies on chemical insecticides (Correa, 2004).

C. pomonella overwinters as full grown larvae in silken cocoons in gaps of the bark of trunks and branches of host trees, in leaf litter, bark mulch and on wooden structures such as fruit bins, woodpiles and sheds. Fruit storage bins have long been recognised as codling moth overwintering sites and hence as a source of codling moth infestation and point of dispersal, when they are placed near the orchard (Proverbs and Newton, 1975). Higbee *et al.* (2001) showed that the emergence and flight activity of CM adults in blocks located close to wooden bin piles extended over a longer period than in orchards without bin piles. In recent years there has been an increasing concern about this codling moth infestation source. There is a perception that bins have become more heavily infested due to the reduced use of insecticides as a consequence of the use of mating disruption (Calkins, 2000 in Higbee *et al.*, 2001).

It is remarkable that exposure of fruit bins to antifungal postharvest treatment with diphenilamine (DPA), does not cause a significant impact on the within overwintering codling moth larvae, even though DPA is a broad spectrum insecticide with cholinesterase inhibitory action (Stadler, unpublished). On the other hand, soft insecticides like spray-oils can kill up to two-thirds of the larvae present in wood props when treated (Newcomer, 1936). We hypothesise that the cocoon silk is a limiting factor affecting penetration of control agents and therefore their efficacy. Hence, complementary to the development of new tools for the control of *C. pomonella* hibernating larvae, it is necessary to recognise the complexity of the interactions between chemicals and the larvae's cocoon silk.

Silk is a proteinaceous polymer that consists of heavy chain fibroin, light chain fibroin and sericins that envelope the fiber core and cement the fibroin fibers together for the cocoon construction (Sehnal and Zurovec, 2004). Early studies of various types of silk show that molecular diversity in silk is greater than in any other fibrous protein (Rudall and Kenchington, 1971). This is probably because functional requirements on silk have been reduced to the formation of a mechanical resistant assembly and its structure is not limited by extracellular or intracellular constraints (Lucas and Rudall, 1968). The structure of fibroin determines properties of silk fiber and the tendency of these hydrophobic surfaces to interact with other substances (Zurovec and Sehnal, 2002). Hydrophobic interactions are dependent on the protein amino acid sequence, the position of hydrogen bonds, the attraction between positively and negatively charge side groups, van der Waals forces, and polar and nonpolar associations appearing as adhesive or cohesive forces (Fedic *et al*., 2003).

A finite contact angle is formed when a drop of liquid is brought into contact with a solid surface (silk); and the final shape of the droplet depends on the relative magnitudes of the molecular forces within the liquid (cohesive) and between liquid and solid (adhesive) (van Oss *et al*., 1980). Hence, the contact angle is a measure of the competing forces of the liquid droplet and a solid, determining whether it spreads over the solid surface or rounds up to minimise its own area.

The objectives of the present work are to gain further insight on the interaction of codling moth silk with different substances, focusing on its differential wettability and its interaction with the wetting substance, determined by adhesion and cohesion forces. The study proposes that complementary to the development of new tools for the control of *C. pomonella* hibernating larvae, the interactions between the codling moth cocoon silk and chemicals or biological control agents have to be understood.

Materials and methods

Insect material

The insects used for the study were obtained from LPE-UNCo rearing facility, and were maintained on artificial diet and standard rearing conditions (Toba and Howell., 1991).

Pairs of 15cm x 5cm x 1.5cm wooden poplar strips taken from dismantled fruit bins were fixed together on one end, leaving a separation of 0.5 cm between them at the other end, to simulate the structure where codling moth characteristically overwinter. Six early fifth instar *C. pomonella* larvae were randomly selected from the rearing media and placed in each pair of strips. Finally, infested strips where placed in an incubator (28 ± 1 °C; 70 - 75% RH) for 72 hours in the dark to allow for cocoon spinning.

Silk material

Fifth instar *C. pomonella* larvae were randomly selected from the rearing media and kept in glass Petri dishes at 28 ± 1 °C; 70 - 75% RH for 72 hours in the dark to allow for cocoon spinning. Silk was gathered from glass surfaces and manipulated by using forceps previously cleaned and wiped with petroleum ether. The morphology of codling moth cocoon silk was examined by a Philips XL series 30 SEM microscope. Samples where pre-treated with Au/Pd.

