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Synergistic and molecular interactions between the formamidine amitraz and copper(II) sulfate, used against the mite *Varroa jacobsoni* O., a parasite of the honeybee *Apis mellifera* L.

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Toxicological field assays have shown that the shock-treatment efficacy of the formamidine pesticide amitraz, used against the parasitic mite *Varroa jacobsoni*, is synergistically improved by the administration of copper(II) sulfate through feeding of the honeybees. Amitraz is autoxidized and this process is accompanied by chemiluminescence. The emission is enhanced in the presence of low concentrations of H_2O_2. A dose-related inhibition of the chemiluminescence by $CuSO_4$ was observed; consistent with the formation of copper–amitraz complexes evidenced *in vitro*. The results suggest the possibility that a protection of amitraz by cupric ions might be at the origin of the enhancement of its toxicity and thus makes a contribution to the observed synergy.

Keywords: amitraz, *Apis mellifera*, copper(II), miticides, synergy, *Varroa jacobsoni*

Introduction

The invasion of western countries by the mite *Varroa jacobsoni*, originating from Java Island (Oudemans 1904), has raised serious problems for bee keepers. The high rate of reproduction and infestation of the parasite on *Apis mellifera* (Ifanditis 1983, Schultz 1984) together with the difficulties in finding appropriate treatments (Merchetti & Barbattini 1984) have considerably complicated the problem. During recent experiments, long-term systemic treatments using cupric salts given in sucrose syrups have proved efficient against the parasite (Guiraud *et al.* 1990). In some cases, the acaricide–insecticide amitraz (AMZ), a formamidine molecule (Tosi *et al.* 1984), was also used either as a short-term shock treatment (Faucon *et al.* 1986) or as a means of rapid evaluation of the levels of infestation of the hives by the mite.

Since AMZ, as with many other insecticides–miticides (usually not specific), is toxic for the honeybees on which it acts as an α-aminergic agonist (Cascino *et al.* 1989), any improvement of the efficacy of treatments should be worthy to be mentioned.

The conjugation of AMZ and copper salts treatments most likely results in the simultaneous penetration of these compounds in the body of the mite. This prompted us to examine the possibility of an interaction between these two treatments.

It has recently been found (Kruk & Bounias 1992) that the oxidation of AMZ in the presence of Cu^{2+} ions leads to the generation of electronically excited light emitting products. All the evidence indicates that cytotoxic oxygen species, such as singlet molecular oxygen ($^1O_2$), H_2O_2, hydroxyl ($HO^{\cdot}$) and superoxide ($O_2^{\cdot -}$) radicals, which can be toxic for all forms of life, are formed during AMZ oxidation. Furthermore, we have also found (Kuriata *et al.* 1992) that AMZ forms complexes with Cu(II) ions. Structures for the Cu(II) complex for AMZ and $CuSO_4$ and AMZ and $CuCl_2$ were proposed in the above paper.

The aim of this work is first to analyze the influence of the simultaneous presence of copper and amitraz on the final efficacy of treatments, and

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then to investigate on the possibility of direct molecular interaction between these compounds using the high sensitivity chemiluminescent method, prior to any other mechanisms.

Materials and methods

Reagents

AMZ [*N*-methyl-bis-(2,4-xylyliminomethyl)-amine] (molecular weight = 293; over 98% pure) was provided by Cluzeau Infolabo (France). Other reagents were of analytical grade from Sigma.

Toxicological experiments

Prior to AMZ treatments, hives were given 5 l of sucrose syrups, either pure or added with $CuSO_4$ (250 mg/l) as previously described (Guiraud *et al.* 1990). Three months later, after monitoring the rate of death of the parasites in both cases, a standard shock treatment by AMZ evaporation was applied to each hive. The number of parasites killed within 24 h was first recorded. Then the hives were gassed and the parasites remaining on adult bees and also on the brood were exhaustively counted in order to allow the final calculation of actual efficacies. Let S be the calculated effects of systemic treatment, from averaging over 24 h, A be the number of mites killed within 24 h following AMZ treatment and R be the residual number of parasites remaining in the hives after gassing the colonies. Then the actual effect of pure AMZ is given by $E_A = (A - S)/(R + A)$.

Hives were of the 'Dadant' model with 12 frames.

Chemiluminescence studies

Chemiluminescence intensity was measured by means of a M12FQC51 photomultiplier with a S20 cathode operating jointly with a Zeiss K-200 recorder (Germany). Chemiluminescence assays were performed in a glass cuvette mounted on the front of the photomultiplier and maintained at 295 K. Solutions were always freshly prepared for each series of experiments. The reaction mixture was composed of various combinations of AMZ, $CuSO_4$ and H_2O_2 in the methanol–0.1 M carbonate buffer, pH 9.6 mixture (1:1). Light emission during peroxidation was started by the rapid injection of H_2O_2. Reagents in the cuvette were always bubbled with air (CO_2 free). Each experiment was repeated three times. The intensity of the light emitted in a reaction was monitored as a function of time: $I = f(t)$. The light sum (ΣI) is the integrated relative intensity equal to the area under the kinetic curve of chemiluminescence.

$$\Sigma I = \int_0^{20\ \mathrm{min}} I(t)\mathrm{d}t$$

The quantum yields of chemiluminescence were determined according to Stauff & Schmidkunz (1963).

