2003-2004

RESEARCH GRANT PROPOSAL FINAL REPORT

PROJECT TITLE: Screening of Plant Essential Oils Against Oxytetracycline-Resistant Strains of the American Foulbrood Pathogen *Paenibacillus larvae.*

Project Number:03-GD-02

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Problem and its Significance:

American Foulbrood disease (AFB) is a severe infectious disease of the larvae of the honey bee, *Apis mellifera*, that is transmitted by the spores of the bacterium *Paenibacillus larvae*, subsp. *larvae* (formerly *Bacillus larvae*) (1).

AFB is generally regarded as one of the most destructive and serious brood diseases of honey bees because colonies with clinical symptoms normally die if treatment is not carried out. Therefore, the disease can cause substantial economic losses in millions of dollars for both beekeepers and producers such as almonds growers that depend on pollination (1,5).

There are many different techniques used by the beekeepers for prevention, controlling and treatment of colonies infected with AFB. In order to salvage the bees and/or the equipment, the disease is controlled by either burning the infected colonies or by the use of antibiotics, such as oxytetracycline hydrochloride (OTC= Terramycin) to suppress and eradicate AFB from the colonies (6).

Currently, oxytetracycline hydrochloride (OTC= Terramycin) remains the only approved drug treatment available in the United States for the prevention and control of AFB (2,4). As the utilization of this antibiotic grew in popularity, several outbreaks of resistant strains have been reported (2,4). Thus, resistance of the American Foulbrood pathogen, to this antibiotic has become a major problem for beekeepers in the United States (4,5). There is, therefore, a desperate need for new antibiotics to treat this disease. The high costs of developing such products and the difficulty in registration of new products for use in an environment where they might come in contact with humans, has pushed researchers to explore alternative antimicrobial products.

Our proposal seeks to determine whether the utilization of alternative antimicrobial agents, such as plant essential oils, can be effective in the control of pathogens resistant to oxytetracycline and also can be used as prophylaxis.

Objectives:

1) **Determine whether different essential oils and/or their derivatives in the slow-release and emulsified formulations can inhibit oxytetracycline-resistant strains of AFB.**
2) **Determine the minimum inhibitory concentration (MIC) of the oils *in vitro*.**

Objective 1: Determine whether different essential oils and/or their derivatives in the slow-release and emulsified formulations can inhibit oxytetracycline-resistant strains of AFB.

We conducted the first set of experiments to determine the dosage levels of each of the oils needed to control the specific pathogen. For this trial the susceptible strain of *Paenibacillus larvae*, subsp. *larvae* obtained from the American Type Culture Collection (ATCC), was used as a standard control. The bioassays were performed using the existing slow-release oils and the emulsified oils that we successfully emulsified in our laboratory. We found that the addition of essential oils to the culture medium, in any of the two different formulations tested, produced a drastic reduction in the number of colonies observed when compared to media without essential oils. However, the effectiveness in controlling and/or inhibiting the growth of *P. larvae* varied with the different oils utilized. As an example, the results obtained with Thymol and Origanum are shown (Figures 1 and 2).

Objective 2: Determine the minimum inhibitory concentration (MIC) of the oils *in vitro*.

Once we determined the effectiveness of the oils, the sensitivity of the microorganism to the active agent was determined by the MIC.

For this bioassay, serial dilutions of the different oils were incorporated into freshly melted brain infusion agar medium (BHI) fortified with thiamine hydrochloride (0.1mg/L) and adjusted to pH 6 and poured in Petri dishes. The concentrations of essential oils used were: 0.001%; 0.01%; 0.02%; 0.03%; 0.06%.

Once the medium solidified in the dishes, 0.2 ml of a bacterial cell suspension in the stationary phase of growth was spread over the surface of the agar media. The plates were incubated 18-24 hours at 32C in the dark and the number of viable colonies in each dilution plate was recorded. The results obtained are shown in Table 1.

Table 1. MIC of essential oils utilized in this proposal

Essential Oil	MIC Slow Release (%)	MIC Emulsified (%)
Thymol	0.02	0.01
Origanum	0.03	0.01
Cinnamon	0.06	0.03
Bay	*	0.06
Clove	*	0.06

*** For these treatments no inhibition was detected even at the higher concentration tested (0.06%)**

The results obtained with the first set of experiments allowed us to identify the dosage level and effectiveness of the oils and formulations tested and also the sensitivity of the microorganism to the active agent. For this trial the susceptible strain of *Paenibacillus larvae*, subsp.*larvae* obtained from the American Type Culture Collection (ATCC), was used.