Oils and Diphenilamine

Winter and summer spray-oils, vegetable oils, water and water emulsions of Diphenylamine (DPA) were used for permeation assays (table 1).

Table 1. Description of the products used for the permeation assays and toxicity tests. Dynamic viscosity was assessed with a Rotoviscosimeter and surface tension was assessed with a Krüss tensiometer. Diphenylamine : CAS Number: 122-39-4 ; Molecular Weight: 169.2300; Use Type: Fungicide, Insecticide, and Plant Growth Regulator; Toxicity 2: Carcinogen, Cholinesterase Inhibitor; Developmental or Reproductive Toxin, Endocrine Disruptor.

Product	Type	Origin	Viscosity mPa-s, 20ºC	Surf. tension dynes/cm, 20ºC
Soya oil	pure former extract	AGDeheza - Arg.	74.16	33.83
Peanut oil	pure former extract	AGDeheza - Arg.	92.12	32.66
Jojoba oil	pure former extract	EcoOil - Arg.	63.78	33.66
YPF	summer spray-oil	YPF- Arg.	20.38	30.50
Total DRV	summer spray-oil	Total Elf - Arg.	21.04	29.83
Total DRI	winter spray-oil	Total Elf - Arg.	60.23	30.02
PEX 9	high refined spray-oil	Total Elf - Canada	40.12	29.83
Castor oil	pharmacopeia	Ballester S.A.- Arg.	805.6	35.17
Diphenilamine	25000ppm in water	Wassington - Arg.	14.14	36.70
Water	distilled water	Lab. Distilled	0.897	72.75

Contact angle measurement

Contact angle measurement is the simplest and most accurate way for characterising the surface properties of solids and determining the interaction energy between a liquid and a solid at minimum equilibrium distance (Neumann and Good, 1979). The sessile drop technique was chosen to measure the contact angle of those substances in which $\theta > 0$, one minute after placing the drop on the silk surface. In this technique, a droplet of each liquid is placed on a solid surface and the angle (θ) is measured on the liquid phase. For substances that permeate silk actively ($\theta \approx 0$), one minute after placing the drop on the silk surface the method chosen was the capillary rise method.

Material conditioning

Under standard laboratory conditions many polymers absorb significant levels of moisture. Therefore, previous to contact angle measurement, the water absorption capacity of CM silk was assessed following the standard ISO 62 (1999) procedure, which is a suitable method to use for polymers.

Silk samples were dried in an oven maintained at 40 ± 1 °C for 72 hours. After that time, samples were hot-weighed and then allowed to cool to room temperature in a steady-state environment at 40 ºC and 75% RH, before being weighed again. Any change in weight between hot and cool samples due to water uptake was recorded (Anonymous, 1999).

Contact angle measurement by the sessile drop method

A simple experimental setup based on the projection of the droplet on a screen was used to measure the contact angle. A digital camera was used to record the images. The contact angles were then calculated from data of three consecutive images after sixty seconds of depositing the droplet on the silk surface. The contact angle "θ" was measured on the tangential line formed between the solid surface and the sessile drop profile where the drop intersects the surface (figure 1). Contact angle of Castor oil, 2500ppm DPA-water emulsion, and distilled water drops were measured by this method.

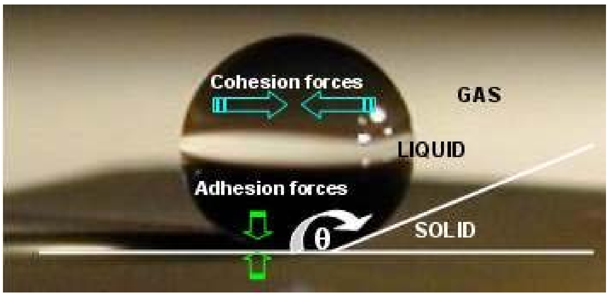

Figure 1. The figure shows the contact angle "θ" at the solid/liquid interface which is determined by the values of the work of adhesion of the liquid to the solid, and the work of cohesion of the liquid.

