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Advances in the apiary control of the honeybee American Foulbrood with Cinnamon (*Cinnamomum zeylanicum*) essential oil

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Abstract

The activity of *Cinnamomum zeylanicum* Nees essential oil (cinnamon oil) against *Paenibacillus larvae* (Withe) was evaluated in the laboratory and in a field experiment in order to improve the biological control of the *Apis mellifera* L. disease American foulbrood (AFB). The MICs (Minimal Inhibitory Concentration) against *P. larvae* were determined by the tube dilution method. Bee lethality was estimated using cages with approximately 374 bees, fed with the essential oil at different concentrations diluted in ethanolic syrup. The security index was calculated as LD50 of *A. mellifera*/MIC of *P. larvae* strain at 24, 48 and 72 h in both tests. The apiary trial, using three groups of five hives each, was carried out in April-May 2006 in an experimental apiary of J. J. Nágera coastal station (Mar del Plata, Argentine). The first group was treated with cinnamon oil (two doses of 1000 µg/ml per hive), the second with oxytracycline-HCl (three doses of 0.4 g per hive) and the third was left untreated as a control. All of the treatments were performed at 7-day intervals. The evaluation of treatment efficacy was made by counting the number of infected brood cells in both sides of a central comb, using a brood surface of 360 cm^2 (18 x 20 cm). For the Mar del Plata AFB strain the *C. zeylanicum* essential oil and antibiotic MICs were 50 µg/ml and 3.125 µg/ml, respectively. Regarding the systemic administration method, bees LD50 at 24 h was 456.07 µg of essential oil of cinnamon/bee. LD50 estimated at 48 h showed a slight decrease with respect to that recorded at 24 h. Security index was 9.1214 (ml/bee) for 24 h. In relation with field trials, after 24 and 31 days from the beginning of treatments the cinnamon oil-treated hives showed a lesser incidence of infected larvae (7.89% and 52.42%) than the control group highlighting a clear efficient control. No significant differences between the two treated groups were recorded. These results represent a further proof of the potential of cinnamon oil to control American Foulbrood with only minor toxicological risks to bees. Furthermore, the use of cinnamon oil avoids problems with antibiotic residues in honey. This is important in marketing honey in the EU where antibiotics in honey are generally forbidden by law.

Key words: Cinnamon oil, oxytetracycline, antimicrobial activity, American Foulbrood control, apiary trial.

Introduction

The American Foulbrood (AFB) is considered the most serious bacterial disease of honeybees (Shimanuki and Knox, 1997). It is caused by the spore-forming bacterium *Paenibacillus larvae* (Withe) (Genersch *et al*., 2006) that affects the larval stage of honeybees (*Apis mellifera* L.), with a rapid widespread and destructive effects on the colony. The spores are extremely infectious and resistant to physical and chemical agents (Haseman, 1961). A dead larva may contain billions of spores (Hansen and Brødsgaard, 1999). The use of antibiotics in hives is illegal in some EU countries because up to now there are no formulations that have obtained the necessary Ministerial registration for manufacture, transport, and sale. Moreover, there are no MRLs (Maximum Residue Limits) of tetracyclines and sulphonamides established for honey according the European Community regulations (Mutinelli, 2003). Only in exceptional cases, the veterinary authority can authorize in the prophylaxis of this disease the employment of antibiotic formulations registered for other veterinary use alone or in association with manipulative treatments, such as the "shaking" method, consisting in transferring bees from diseased colonies onto new combs into empty box. However, affected honeybees colonies often have to be destroyed and the equipment adequately decontaminated (Bailey and Ball, 1991). In other countries as in the USA, Canada and Argentina, preventive treatments with antibiotics are allowed, being considered a routine procedure to prevent outbreaks of AFB (Lindstrom, 2006). Consequently, various strains of *P. larvae* showing resistance to antibiotics, such as oxytetracycline-HCl (OTC), have been discovered in Argentina (Alippi, 2000) as well as in many United States areas (Miyagi *et al*., 2000). Moreover, the extensive use of antibiotics leads to an accumulation of residues in beehive products (especially in honey), decreasing their quality and making their marketing more difficult (Fuselli *et al*., 2005).

Because of legal and biological issues associated with antibiotic use in hives, we have been examining natural antimicrobial products for AFB management. Based on previous experiences (Gende *et al*., 2008b), we chose to study the antimicrobial activity of cinnamon oil against a specific Argentinean strain of *P. larvae* and the oils toxicity to *A. mellifera* using both laboratory and field tests.

