ISOLATION OF POTENTIALLY PATHOGENIC MICROORGANISMS FROM VARROA MITES IN ARGENTINA

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Introduction

Varroosis is considered the parasitic disease responsible for most of the losses in argentine apiculture (Bacci, 2010).

The use of chemical acaricides has resulted in the generation of tolerant varroa populations and contamination of bee products (SENASA, 2005).

In Argentina, populations of tolerant varroa to coumaphos and amitraz, two of the most commonly used miticides, has been reported (Maggi *et al*., 2009; Maggi *et al*., 2010).

Biological methods, such as the use of entomopathogenic bacteria and fungi as biocontrol agents, are alternative strategies for the control of varroosis. Some fungal species whose pathogenicity against varroa has been evaluated in laboratory are *Hirsutella thompsonii* (Kanga *et al*. 2002, Peng *et al*. 2002), *Metarhizium anisopliae* (Kanga *et al*., 2003, Rodríguez *et al*., 2009; Hamiduzzaman *et al*., 2012) and *Beauveria bassiana* (Rodríguez *et al*., 2009, Espinoza-Ortiz *et al*., 2011; Hamiduzzaman *et al.,* 2012). The effectiveness of these two species has also been evaluated with hives in field trials (Kanga *et al.,* 2006, Meikle *et al*., 2009; Kanga *et al.,* 2010; Molina Campos, 2010). In Argentina *Beauveria bassiana* strains were isolated from collected mites (Lecuona *et al*., 2010).

Other fungi evaluated as biocontrol agents in laboratory under field conditions, some of which had been isolated of from varroa, were: *Lecanicillium lecanii* (Gerritsen and Cornelissen, 2006*), Verticillium lecanii, Paecilomyces spp* and *Tolypocladium spp* (Shaw *et al*., 2002), *Aspergillus flavus*, *Mucor ramosissimus, Mucor indicus, Mucor hiemalis, Penicillium multicolor* and *Penicillium simplicissimum* (Hrabak, 2003).

Bioassays to evaluate bacterial effectiveness in controlling *Varroa destructor* were performed with *Bacillus thuringiensis* (Van der Geest *et al.,* 2000; Marquez *et al*., 2002), and strains of *Enterobacter cloacae*, *Staphylococcus aureus* (Hrabak, 2003), *Bacillus* sp. and *Micrococcus* sp., isolated from varroa (Tsagou *et al*., 2004).

Effectiveness of biological control methods may be dependent of environmental conditions under which it applies (Davidson *et al.* 2004). Therefore, isolation of native strains with acaricide properties is important because these strains could be better adapted to the conditions in they must act.

The aim of this work is to isolate autochthonous microorganisms that can be used as biological control agents of *Varroa destructor* in *Apis mellifera* colonies in Argentina.

Materials and methods

To collect varroa fall naturally, trap floors were placed in experimental beehives of National University of Luján. Varroas were colleted at intervals equal or less than 24 h, separated and grouped into batches identified by date of collection. Mites were stored under refrigeration (2-8°C) until they were processed. For isolating microorganisms, mites were surface disinfected with 70% ethanol and two washes with sterile distilled water. Subsequently, they were subjected to two techniques for isolation of potentially pathogenic microorganisms:

a) suspension of mites in sterile physiological saline solution (0.8% w/v NaCl), pre-incubation at 37°C ± 1°C for 2 h , and streaking of the suspension on Tryptone Soy agar (TSA, Biokar Diagnostic, France) for isolation of bacterial microbiota, Sabouraud Dextrose agar (SA, Biokar Diagnostic, France) and Yeast Extract Glucose Chloramphenicol agar (YGC, Merck KGaA, Germany) for isolation of fungal microbiota.

b) seeding mites directly on TSA or SA in Petri dishes.

TSA plates were incubated at 37°C ± 1°C for 24-48 h; SA and YGC plates at 25°C ± 1°C during 10 days, with daily inspection to detect fungal growth.

Colonies grown on TSA were isolated by inoculation in Tryptone Soy Broth (TSB, Biokar Diagnostic, France) and incubation at 37°C ± 1°C for 24 h, and were subjected to re-isolation in TSA and staining to corroborate purity. The stock cultures were maintained at -20 °C in TSB supplemented with 10% v/v glycerol (Sintorgan Argentina).

Colonies grown on YGC and AS plates were re-isolated in Potato Dextrose Agar (PDA, Britania, Argentina) for identification and incubated at 24°C ± 1°C with 12:12 (L:D) h photoperiod for 7 days. The stock cultures were maintained at 5°C in PDA slants.

Tests applied for bacterial identification were:

• Enzymes presence: catalase, oxidase, urease, gelatinase, β-galactosidase, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase

• Fermentation of carbon compounds: glucose, sorbitol, mannitol, inositol, rhamnose, sucrose, melibiose, arabinose, amygdalin

• Indole production, hydrogen sulfide production, Voges-Proskauer reaction, motility, citrate utilization, Gram stain, dextrose oxidation/fermentation.

Fungal isolates were identified preliminarily by morphological studies: colony shape and pigmentation on PDA (front and back), mycelium aspect, microscopical characteristics of conidiophores, conidiogenous cells and conidia.

Results

17 bacterial strains were isolated, 15 belong to the family *Enterobacteriaceae*, 1 to *Bacillaceae* family (strain 24) and 1 to *Pseudomonadaceae* family (strain 25). The results of tests for identification of bacteria are shown in Table 1.

Table 1. Tests for bacterial identification

	1	**3**	**4**	**5**	**7**	**8**	**9**	**10**	**11**	**14**	**17**	**19**	**20**	**22**	**23**	**24**	**25**
Gram	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Gelat.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
β Galact	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Lysine D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Ornithine D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Arginine DH	+	-	+	-	-	+	-	+	+	+	-	-	+	-	+	+	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Melibiose	-	-	-	-	+	-	-	+	-	-	+	-	-	-	+	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
O/F	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/-

Gelat.: gelatinase; β Galact: β galactosidase; Lysine D: lysine decarboxylase; Ornithine D: ornithine decarboxylase; Arginine DH: arginine dihydrolase; H_2S: hydrogen sulfide production; VP reaction: Voges-Proskauer reaction; O/F: dextrose oxidation/fermentation

Biochemical reactions with higher discriminatory power between strains belonging to family *Enterobacteriaceae* were the presence of arginine dihydrolase enzyme and melibiose fermentation.

Furthermore, some strains further were subjected to reactions to detect presence of beta-glucuronidase, beta-xylosidase and pyrrolidonyl-aminopeptidase, and ability for adonitol and xylose fermentation. Of these tests, alone the detection of beta-xylosidase had discriminatory power, because it was detected in only one strain (strain N° 10).

Analysis of identification profile of most of the strains belonging to the family *Enterobacteriaceae* were corresponded with profiles of atypical strains of *Serratia marcescens*.

With regard to isolation of fungi, nine strains have been isolated, which have been classified, according to the macroscopic and microscopic morphological characterizations, as belonging to the genera *Penicillium* and *Trichoderma*.

Conclusion

Bacterial strains belonging to the families *Bacillaceae, Pseudomonadaceae* and *Enterobacteriaceae*, and fungal strains belonging to the *Penicillium* and *Trichoderma* genera, which are under process identification to species level, have been isolated from varroa mites collected in beehives located in Buenos Aires (Argentina). In subsequent trials their pathogenicity on varroa will be evaluated under laboratory and field conditions, in order to determine their viability as biological control agents against *Varroa destructor*.

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