The concept of the equilibrium of the surface forces is expressed mathematically by Young's equation (in: van Oss, 1994):

γ_L = water surface tension
γ_S = solid surface tension
$\boldsymbol{\theta}$ = contact angle
γ_{SL} = liquid-solid interaction forces

$$\gamma_L \cos \theta = \gamma_S - \gamma_{SL}$$

Contact angle measurement by the capillary rise method

Contact angles of the mineral and vegetable oils were measured by the capillary rise method. The silk was packed into a capillary tubing with a known radius ($r = 0.1$ cm) and packed down by a load of 8 N with a Teclock GS 709G Type "A" durometer. Further, one end of the capillary tube was immersed into the liquid that rose through the capillaries formed between the silk fibers within the tube (figure 2). The distance drifted by the liquid (L) as a function of time ($t = 60$ sec) was measured. The contact angle (θ) was calculated using the *Washburn* equation (Adamson and Gast, 1997).

Toxicity assessment of DPA and oils to codling moth larvae

Two types of bioassays were conducted to determine the toxicity of the products to CM larvae. On one hand, contact toxicity bioassays "in vitro" were developed to determine the toxicity of the product applied directly on the larvae's body. On the other hand, trials were developed "in toto" using sentinel cocooned codling larvae between wooden strips. This was done to determine if the silk cocoon acts as a barrier to a chemical treatment.

"In vitro" contact toxicity bioassays

Seventy two hours after incubation of the infested wooden strips, sentinel codling moth larvae were carefully removed from their silk cocoon. Tests were performed with three replicates of thirty sentinel larvae for each product: 2500ppm DPA-water emulsion, pure vegetable oils (castor-, soy-, jojoba-, and peanut-oil), and mineral oils (DRV, DRI, Pex 9 and YPF). Larvae were treated as follows:

A- oils : applied topically utilizing a Hamilton repeating dispenser PB 600, supplied with a 50µl syringe. One micro liter was delivered on the dorsal body-surface of the codling moth larvae.
B- DPA "dip test" : CM larvae (LV) were dipped in the 2500ppm DPA-water emulsion during 30 seconds.

After the treatment, the larvae were placed on filter paper (Whatman No. 1) in Petri dishes and stored at constant temperature and humidity (28 ± 1 ºC; 70 - 75% RH). Controls were dipped in distilled water and handled in the same way as treated insects.

