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ORIGINAL RESEARCH ARTICLE

Preventive and antimicrobial activities of alkoxysilane against the American foulbrood pathogen *Paenibacillus larvae* in *Apis mellifera* L.

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Summary

The aim of this study was to test the hydrolysis product of a quaternary amine-containing organosilicon salt (Si-QAC) for its preventive and antimicrobial activity against the American foulbrood (AFB) pathogen *Paenibacillus larvae*, a major cause of colony losses worldwide. The effects of Si-QAC were examined on 28 different local strains of *P. larvae* in the spore, vegetative and spore-vegetative forms, as well as the *P. larvae* strain ATCC 9545. The experiment was set up for spore and vegetative forms in four different ways. The results show that Si-QAC inhibits bacterial growth significantly in *in vitro* experiments. The extended studies were completed by cage experiments to identify toxicity levels. It was found that there is no toxic effect on honey bees by direct or indirect contamination with Si-QAC. These studies suggest that the preventive activity of Si-QAC against *P. larvae* is most effective as a disinfectant.

Actividades preventivas y antimicrobianas del alcoxisilano contra el patógeno de la loque americana *Paenibacillus larvae* en *Apis mellifera* L.

Resumen

El objetivo de este estudio fue probar el producto de la hidrólisis de la amina cuaternaria que contiene sal de silicio orgánico (Si-QAC) como actividad preventiva y antimicrobiana contra el patógeno de la loque americana (AFB) *Paenibacillus larvae*, la mayor causa de pérdida de colonias a nivel mundial. Los efectos de Si-QAC fueron examinados en esporas, en formas vegetativas y en formas de esporas vegetativas de 28 cepas locales diferentes de *P. larvae,* así como en la cepa ATCC 9545. El experimento se estableció para las formas espora y vegetativa de cuatro maneras distintas. Los resultados mostraron que el Si-QAC inhibe significativamente en crecimiento bacteriano en los experimentos *in vitro*. Los estudios fueron completados mediante experimentos en caja para identificar los niveles de toxicidad. Se encontró que no había efecto tóxico en abejas por contaminación directa o indirecta con Si-QAC. Estos estudios sugieren que la actividad preventiva de Si-QAC con *P. larvae* es la más efectiva como desinfectante.

Keywords: alkoxysilane, American foulbrood, disinfectant, organosilicon, *Paenibacillus*

Introduction

Beekeeping is an important agricultural activity, not only for producing honey and hive products, but also for agricultural pollination. One of the most serious pathogens of the honey bee *Apis mellifera* L. worldwide is American foulbrood (AFB). It is very common among the honey bee colonies in Turkey, and has a tremendous economic impact on the Turkish Beekeeping Industry. AFB is caused by *Paenibacillus larvae*, a spore-forming, gram-positive, microaerophilic bacterium. *P. larvae* has been isolated from approximately 20% of all honey bee colonies in Turkey (Özkırım and Keskin, 2002). For many years an alternative to antibiotics has been sought to prevent the distribution

of AFB (Lauro *et al.*, 2003, Antunez *et al.*, 2004, Özkırım and Keskin, 2005). In apiaries, honey bees are frequently in contact with the surface of combs and hives. They may carry pathogens into the hive from outside, from nectar and pollen sources, or from other colonies. Hive disinfection is very important to protect colonies from pathogens, particularly in spring and autumn. Larvae and newly emerged bees come in contact with comb and hive surfaces and may be exposed to pathogens.

For over a decade, alkoxysilanes have been used in a number of industries as coupling agents to reinforce or impart desirable properties to a variety of materials. In addition, a number of enzymes are known to remain biologically active when bonded to inorganic surfaces by alkoxysilanes (Vetter *et al.*, 2009). Their activity persists even after repeated washing. Si-QAC (3-(trimethoxysilyl)-propyldimethyloctadecyl ammonium chloride) is an alkoxysilane that acts on membrane phenomenon, i.e., membrane lysis, membrane enzyme inactivation, or interference with ion transport (Lawrence, 1968). The antimicrobial activity of Si-QAC is thought to be due to the disruption of membrane function with possible cell lysis, caused by the high concentration of charged chemical (quaternary ammonium chloride) on the substrate (Vetter *et al.*, 2009). We tested Si-QAC for antimicrobial activity against *P. larvae* by *in vitro* tests and *in vivo* laboratory cage experiments.