Statistical treatment of data

Means ± SD obtained from (N) determinations were compared using Student's t-test and the probabilities of significance associated to the conclusion (i.e. risk levels) were calculated from the equation of Student's t-distribution. In the case of large SDs, data have been compared using the non-parametric Mann–Whitney method: in this case, corresponding probabilities have been calculated from the equation of the unit normal distribution.

Results

Toxicological assays

Table 1 indicates the punctual (within 24 h) efficacy of treatments by either $CuSO_4$ alone or AMZ alone, compared with the association of both, in the case of hives without operculated brood. The immediate efficacy of the formamidine, which is a shock treatment, is indeed much higher than that of $CuSO_4$ ($P = 0.013$). The combination of both treatments yields a 90.5% higher efficacy, but with low significance, due to the large variability following treatments with AMZ alone.

Table 2 gives the results obtained in the case of hives containing operculated alveoli. In this case, no significant difference was observed between individual treatments, whereas the combination of both gave significantly higher levels of efficacy.

Table 1. Action of $CuSO_4$ feeding on a punctual AMZ treatment in hives without operculated brood

$CuSO_4$ alone	AMZ alone	AMZ and $CuSO_4$
1.11 . [6]	45.0 [38]	94.0 [80]
0.97 [8]	95.8 [71]	91.5 [174]
0.87 [9]	6.0 [85]	99.0 [194]
0.99 [19]	7.5 [188]	98.6 [327]
0.74 [187]	94.6 [222]	98.8 [340]
2.48 [69]	28.0 [654]	
	18.0 [1019]	
1.193 ± 0.642	42.128 ± 38.570	96.380 ± 3.432
(N = 6)	(N = 7)	(N = 5)
t-test	⟵ $P < 0.001$ ⟶	
	⟵ $0.001 < P < 0.01$ ⟶	
Mann–Whitney test	⟵ $0.001 < P < 0.01$ ⟶	
	⟵ $0.02 < P < 0.03$ ⟶	

The total numbers of parasites involved are given in square brackets. Means ± SD are calculated for (N) hives. All data are equivalent to percents of parasites killed within 24 h in one hive.

Table 2. Action of $CuSO_4$ feeding on punctual AMZ toxicity to *V. jacobsoni* in hives with operculated brood

$CuSO_4$ alone	AMZ alone	AMZ and $CuSO_4$
0.88 [24]	2.7 [368]	40.0 [5]
0.97 [23]	6.6 [878]	33.0 [3]
0.65 [17]	0.8 [126]	14.3 [7]
0.71 [22]	1.1 [260]	12.5 [8]
0.97 [39]	0.5 [386]	40.0 [5]
0.84 ± 0.15	2.34 ± 2.53	27.96 ± 13.61
	← 0.001 < P < 0.01 →	
← 0.001 < P < 0.01 →		

The total numbers of parasites involved are given in square brackets. Means ± SD are calculated for (N=5) hives. All data are equivalent to percents of parasites killed within 24 h in each hive.

It is noteworthy that the presence of operculated alveoli (i.e. of parasites protected from the shock treatments) strongly decreases the efficacy of AMZ, either alone ($P = 0.016$) or in combination with copper ($P = 0.024$).

Molecular mechanisms

The autoxidation of AMZ in the presence of oxygen is accompanied by a weak light emission. The quantum yield of the emission was estimated to be about 10^{-12} photons molecule^{-1} of AMZ. The addition of 0.05 mM $CuSO_4$ results in a decrease of the chemiluminescence intensity. In contrast, the addition of 2 mM H_2O_2 to the above mentioned solution of AMZ leads to a large increase in both of the light intensity (by a factor of 10) and of quantum yield (10^{-9} photons molecule^{-1} of AMZ). This emission is also decreased in the presence of $CuSO_4$. Courses of the chemiluminescent reactions are shown in Figure 1.

The light sum of the chemiluminescence accompanying the oxidation of AMZ is strongly dependent on the AMZ and H_2O_2 concentration (Figure 2). These data allow us to choose, considering high sensitivity and reproducibility, the optimum conditions for the luminescent reaction during the study of the influence of $CuSO_4$ concentration on the light emission. Figure 3 shows the dose-related quenching effect of $CuSO_4$ on the chemiluminescence during peroxidation of AMZ.