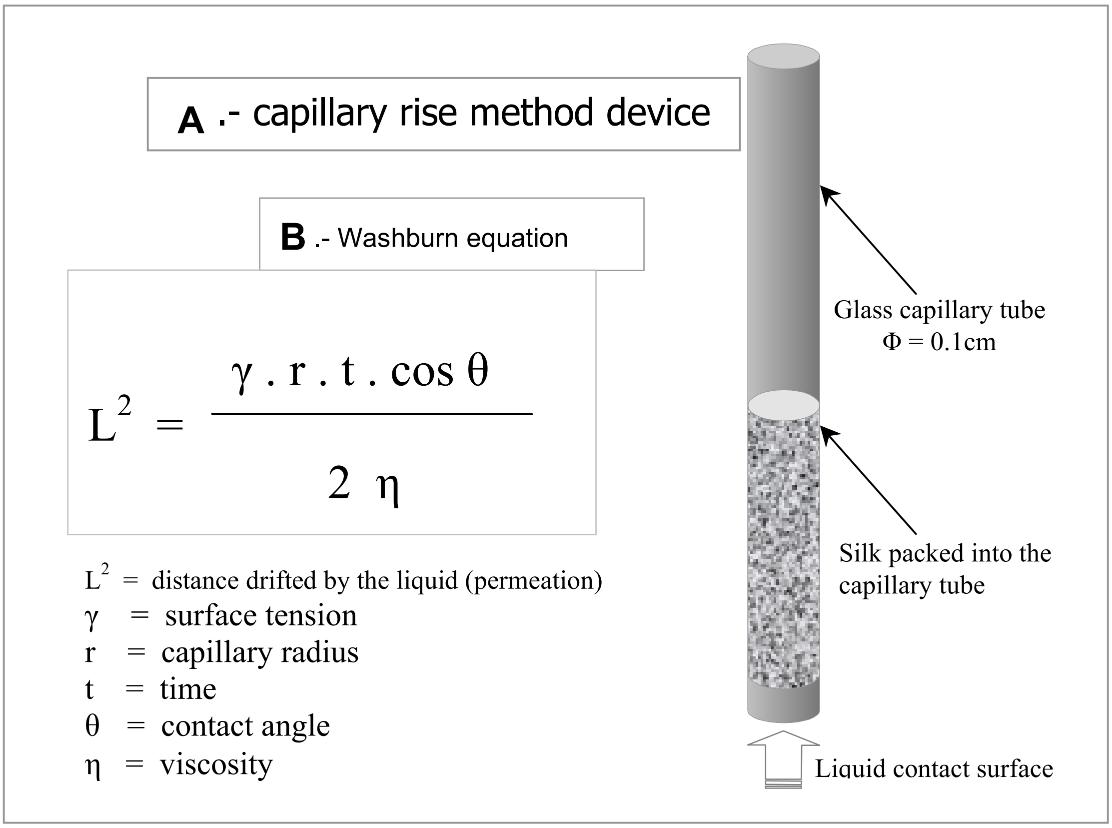

Figure 2. The silk is packed into a 0.1cm Φ glass capillary tube, one end of which is subsequently immersed into the product. The liquid rises through the capillaries formed in between the fibers within the tube. The distance "l" drifted by the liquid as a function of time "t" is measured. Knowing the mean radius "r" of the capillaries present in the tube, it is possible to calculate the contact angle using the Washburn equation.

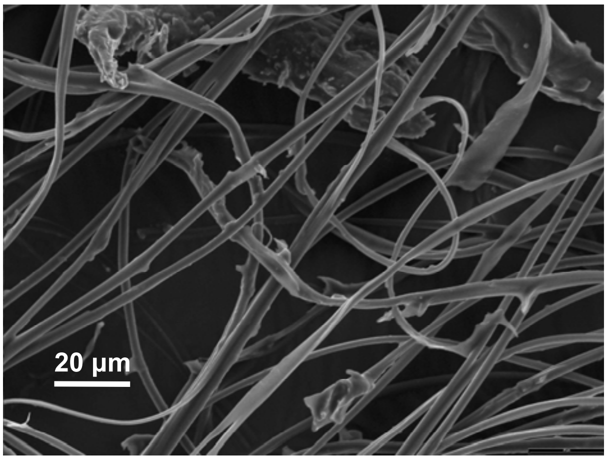

Figure 3. SEM micrograph of the codling moth cocoon silk. Magnification 756, scale 20 μm.

The endpoint of the toxicity test was mortality at adult hatching. Complementary observations of larval and pupal mortality were performed after the treatment in 48 hours intervals.

"In toto" contact toxicity trial

Seventy two hours after incubation of the infested wooden strips, five pairs of these strips were sprayed profusely (500 cm³/m²) with each product, five were sprayed with tap water, and five were left untreated (control). This was replicated three times for each treatment. The excess product was drained off and four days later each pair of boards was stored in a plastic bag and placed in the incubation room (28 ±1 °C; 70 - 75% RH). The endpoint of the trial was mortality at adult hatching. Further complementary observations of pupal and larval mortality were performed by splitting each pair of strips.

Results and discussion

Contact angle measurement

Codling moth silk characteristics

As shown in figure 3, *C. pomonella* cocoon silk fibers are smooth and a series of minor defects may be found in cocoon filaments such as loops, split-ends, nibs and hairiness. The diameter of fibers is approximately 3.2 to 3.5 μm.

Material conditioning

The exposure of pre-dried silk samples to a steady-state environment for 72 hours did not cause a moisture gain in the tested silk samples. Thus, moisture content in codling moth cocoon silk was not considered to be a source of variation in contact angle measurement.

Contact angle measurement by the sessile drop method and the capillary rise method

The Washburn equation was used to calculate permeation (L^2) of the products tested. The data used to calculate L^2 were: viscosity, surface tension, and cosine of the wetting angle for each product.

The contact angle (θ) of substances that do not permeate silk actively, like Castor oil, 2500ppm DPA-water emulsion and water ($\theta > 0$), was measured with the sessile drop method (SDM) (table 2). For substances that actively penetrate silk, wetting angle ($\theta \approx 0$) was assumed to be zero.