Materials and methods

Isolation of *P. larvae*

From different provinces of Turkey, 28 *P. larvae* strains were cultured from brood combs showing clinical symptoms and testing positive for AFB (Table 1). The cellular suspensions obtained from brood samples were transferred to two small glass flasks, each containing 20 ml of Brain Heart Infusion Broth (BHI-B), and incubated at 37°C for 24 h. Once activated, 0.1 ml of the *P. larvae* culture was transferred to tubes containing Brain Heart Infusion Agar (BHIA) and Nalidixic acid (3 µg ml^{-1}) and incubated for 48 h at 37°C (Alippi, 1999). The tubes were stored 4°C for two days and spore-vegetative forms of *P. larvae* were obtained.

Sporulation of *P. larvae*

Tubes containing *P. larvae* were kept at 4°C for 10 days to induce sporulation. Bacterial cultures were suspended in to 5 ml of BHI-B and shocked thermally at 80°C for 10 min. This process was repeated five times. This process induced sporulation and eliminated other vegetative forms of bacteria. Cultures containing the spore forms of *P. larvae* were used for our experiments.

Table 1. Twenty eight local strains of *P. larvae* from different provinces of Turkey.

P. larvae **Strain Code**	**Province**
01/15	Adana
01/17	Adana
01/33	Adana
01/37	Adana
01/38	Adana
31/09	Hatay
31/28	Hatay
31/27	Hatay
31/08	Hatay
31/11	Hatay
01/41	Adana
48/38	Muğla
31/18	Hatay
48/35	Muğla
31/32	Hatay
01/29	Adana
48/07	Muğla
31/24	Hatay
31/02	Hatay
01/19	Adana
48/27	Muğla
01/23	Adana
31/26	Hatay
48/31	Muğla
01/40	Adana
31/21	Hatay
01/43	Adana
31/16	Hatay
9545	ATCC

Testing the preventive and antimicrobial activities of Si-QAC

Si-QAC (supplied by Nanotechnology Company; Istanbul, Turkey) was tested against 28 different local strains of *P. larvae* (including spores vegetative, and spore-vegetative forms) and the *P. larvae* strain ATCC 9545 (Table 1). All strains were inoculated in BHI-B Medium (42 gl^{-1}, Sigma) and incubated overnight. Following incubation, 0.1 ml of bacterial culture (1×10^8 CFU ml^{-1}) was transferred to BHIA Medium. Si-QAC was tested on both spore and vegetative forms of *P. larvae.*

Sulbactam-ampicillin (SAM) was used as positive control (Özkırım and Keskin, 2005) and three plates for negative control (only the bacteria). All plates were incubated at 37°C for 48 hours.

In order to measure the preventive activity of Si-QAC against the three forms of *P. larvae*, the surface of the plates were washed using 1 ml of sterilized saline solution (0.9% NaCl) and cleaned with a sterilized cotton stick. The cleaning saline solution collected in a micro -tube and the cotton sticks were suspended in the solution. The number of cells in 0.1 ml of the solution was measured using a haemocytometer and compared to the number of bacteria counted in the negative controls.

The disc diffusion method (Antimicrobial Disc Susceptibility Test) was used (NCCLS, 1997) to measure the antimicrobial activity of Si-QAC (Madigan *et al.*, 2009). Sterilized absorbent paper was prepared like antibiotic discs of 35 mm diameter. After inoculation of 0.1 ml *P. larvae* bacterial solution, the paper disks were placed the middle of the plates and inoculated with 0.5 ml of the Si-QAC solution. All plates were incubated at 37°C for 24 h.

