POTENTIAL OF DESTRUXINS FROM METARRHIZIUM ANISOPLIAE FOR THE CONTROL OF THE HONEYBEE PARASITIC MITE *VARROA DESTRUCTOR*

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Introduction

Varroa destructor Anderson & Trueman (2000) is an ectoparasitic mite which causes severe damage to *Apis mellifera* populations worldwide. Adult females of *V. destructor* feed directly on the haemolymph of honey bee pupae and can activate and transmit honeybee viruses. Infestation causes an increased prevalence of bee diseases and colony mortality, resulting in decline of honey production and of pollination efficiency.

At present, chemical control is the most effective and widely used means of reducing the negative impact of *V. destructor* infestation. However, the number of approved products available in individual countries is limited and populations of mites resistant to pyrethroid and organophosphorous acaricides are already established in some areas and their distribution is increasing (Lodesani and Costa, 2005). Repeated use of chemical treatments also poses the risk of accumulation of residues in hive products (Wallner,1999). Alternative means of controlling mite populations such as the removal of infested drone brood and the use of organic acids and volatile essential oils are labour-intensive and of variable efficacy (Fries, 1997; Thomas, 1997).

These problems have highlighted the need for alternative control strategies including biological and microbial control. No natural enemies have been observed causing population declines of *V. destructor* in honeybee colonies. However, entomopathogenic fungi, which kill other mites and ticks in nature, have been identified as potential microbial biopesticides (Chandler et al., 2000; 2001).

Recent studies showed that *V. destructor* is susceptible to entomopathogenic fungi. Some isolates of fungi seem to be able to kill mites in laboratory bioassays performed under the same conditions of high humidity and high temperature that occur within honeybee colonies. In particular, several isolates of *M. anisopliae* showed a significant effectiveness against *V. destructor*, but also caused significant mortality of adult honey bees in maximum challenge bioassays (Shaw et al, 2002), although these results should be interpreted with caution due to high control mortality, as suggested by several authors (Bailey et al., 1983; Butt and Goettel, 2000).

Entomopathogenic fungi use different strategies to penetrate the outer surface of their targets and kill them. One important aspect of the mode of action of these pathogens is the production and release of mycotoxins. Among the metabolites produced by entomopathogenic fungi, destruxins are of particular interest because these components are the only mycotoxins detected in the insect body when advanced stages of infection cause death (Hu et al., 2007).

Destruxins (Dtx; Fig. 1) were first isolated from *Metarrhizium anisopliae*. These compounds are typically composed of five amino acids and a α-hydroxy acid forming a cyclic hexadepsipeptide. The general formula of destruxins is cyclo (–D-HA–L-Pro–L-Ile–L-MeVal–L-MeAla–β-Ala-), where HA represents a D-α-hydroxy acid residue. Currently, over 35 different structurally related destruxins have been isolated, and 15 of these were isolated from *M. anisopliae*. Dtxs can be classified into main series, depending on the nature of the pentanoic acid side chain, and into further sub-series according to differences in the amino acid substitution pattern. Dtxs A, B and E have the same amino acid sequence but differ in their hydroxy acid residue and are the most represented. Insecticidal activity of Dtx E is probably due to the reported influence on calcium fluxes and protein phosphorylation (Dumas et al. 1996) and cytotoxic inhibition of ATP-dependent acidification of endosomes and lysosomes (Naganuma et al.1992). The mode of action is still under investigation, but Dtx E is known to inhibit vacuolar-type H(+)-ATPase activity (Yoshimoto and Imoto 2002). In addition to the insecticidal activity, Dtx E has been reported to prevent the formation of osteoblasts (Nakagawa et al. 2003), inhibit the proliferation of certain cancer cell lines (Kobayashi et al. 2004), and intervene during the disease by an immune-inhibitory effect (Vey et al., 2002).

Entomopathogenic fungi could represent a promising alternative to chemical insecticides. However, a major hurdle concerning the registration of these fungi as plant protection agents is the possible toxicity of secreted metabolites, especially secondary metabolites.