Materials and methods

Essential oil

Cinnamon essential oil, was furnished by Cruciani Company (Rome, Italy) and derived from the bark of *Cinnamomum zeylanicum* Nees, the essential oil/product was the same previously employed and analysed by Floris *et al*. (1996).

Antimicrobial assay

A bacterial strain of *P. larvae* was isolated from honeybee brood combs of colonies with clinical symptoms of AFB; these were collected in hives located in Mar del Plata (38°0'S-57°33'W), Buenos Aires province. The Argentinean strain of *P. larvae* was previously identified in laboratory by biochemical assays (Alippi, 1992) and tests based on PCR and restriction fragment analysis of the 16S rRNA genes (rDNA), using the primers PL 5 (5'-CGAGCGGACCTTGTGTTTCC-3') and PL 4 (5'-TCAGTTATAGGCCAGAAAGC-3), which amplify a fragment of the *P. larvae* 16S rRNA gene (Piccini *et al*., 2002; D'Alessandro *et al*., 2006). The pure strain was maintained on MYPGP agar with 15% v/v glycerol until used. Vegetative cells of *P. larvae* were grown on MYPGP agar for 48 h at 35 ± 0.5 °C, and then suspended in double distilled autoclaved water. To measure antimicrobial activity with serial dilution method, microbial biomass concentration was adjusted to 0.5 of MacFarland scale (Scott, 2006). The minimal inhibitory concentration (MIC) of cinnamon essential oils and antibiotic (oxytetracycline-HCl) separately, was directly assessed by turbidity observation. Stock solutions of each antimicrobial agent in sterile water were made; essential oil of cinnamon was emulsified with 8% v/v propylene glycol (1-2 propanediol). One milliliter of each stock solution was added to MYT broth (Gende *et al*., 2008a) and serially diluted (final range 12.5-2000 µg/ml from essential oil and 0.000315-100 µg/ml for antibiotic). Microbial biomass suspension was then added to each serial dilution tube with agitation, at room temperature, using a Vortex dispersing tool (Fbr® by Decalab Srl). All sample tubes (as well as positive and negative controls) were incubated at 35 ± 0.5 °C for 48 h in order to determine MIC values under microaerobic conditions (5-10% of CO_2). All MIC tests were performed by triplicate for microbial agent and strain.

Bees lethality test

To evaluate the oil effect on *A. mellifera*, an average of 374 ± 45 adult worker bees taken from the nest were placed in each cage (16 x 12 x 6 cm) with nylon mesh on the walls for air circulation. After being starved for 4 hours, to allow bees to feed during the experiment, a cylindrical feeder (8 x 3.5 cm) were placed inside each cage (Maggi *et al*., 2009) with 10 ml of sugar-water syrup (2:1) in 70% ethanolic solution containing 2000, 4000, 8000, 16000 µg/ml of cinnamon oil concentrations, respectively. Bees in cages fed with sugar syrup in 70% ethanolic solution without oil and other treated with dimethoate were also included as controls. Each treatment was replicated five times. After 24 h, the cages received only 2:1 syrup, depending on demand of bees. All cages were maintained at room temperature (22 °C and 65% RH) and a synthetic queen pheromone was also introduced in them. Bee mortality was recorded at 24, 48 and 72 h. Once the experiment finished, the bees were killed by immersing the cages in a 70% alcoholic solution container. The final number of bees was recorded to calculate the proportion of dead bees attributable to each time (24, 48 and 72 h), and statistical analysis was made by linear regression method.

The *Security Index* was determined as LD50 of *A. mellifera/* MIC of *P. larvae*, modifying the expression given by Lindberg *et al*. (2000).

Apiary trial

The field trial was carried out from April to May 2006 in the Arthropods Laboratory at the Universidad Nacional de Mar del Plata (Argentine) and in an experimental apiary of J. J. Nágera coastal station placed on route 11 Km 32 (38°10'06"S, 57°38'10"W).

Twenty days before treatments, 15 nuclei of homogeneously strong-bees were prepared; each colony consists of 5-6 combs of bees. Capped and open broods as well as food reserves were included in same proportion. The colonies from which these combs were extracted had not been treated with antibiotics during the 24 months prior the experiment. The young queens were marked to be easily recognized. Hives were divided into three groups of five. All the hives were artificially infected with the same number of scales of pre-pupae or pupae affected by AFB. Inoculation was made with brood comb sections (5 x 5 cm) collected from colonies showing AFB clinical symptoms. Each infected comb section had 45 ± 5 scales and they were introduced into nuclei in central position of a nest central comb. After 3 days, the piece of incorporated brood comb was modified in its entirety by the bees and converted with new wax, giving up the comb of experimentation with normal and similar characteristics to the remaining breeding ones. The Argentinean strain of *P. larvae* used in the artificial inoculation was the same that had been analyzed previously.