The permeation (L) of substances that actively penetrate silk was measured using the capillary rise method (CRM). Also, the Washburn equation was used to calculate permeation (L^2) of these products; assuming for calculation purposes, that in these cases wetting angle was zero ($\theta = 0$). In the case of 2500ppm DPA-water, and water, where silk permeation could not be measured, it was calculated using the Washburn equation. For this purpose, the contact angle ($\theta > 0$) was measured by the sessile drop method, and the $\cos \theta$ value was used to calculate (L^2) (table 2).

The highest permeation activity was shown by summer sprayoils. Winter sprayoils have a lower permeation activity when compared to summer sprayoils and this is probably due to the significant differences in viscosity between both groups: Total DRI and Pex 9 vs. Total DRV and YPF (table 2).

Vegetable oils are not as effective in wetting codling moth cocoon silk as mineral summer sprayoils. Despite the substantial differences in viscosity (η) among vegetable oils, there is no significant difference between them in their silk permeation capacity (table 2).

“In vitro” and “in toto” toxicity assessment of DPA-water emulsion and oils to *Cydia pomonella*

“In vitro” contact toxicity bioassays

Mineral oils can be ranked based on their contact toxicity to codling moth larvae, as follows : Total DRV > YPF > Total DRI > Pex 9. Vegetable oils can also be ranked as follows : Castor oil > Soybean oil = Jojoba oil = Peanut oil. Average mortality caused by mineral oils ranged from 53% to 93%. The 2500ppm DPA-water emulsion caused an average mortality of 77% to CM larvae in the dip test (table 3A).

“In toto” contact toxicity trial

Mineral oils can be ranked based on contact toxicity to codling moth cocooned larva as follows : Total DRV > YPF > Pex 9 > Total DRI. Vegetable oils can also be ranked as : Jojoba oil > Soybean oil = Peanut oil > Castor oil. Average mortality caused by mineral oils to cocooned larvae ranged from 33% to 86%. The 2500ppm DPA-water emulsion caused an average mortality of 10% to codling moth larvae in the dip test (table 3B).

A *t* test was conducted to compare the toxicity of the products when they were applied in vitro to when they were applied to the cocooned larvae. Significant differences in mortality were observed between the two types of toxicity tests ($P < 0.05$).

It was shown that mortality caused by 2500ppm DPA-water emulsions and castor oil to *C. pomonella* fifth instar larvae when directly exposed in the “in vitro” bioassays, (with no cocoon) is about 77% (table 3A). In contrast, spraying of codling moth larvae infested poplar boards with a 2500 ppm DPA-water emulsion and castor oil, cause only 10% mortality to the larvae in their cocoons (table 3B).

Table 2. Results from CM silk permeation assays; CRM = capillary rise method, L measured with the CRM when $\theta \approx 0$, one minute after placing the drop on the silk surface ; SDM = sessile drop method, θ^o measured with the SDM when $\theta > 0$, one minute after placing the drop on the silk surface ; $\sqrt{L^2}$ calculated by the Washburn equation using $\cos \theta = 1$ when $\theta \approx 0$ and $\cos \theta$ (SDM) when $\theta > 0$; * measured at 20 ºC; Physical properties in units as used for calculations.

Product	Physical properties		Silk permeation		
	Viscos. η	Surf. tension	Measured		Calculated
	dyn-s/cm² *	dynes/cm *	SDM (θ^o)	CRM (L cm)	$\sqrt{L^2}$ by Washburn
YPF	0.20	30.50	-	10.2	22.0
Total DRV	0.21	29.83	-	9.7	21.0
Pex 9	0.40	30.00	-	6.5	15.0
Jojoba oil	0.63	33.50	-	5.1	12.5
Soybean oil	0.74	33.83	-	4.5	12.0
Peanut oil	0.92	32.66	-	3.5	12.5
Total DRI	0.60	30.02	-	2.2	8.6
Castor Oil	8.05	35.17	89	0.0	0.5
DPA-water 2500ppm	0.14	36.70	109	0.0	-15.9
Water	0.10	72.75	113	0.0	-29.2

Table 3. Codling moth 5th instar larvae mortality in contact toxicity bioassays with a single dose of each product tested. A. - toxicity assessment of substances tested "in vitro" by topical application and dip-test on *C. pomonella* fifth instar larvae. B. - toxicity assessment of substances tested "in toto" by spraying on poplar boards infested with C. pomonella fifth instar larvae. STD = Standard deviation. * Mortality was assessed until adult emergence.