A one-way ANOVA ($p < 0.05$) was used to compare the preventive and antimicrobial activity of Si-QAC against all forms of *P. larvae*. The Wilcoxon rank-sum test was used to determine significance of CFU difference ($p < 0.05$) between all forms of *P. larvae* and the control groups. A one-way ANOVA ($p < 0.05$) and the Duncan test were used to check significant differences in the inhibition zones of Si-QAC discs between all forms of *P. larvae* and the positive control. The diameters of discs were not included in the measurements for statistical tests.

Toxicity tests using laboratory cages

A total of 20 cages, each with 50 newly emerged bees, were used. Of these, 10 were used as a treatment group and the other 10 served as a control group without any chemical or disinfectant added. Si-QAC solution (1% w/v) was sprayed onto all sides of the 10 wooden cages of the treatment group. Specifically, 10 ml of the solution was sprayed onto 200 cm^2 of the cage surfaces (20 cm^2 ml^{-1}). After 10 min of drying, 50 newly emerged bees were put inside each cage. All cages were observed over 18 days. The honey bees were fed 5 ml sugar syrup by syringe and 3 g pollen cake in small caps per day. Dead bees were counted every two days. The goal was to determine the toxicity of Si-QAC on the honey bees.

Results

In vitro laboratory experiments

The *in vitro* laboratory experiments showed that Si-QAC has antimicrobial activity and that the activity differs among the three forms (spore, spore-vegetative, vegetative) of *P. larvae* (one-way ANOVA, $R^2 = 0.061$, degrees of freedom [df] = 4, $p = 0.02$). The number of cells in the Si-QAC treated plates (CFU ml^{-1}) were significantly different from the control groups in all three forms of *P. larvae* (Wilcoxon rank-sum test, $Z = 11.65$, $p = 0.001$) (Table 2). The reduction rates of CFU ml^{-1} in spore, spore-vegetative, and vegetative forms were similar. Accordingly, there were no differences between the experimental groups (one-way ANOVA, $R^2 = 0.07$, df = 3, $p = 0.66$).

Table 2. The number of cells in the Si-QAC treated Plates (CFU/ml). % reduction = (control – sample no / control x 100).

P. larvae **Strain Code**	**Spore form**	**Spore-vegetative form**	**Vegetative form**
01/15	310	303	225
01/17	348	308	223
01/33	220	309	276
01/37	245	344	253
01/38	318	322	251
31/09	356	277	224
31/28	344	256	223
31/27	256	324	227
31/08	238	356	219
31/11	267	322	250
01/41	332	244	263
48/38	355	213	216
31/18	343	244	218
48/35	326	255	291
31/32	287	231	235
01/29	267	278	281
48/07	265	300	227
31/24	301	277	216
31/02	255	212	212
01/19	286	200	209
48/27	299	210	207
01/23	381	233	229
31/26	305	212	245
48/31	365	276	249
01/40	356	298	273
31/21	377	301	232
01/43	311	306	261
31/16	254	220	251
9545	201	225	183
% Reduction	98.2	99.1	99.3

The antimicrobial activity of Si-QAC was measured by analysing its inhibition zones around discs. No differences were found between the three forms of *P. larvae* (one-way ANOVA, $R^2 = 0.05$, df = 2, $p = 0.75$). In contrast, comparing data from experimental groups and positive control (Table 3), the positive control (SAM) had significantly more antimicrobial activity on vegetative and spore-vegetative forms of *P. larvae* (one-way ANOVA, $R^2 = 4$, df = 5, $p = 0.001$, and Duncan table).

Table 3. Duncan Test Analysis. *a,b,c are significantly different groups.