Our results showed that the emulsified formulation is more effective than the slow-release formulation. For this reason in the second phase of the project, we tested the effectiveness of emulsified oils to control the oxytetracycline (OTC)-resistant strains of AFB. The OTC-resistant strain # 3b was a field sample isolated in our lab. The results obtained with the emulsified formulations of Thymol and Origanum (Figures 3 and 4); indicate that OTC-resistant strain is as sensitive as the OTC-susceptible strain to these formulations.

Conclusions:

The oils included in this study were Thymol, Origanum, Cinnamon, Bay and Clove. The analysis of the MIC (Minimum Inhibitory Concentration) of the different oils shows that Thymol and Origanum were the most effective to control the bacterial growth at the concentration 0.01% for the emulsified formulation and 0.02% for the slow-release formulation. Our results also showed that the emulsified formulation is more effective than the slow-release formulation to control bacterial growth in *in vitro* trials. We found that the addition of essential oils to the culture medium, in any of the two different formulations tested, produced a drastic reduction in the number of colonies observed when compared to media without essential oils. However, the effectiveness in controlling and/or inhibiting the growth of OTC – susceptible or resistant strains of *P. larvae* varied with the different oils utilized. These observations are very encouraging and suggest that plant essential oils can be successfully utilized to control the growth of AFB even when resistance to OTC has developed in the colony.

The long-term goal of this research was to develop an alternative treatment to control OTC – susceptible or resistant strains of *P. larvae* based on the utilization of plant essential oils as antimicrobial agents. We believe that the use of these oils as treatment and prophylaxis is a key element in the beekeeping industry and will provide products that can be alternated to minimize the chance of resistance. We are hoping that the treatment with these antimicrobial agents will not only reduce pathogens but also enhance hive vigor by improving larval health and survivorship.

REFERENCES:

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0. Control no oil.
0.001%-0.02% oil concentrations

Figure 1

Effect of Thymol on the growth of *P. larvae in vitro*

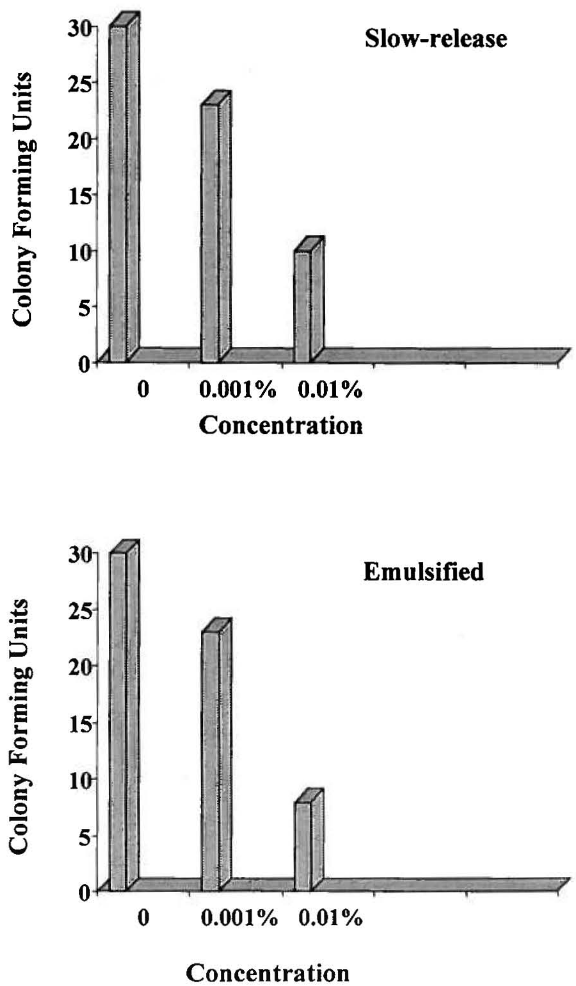
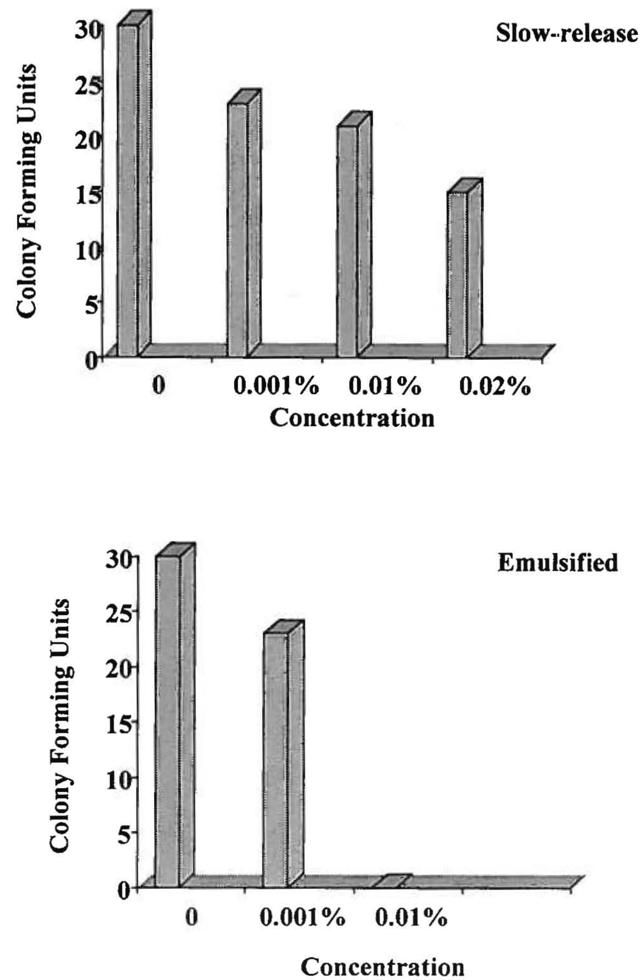