The first group of hives (O) was treated with oxytetracycline-HCl and received three weekly doses, each of 0.4 g antibiotic mixed with an equal quantity of powdered sugar. The second group (Ci) received two weekly treatments of 250 ml syrup (solution 2:1 of sugar/water) added with cinnamon oil at a concentration of 1000 µg/ml. In this case, it was also necessary to use an emulsifier of 70% ethanol in the syrup. The third group (Co) was left as a control and received only ethanolic syrup (solution 2:1 of sugar/water with ethanol).

The evaluation of AFB disease incidence following the treatments was made by counting the number of infected brood cells in both sides of a central comb portion of 360 cm^2 (18 x 20 cm). For each hive, the number of cells with infected larvae and/or scales and the number of cells with healthy larvae were weekly counted until the 31st day after the experimental infection.

Statistical analysis

Experimental data were analyzed by analysis of variance (ANOVA) after arcsine $\sqrt{y/100}$) transformation, in the case of percentages, to reduce the heterogeneity of

Table 1. Medium lethal dose (LD50) (µg of cinnamon essential oil/bee) at different exposure-time intervals.

Hours	Regression equation	R^2	LD50 (µg essential oil/bee)
24	$y = 0.0031\,x - 2.82144$	0.5419	456.07
48	$y = 0.00316\,x - 1.01723$	0.5327	432.13
72	$y = 0.00316\,x - 0.44988$	0.5319	427.32

Table 2. Security index values (SI), ratio between medium lethal concentration (µg of cinnamon essential oil/bee) and minimal inhibitory concentration (MIC, µg/ ml) of essential oil against *P. larvae*.

Hours	LD50 (µg essential oil/bee)	MIC (µg/ml)	SI (ml/bee)
24	456.07	50	9.1214
48	432.13	50	8.6426
72	427.32	50	8.5464

Table 3. Mean values (percentage ± s.d.) of infected larvae at different days from experimental infection.

Days	Control	Essential oil	Antibiotic	F-value
17	0.59 ±0.30 a	0.14 ± 0.13 b	0.23 ± 0.31 b	4.54 (P = 0.0341)
24	13.08 ± 5.23 a	5.19 ± 2.95 b	4.75 ± 3.41 b	6.82 (P = 0.0105)
31	83.91 ± 13.39 a	31.49 ± 5.84 b	19.06 ± 6.3 b	29.39 (P = 0.0000)

Means in each line followed by different letters are significantly different (LSD test, $P < 0.05$).

the variance. When the F-tests were significant, means were separated applying the least significant difference (LSD) test ($P < 0.05$) (Statgraphics Plus 2001). Tables show non-transformed values.

Results

Antimicrobial assay

Against *P. larvae* strain used in these experiments, MIC values were 50 µg/ml and 3.125 µg/ml respectively for the essential oil of *C. zeylanicum* and for oxytetracycline-HCl.

Bees lethality test

Estimated *A. mellifera* LD50s were 456.07 µg/bee, 432.13 µg/bee and 427.32 µg/bee at 24 h, 48 h and 72 h, respectively (table 1). Referring to the ICBB (1985), the theoretical LD50 values for the bees of specific active ingredient, like dimethoate, range between 0.1-0.3 µg/bee; this level corresponds to a highly toxic compound (Gough *et al*., 1994). Therefore, the highest LD50 value for the *C. zeylanicum* essential oil, would correspond to a "virtually non-toxic" product, according to the ICBB (1985).

As an adaptation of the methodology used by Lindberg *et al.* (2000) the *security index* was employed to estimate a safe factor of the colony (table 2). This parameter diminished after 24 h, because LD50 values decreased over time, while MIC values remained stable. This trend shows that when oil exposure-time of bees increases, toxicity is expected to grow.

Apiary trial

After the artificial infection of bee colonies, the experimental model allowed to follow the development of the disease in all beehives. From the 3rd to the 5th day after the scales inoculation, the bees cleaned the infected cells and the queen laid eggs inside. After the 10th day, infected larvae were not detected during inspections. From the beginning of the treatments to the 17th day, colonies treated with essential oil showed an average of 0.14% infected larvae, which is similar to levels recorded in colonies treated with antibiotics, but lower than untreated ones. After the 24th day, colonies treated with essential oil showed a higher number of infected larvae than those treated with antibiotics, but even larger numbers of infected larvae were recorded in the control hives. Significant differences ($P < 0.05$) among treated and control groups were verified (table 3). From the beginning of the treatments to the 24th and 31st day, the essential oil-treated hives group showed a reduction in infected larvae percentages of 7.89% and 52.42%, respectively, in comparison with the untreated ones. By contrast, these values increased of 0.44% and 12.43%, respectively, when compared to the antibiotic treated group. However, no statistical differences were recorded between the two treated groups (table 3).