		Inhibition zone diameter (Av.) mm* (Standard Deviation-SD)
Duncan	**Si-QAC spore form**	14.0476 (11.5926)[a*]
	Si-QAC sporo-vegetative form	15.8905 (10.3080)[b]
	Si-QAC vegetative form	15.9762 (12.6192)[b*]
	SAM	24.0238 (17.8319)[c*]

In vivo cage experiments

In the *in vivo* cage experiments, we found no statistical difference between the control and Si-QAC treated cages on honey bee mortality rate (one-way ANOVA, $R^2 = 0.6$, $p = 0.43$). It was observed that the 50 newly emerged bees in each cage consumed approximately 3.5 ml of sugar syrup and 1.8 g of pollen cake per day. The comparison results between the two groups demonstrated that there is a correlation between their lines on the graph. There was thus no evidence of a toxic effect of Si-QAC on honey bees (Fig. 1).

Discussion

This is the first published data describing the preventive and antimicrobial activities of alkoxysilanes in apicultural research. We examined the preventive activity of Si-QAC against *P. larvae*, and comparison of the results across all three groups shows that Si-QAC has the greatest preventive and antimicrobial activities against the spore-vegetative and vegetative forms of the bacterium. During the infection period, all spores become vegetative forms which can then infect honey bee larvae. This process occurs within the hive, which is the most suitable medium for *P. larvae* vegetative growth (Genersch, 2010). At the onset of germination, calcium dipicolinate in the spore-wall is released. It is possible that this alteration of the spore wall allows Si-QAC to be more effective as an antimicrobial on the spore-vegetative and vegetative forms of *P. larvae*. Although Si-QAC is effective on vegetative forms, the vegetative forms of *P. larvae* mostly grow inside the larvae (de Graaf *et al.*, 2006), so it is not likely that Si -QAC will be able to come into contact with this form within the hive. It is, however, the spore-vegetative forms that are the highest risk for spreading AFB rapidly.

Si-QAC was tested on the three forms (spore, vegetative, and spore-vegetative) of *P. larvae* by two methods, which accounted for different infection scenarios within the hive. Beekeepers could use Si-QAC to clean beekeeping equipment such as hives, combs, wax, etc. It was observed that Si-QAC has antimicrobial activity on both experimental groups. In the first group, the bacterial infection occurred first followed by application of Si-QAC later. We believe that the application of Si-QAC covered the bacterial cells and changed the chemical composition and osmotic pressure of the BHIA Medium. It is known that *P. larvae* is a microaerophilic bacterium (Loncaric *et al.*, 2009), so was unable to tolerate these changes in the medium and could not grow. In the second group, the medium was treated with