Metarhizium species produce a variety of secondary metabolites other than destruxins. Recently, two polyenesubstituted pyrrolidinones with mutagenic activity have been isolated from mutants of a wild-type strain of *M. anisopliae*. These compounds have yet to be documented from isolates of *M. anisopliae* other than the mutant and its laboratory derivatives. Nonetheless, this possibility represents a potential risk for the use of *M. anisopliae* as a biocontrol agent. Thereby the isolation and purification of the toxins causing insect death could by-pass these uncertainties and provide a safer biocontrol product. Also, the temperature and humidity conditions in the hive do not correspond to the ideal growth conditions for the fungus, and efficacy is therefore limited by reduced proliferation, while the purifies toxins would not suffer from these limitations.

Aim of the project
Our project aimed at investigating the possible application of purified destruxins from *M. anisopliae* as agents to control Varroa infestation levels. To this aim, the purified and fully characterized compounds are being used for bio-assays for testing destruxin acitivity against *V. destructor.*

Materials and methods
An isolate of Metarhizium anisopliae (ARSEF: 4556 [DAT 506]) was grown in Czapek culture broth + 0.5 % peptone for about 10 days at 26° C and 200 rpm in a rotary shaker. The culture was filtered before the extraction, purification and structural characterization of destruxins, according to Pais et al. (1981) and Liu et al. (2004).

In a first stage, a new protocol for the extraction and the pre-fractionation by HPLC of destruxins from the culture broth was developed. In a second stage the purification of single destruxins from each fraction was then performed using preparative and semi-preparative chromatography.
Dose–response assays of the isolated metabolites against *V. destructor* and their impact on individual honey bee workers were carried out over a 2 year period in several kinds of trials:

- **Trial 1:** the crude dtx extract was dissolved in water. Mites were treated with three 10-fold decreasing concentration levels (highest 0.35mg/ml). 0.02 ml of dtx solution were sprayed on each dish (Fig. 2). Bees (~100 per cage) were sprayed with 0.2 ml of a 0.25 mg/ml dtx solution.
- **Trial 2:** the crude dtx extract was dissolved in a 20 % ethanol solution, and two 10-fold decreasing concentration levels (highest 5mg/ml) were used to spray the mites and the bees.
- **Trial 3:** separated dtx fractions (dtx B and CE) were dissolved in 25% acetone and 75% of 0.05% Tween 80 solution. Two 10-fold decreasing concentration levels (highest 5mg/ml) were used. The administration mode for mites and bees consisted in submitting them to the aerosol-vaporised solution in an air-tight 10 dm3 jar (Fig. 3).

For all trials female adult varroa mites were collected from sealed worker brood cells (pupae at *pw* stage) from untreated hives of the CRA-API apiary in Reggio Emilia (northern Italy). The mites (6 per repetition) were placed in Petri dishes (35 mm diameter x10mm high) which had previously been filled with sterile and residue free wax, before treatment with the dtx extracts.

According to the trial, crude or fractionated dtx, dissolved with water, ethanol or acetone were sprayed with a small volume perfume sprayer or subjected to a saturated vapour in an aerosol chamber.

Then mites were left for 2 hours before being fed with spinning larvae (*l5* stage). The larvae (2 per dish) were substituted every 24 hours. The trial dishes were incubated in an incubator at 26°C and 70% R.H., in the dark. Observations were carried out every 24 hours in order to count and collect the dead mites, until all mites were dead.

Adult worker honey bees were collected from the supers of a single hive and placed in laboratory hoarding cages, fed with 50 % sucrose syrup and incubated at 32 ° C and 75 % RH.

Statistical analyses of the data were performed using Statistica 8.0 (Stat Soft) software.

Results
The crude extract and each purified fraction was characterized using mass spectrometry and at least twelve compounds belonging to the destruxin group were identified by comparison with structural data already reported in the literature.

Although the growth conditions of *M. anisopliae* were standardized, the production of destruxins was found to be strongly susceptible to the environmental parameters, thus showing a strong variability in the relative

amount of each destruxin in the crude extract. For this reason, after initial trials with the crude dtx, in the final stages the separated fractions B and C+E were used.