PRODUCT	A. "*in vitro*" bioassay					B. "*in toto*" bioassay						
		Number of larvae		Mortality			No. of	Number of larvae			Mortality	
	Test	per replicate	per product	% *	STD	Test	poplar strips	per p. Strip	per replicate	per product	% *	STD
YPF	Topical Application	30	90	80	5.7	Spray	15	6	30	90	73	3.3
Total DRV		30	90	93	3.3		15	6	30	90	86	6.6
Pex 9		30	90	63	3.3		15	6	30	90	63	3.3
Jojoba Oil		30	90	53	5.7		15	6	30	90	50	8.8
Soybean Oil		30	90	53	6.6		15	6	30	90	46	8.8
Peanut Oil		30	90	53	6.6		15	6	30	90	46	5.7
Total DRI		30	90	77	5.7		15	6	30	90	33	3.3
Castor Oil		30	90	77	3.3		15	6	30	90	10	7.7
DPA-water 2500ppm	Dip-test	30	90	77	3.3		15	6	30	90	10	5.1
Control 1 (water)		30	90	10	3.8		15	6	30	90	6	5.1
Control 2 (untreated)	-	30	90	6	3.3	-	15	6	30	90	6	3.3

The differences in mortality observed between the “in vitro” bioassays and “in toto” trials for three compounds with similar “in vitro” toxicity (Castor oil, Total DRI and DPA-water) (table 3A; figure 4), clearly suggest that the lack of efficacy of these products on the cocooned larvae is linked to the presence of the silk cocoon.

It was shown that silk is a selective barrier that protects the codling moth larvae. The capability of a substance to permeate and penetrate the codling moth silk depends on its physical characteristics. That could explain why chemicals of common use in fruit drenching, which are potential insecticides, do not harm the *C. pomonella* larvae when it’s protected inside the cocoon (because of silk hydrophobicity). On the other hand, lipophilic soft insecticides like sprayoils and vegetable oils permeate silk, reaching the codling moth larvae.