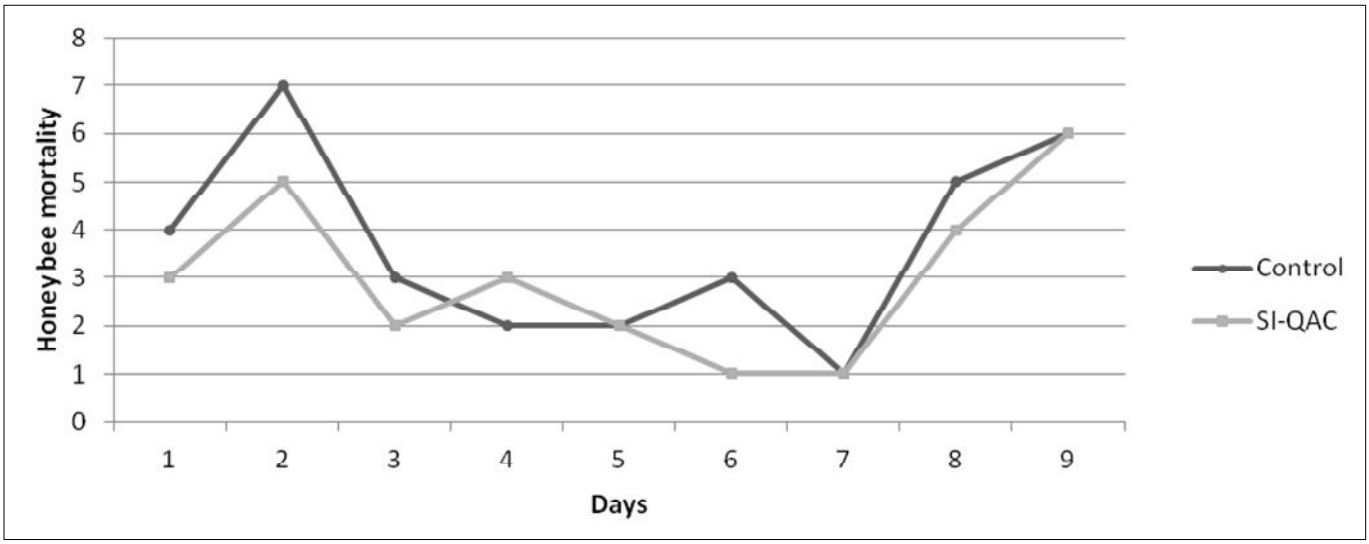

Fig. 1. Comparison of honey bee mortality between Control and SI-QAC treated cages.

Si-QAC initially and inoculated with the bacteria later. Other studies have found that the surface-bonding ability of Si-QAC inhibits spore or vegetative penetration of *P. larvae* to BHIA Medium's surface. Additionally, it is thought that Si-QAC may act as a barrier between bacteria and medium. The presence of Si-QAC inhibited the *P. larvae* from reaching the medium to feed on it, causing the death of bacteria.

To examine the Beekeepers' methods of applying Si-QAC to hives in fields, we tested spraying and dissemination methods. A comparison of the two methods showed that 1 mL of the Si-QAC solution is more effective when sprayed. It is believed that spraying covers the hives with lots of small droplets and allows the solution to dry very quickly. Additionally, the solution is able to reach all outlying points of the hive, increasing its preventive and antimicrobial activity.

Si-QAC was also tested for its antimicrobial activity using the disc diffusion method (NCCLS, 1997; Madigan *et al*., 2009). The appearance of an inhibition zone around a disc means that the product has antimicrobial activity. Its antimicrobial activity level is based on diameter of the zone (Black, 2008). This study supports the hypothesis that the antimicrobial activity of Si-QAC is thought to be due to the disruption of membrane function with possible cell lysis, caused by the high concentration of charged chemical (quaternary ammonium chloride) on the substrate (Vetter *et al*., 2009). The highest level of antimicrobial activity was seen on vegetative forms of *P. larvae*. While Si-QAC showed the highest antimicrobial activity on vegetative forms, the infection is spread by the spore forms (Gillard *et al*., 2008). Moreover, when the zone diameters are compared with positive control (SAM) measurements, Si-QAC could not be considered as an alternative antibiotic for treatment of AFB.

Considering the cage experiments, the direct contact with a 1% (w/v) solution of Si-QAC did not cause toxicity to honey bees. This study emphasized that Si-QAC inhibits the bacterial growth by means of biocidal and biophysical effects only directly to the place where it is applied (Vetter *et al*., 2009). On the other hand, the results show that Si-QAC has an important role in inhibiting the growth of *P. larvae*. Even a single spore can grow by itself and cause disease, but AFB could be prevented by using Si-QAC in hives. It has furthermore been reported that organosilicon treatments prevent the disfigurement of wood due to moisture (Vetter *et al*., 2009), so Si-QAC could also prevent fungal growth in the wooden hives generally used by beekeepers. It is possible that the use of Si-QAC 1% (w/v) solution, particularly in spring and autumn, could protect honey bee colonies from bacterial and fungal diseases caused by a high level of moisture in hive. For this reason, though it has not yet been tested, it is probable that Si-QAC would be more effective in the field. Additionally, other studies have shown that the Si-QAC solution has antimicrobial activity against other common bacteria as well (Lawrence *et al*., 1968). The preventive use of Si-QAC as a preventive disinfectant in beekeeping is important for addressing the problem of resistance of pathogens to commonly used antibiotics and reducing residues in honey bee products.

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