Figure 2

Effect of Origanum on the growth of *P. larvae in vitro*



0. Control no oil.
0.001%-0.02% oil concentrations
OTC. Oxytetracycline

Figure 3

Effect of Thymol on the growth of *P. larvae in vitro*

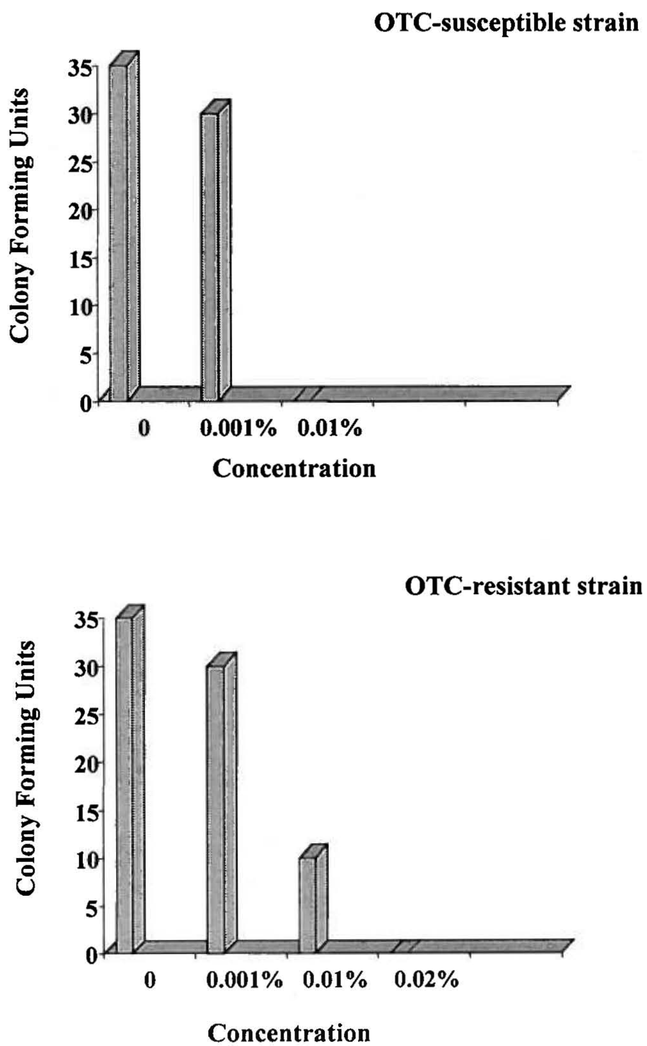


Figure 4

Effect of Origanum on the growth of *P. larvae in vitro*