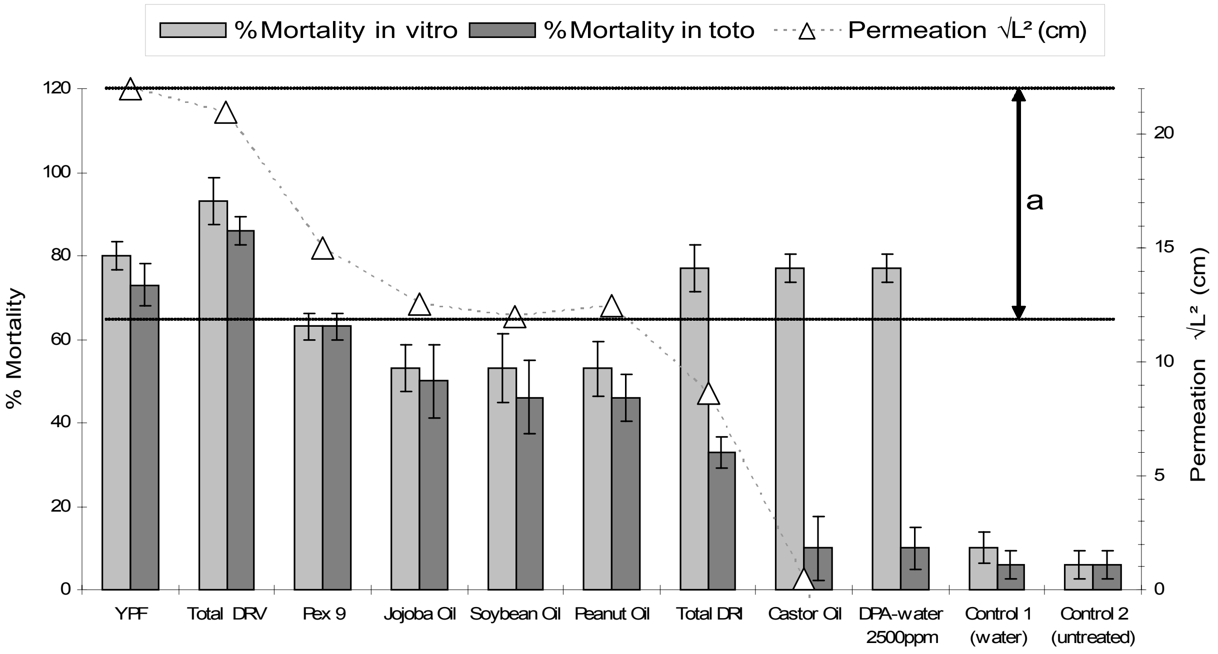

Figure 4. Percentage mortality of *C. pomonella* fifth instar larvae in the "in vitro" bioassays and "in toto" trials using pure mineral oils, vegetable oils and a 2500ppm DPA-water emulsion. Significant differences were observed between toxicity of the products in the in vitro versus the in vivo studies (t = 1.84, df = 14, P = 0.087). Figure also shows permeation measured (L) (Table 2 and 3). (a) Larvae mortality in "in toto" and "in vitro" assays match, for products with permeation values between 13 and 22 cm.

No strong correlation was found between measured silk permeation and viscosity (R = -0.06685) or between permeation and surface tension (R = -0.51074). Hence, the behaviour of a substance on silk surface cannot be predicted by analyzing any one of the physical characteristics studied.

Differential wettability displayed by silk to a wetting substance is determined by adhesion and cohesion forces as van der Waals and electrostatic forces. The calculated permeation ($\sqrt{L^2}$) is shown here as a fairly precise tool to recognize silk wettability because measured permeation (L) correlates with calculated permeation ($\sqrt{L^2}$) (R = 0.97354). For substances with $\theta \approx 0$, the Washburn equation can be used to calculate the permeation capacity.

Larval mortality results from "in toto" and "in vitro" assays are very similar for products with permeation values between 13 and 22 cm. These results suggest that L^2 can provide valuable information to identify a product that penetrates actively the codling moth silk cocoon.

The properties of the CM cocoon silk are a limiting key factor affecting the penetration of insecticides and probably biological control agents into the cocoon. Two properties of codling moth cocoon silk are predominant in determining resistance against wetting by liquid water: adhesion and cohesion energy (a consequence of molecular interactions between the silk surface and water) and fiber density in the cocoon. Thus, silk hydrophobicity prevents larvae from getting wet and keeps it isolated from entomophagous and entomoparasitic organisms, which can be transferred by water layers in a habitat where wet and dry conditions frequently alternate. This fact needs to be considered when developing tactics for the control of hibernating codling moth larvae in fruit harvest bins because the effectiveness of a given control tactic will depend on how well it can penetrate the protecting cocoon. There's little information in the literature about Tortricidae silk since most of the papers deal with silk fibroin from Bombycoidea, Papilionoidea and Pyraloidea (Fedic *et al.*, 2003; Zurovec and Sehnal, 2002; Sehnal and Zurovec, 2004). Therefore, without knowledge on the molecular structure of CM cocoon silk, it is not possible to hypothesise on its electrical charge and interaction energy with other substances. Detailed structural and mechanical studies on *C. pomonella* silk proteins should be performed in the future to enhance our understanding of the relationship between the silk proteins' sequence/structure and their mechanical properties.

Acknowledgements

We thank Lic. Fabián Tricárico for the SEM micrograph from *C. pomonella* cocoon silk. This research was supported by the Argentine National Research Council (CONICET) and developed at the Laboratory of Parasitology and Ecotoxicology (Faculty of Engineering, UNCo).

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Authors' addresses: Teodoro STADLER (corresponding author, e-mail: lpe@uncoma.edu.ar), Laboratorio de Parasitología y Ecotoxicología - Facultad de Ingeniería - Universidad Nacional del Comahue, Buenos Aires 1400 (Q8300BCX) Neuquen, Argentina; Adriana FORNES, Facultad de Ingeniería, Universidad Nac. Cuyo, CC 405 (5500) Mendoza, Argentina; Micaela BUTELER, Department of Entomology, Montana State University, 333 Leon Johnson Hall, Bozeman, MT-USA.

Received April 4, 2005. Accepted May 11, 2005.