- **Trial 1:** The highest concentration (0.35 mg/ml) caused 94 ± 6.4 % mortality of mites 48h after treatment (a.t.), while no differences were observed in mortality of treated bees compared to control 16 days after treatment. Bees sprayed with the dtx solution did not die significantly earlier than control-treated bees.
- **Trial 2:** With a higher dtx concentration (5 mg/ml) dissolved in 20% ethanol solution a higher mite mortality (63.9 ± 14.2 %) compared to control (8.3 ± 5.3 %) was observed 48h a.t.. In the same trial bees sprayed with the same concentration did not die sooner compared to control (water sprayed), and lived longer than bees sprayed with only ethanol solution. However bees sprayed with the lowest concentration lived less than controls.
- **Trial 3:** Due to uncertainty of results, crude dtx were fractionated and used for a further trial. Highest mortality towards varroa 48h a.t was caused by dtx CE: 45.8 ± 4.8 % compared to 18.3 ± 9.5 % in control (Fig. 4). Bees sprayed with the same concentration did not die significantly sooner than control bees.

Conclusions

The presented experiments show that destruxins have a good potential for controlling *V. destructor* infestation levels, as mortality of the mites in laboratory tests is significant. Further research will focus on improving standardization of the extracts obtained from the fungal cultures, in order to decrease variability of effectiveness. Additional investigations are necessary to establish whether one or more of the dtx fractions, at an optimal dosage, can ensure an acceptable combination of varroa toxicity and harmlessness to bees.

Figures

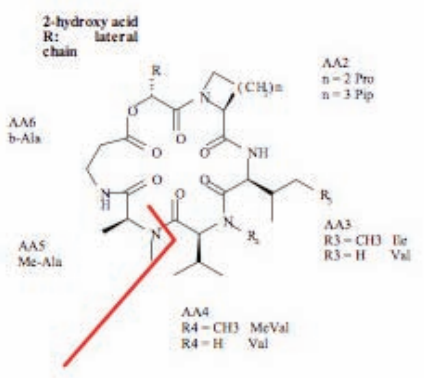

Figure 1: Chemical structure of destruxins.

Figure 2: Experimental dishes containing treated and non-treated Varroa mites and honey bee larvae.

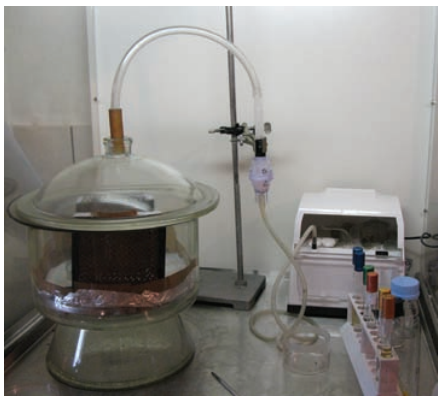

Figure 3: Aerosol chamber used in trial 3 for treatment of mites (in Petri dishes) and bees (in cages provided with metal grid).

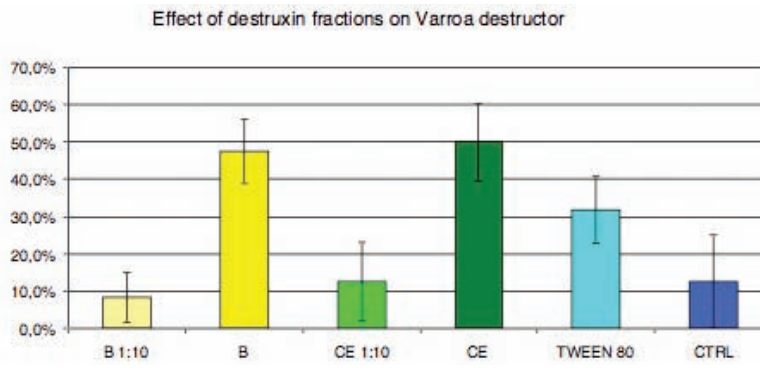

Figure 4: Death rate of Varroa mites treated with different dtx solutions in trial 3.

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