Discussion and conclusions

MIC values of cinnamon essential oil obtained from Mar del Plata AFB strain were similar to those obtained against others *P. larvae* strains isolated from infected honeybee brood combs of Buenos Aires province (Gende *et al*., 2008b). In relation with minimal inhibitory concentration of the antibiotic, the Mar del Plata strain was susceptible by introducing a value of MIC less than 5 µg/ml (Alippi, 1996).

When the essential oil was administered in sugar syrup by systemic way, regression equations presented in table

1 shows that the mortality in bees fed with 1000 µg/ml syrup did not exceed 10.0% on any day. Although the oral toxicity of essential oils has been already studied on the bees (Ebert *et al*., 2007), following the preliminary test carried out by Carta and Floris (1989), to our knowledge this is the first report regarding the use of cinnamon essential oil by oral administration in vitro assays.

In the experimental apiary control beehives showed higher levels of infected cells than treated ones. Therefore, both tested products, the antibiotic and the cinnamon oil, were effective in the control of AFB. However, as already known, the use of antibiotics compared to the essential oil can generate toxicological and biological risks due to an accumulation of residues in beehive products which decreases their quality and can also induce resistance to *P. larvae* strains or other bee pathogens (Miyagi *et al*. 2000; Evans, 2003; Thompson *et al*., 2005; Martel *et al*., 2006). Although we can exclude that the Argentinean strain of *P. larvae* tested is partially resistant to antibiotics (Alippi, 1996), the analogous disease development in the treated groups (O and Co), proved that cinnamon oil may represent an important alternative natural product for AFB control in apiary. Its efficacy was statistically comparable to the tested antibiotic. The results of our experiments support other research findings (Carta and Floris, 1989; Carpana *et al*. 1996; Floris *et al*., 1996; Floris, 2001). Moreover, several recent studies proved the use of cinnamon as a stimulus for honey bee learning (Abramson *et al*., 2006, 2008) or its use against other important mites and disease vectors (Abramson *et al*., 2007). In addition, the apparently lower ability of other natural essence-based products to keep down bacterial infestation levels (Albo *et al*., 2003), encourages the employment of cinnamon oil in the hives. Nevertheless, in our case the experimental infection was based on an initial inoculum corresponding to a very high dosage of bacterial spores in comparison with a natural infection. In fact, a dry scale formed from a diseased larva affected by AFB usually contains approximately 2 x 10^9 spores/bee (Shimanuki and Knox, 1997); in our tests an average number of 45 ± 5 scales corresponding to about 9 x 10^{10} spores/bee, were introduced in the previously healthy beehives. On the other hand, it is generally agreed that the use of naturally infected beehives better mimic the reality and consequently provide more reliable treatment efficacy evaluations. Beyond, in order to avoid the possible loss of honeybee orientation observed by Higes *et al*. (1997), we included only two doses of cinnamon essential oil in two weekly treatments. So that, enhanced efficacy of cinnamon oil in comparison with antibiotic treatments, might have been obtained by either using three different doses or with an experimental infection based on a lower number of scales. Finally further improvements might be obtained employing different and more appropriate treatment application techniques. For instance, manipulative treatments, such as the cited "shaking" method (Bailey and Ball, 1991) aimed at salvaging adult bees from combs containing diseased brood, before the administration of cinnamon oil, is supposed to provide optimal efficacy in preventing AFB. Enhancement of treatment efficacy might be possible also if this oil is used in combination with other active ingredients such as thymol (Fuselli *et al*., 2006); plant extracts, other essential oils and their principal components (Gende *et al*., 2008b; 2008c; 2009) involved in integrated pest management strategies of the hive.

Everything considered, this study represents a further progress of a larger research, involving the chemical composition and the concentration of the main cinnamon essential oil compounds, with special regard to the components inhibiting bacterial growth (Gende *et al*., 2008b). All these findings have helped to define recommended concentrations for apiary application in order to minimize the toxicological risks for bees and to avoid the honey taste threshold (Bogdanov *et al.*, 1999) or other undesirable effects, such as the appearance of resistance strains as a consequence of the indiscriminate use of antibiotics.

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