Discussion

The efficacy of AMZ as determined within 24 h, i.e. a short-term or shock treatment, is almost double in the presence of cupric salt than with sucrose syrup alone in hives without brood. However, the large variability observed with AMZ alone affects the significance level of the comparison (Table 1).

When hives contain operculated brood, the efficacy is much reduced in all cases, since all parasites

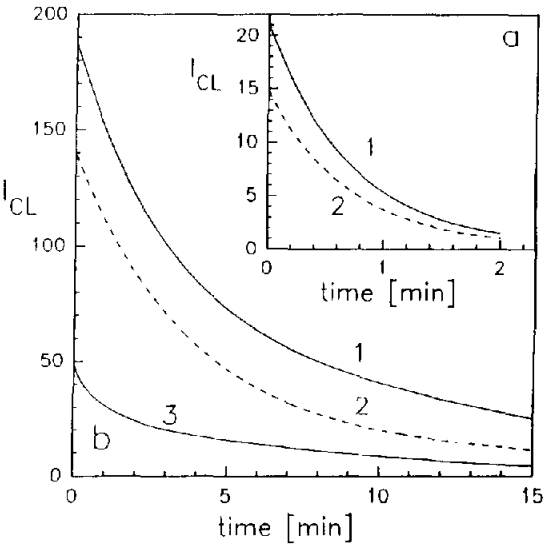

Figure 1. Kinetic curves of chemiluminescence accompanying oxidation of AMZ. (**a**) Curve 1, 1 mM AMZ; curve 2, 1 mM AMZ and 0.05 mM $CuSO_4$. (**b**) The same as (**a**) but in the presence of 2 mM H_2O_2. Curve 3, control reaction in the absence of AMZ.

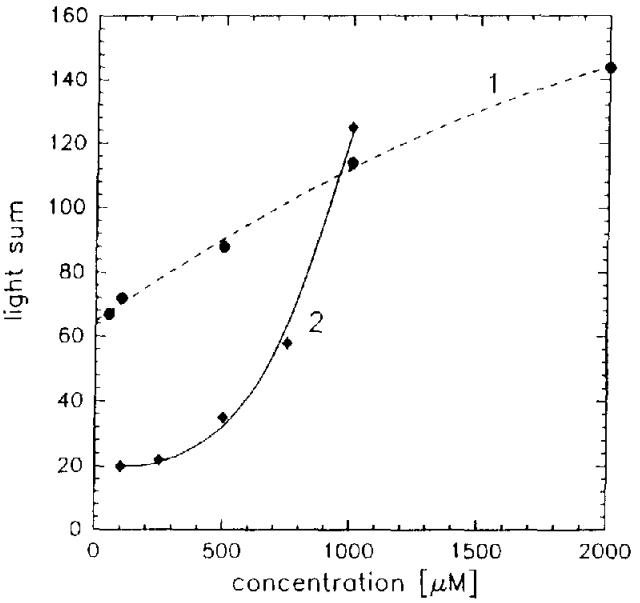

Figure 2. Light sum as a function of concentration of AMZ (curve 1) and H_2O_2 (curve 2). Conditions: curve 1, 1 mM H_2O_2, 0.05 mM $CuSO_4$; curve 2, 1 mM AMZ, 0.05 mM $CuSO_4$.

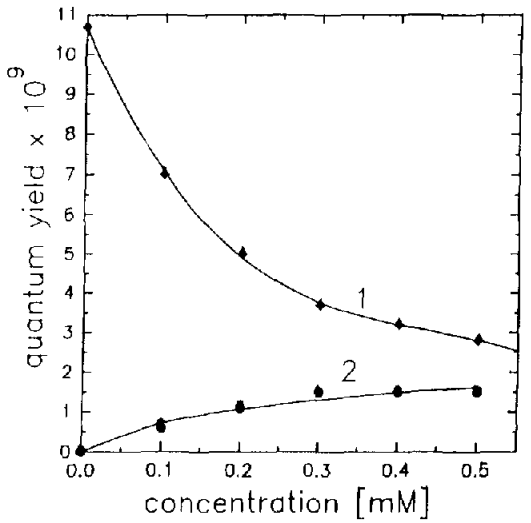

Figure 3. Effect of the $CuSO_4$ concentration on the quantum yield of chemiluminescence emitted during peroxidation of AMZ. Curve 1, 1 mM AMZ and 1 mM H_2O_2; curve 2, control reaction in the absence of AMZ.

in operculated alveoli are protected from the treatment (Table 2). In this case, the synergistic action of Cu^{2+} on AMZ treatment shows a good level of significance. The effect of associated treatments is much higher than the sum of separate ones, so that a true synergistic effect may be involved.

It should be noted that Cu^{2+} action is a long-term one, with a complete efficacy over several months (about 90% over 3 months) and that the toxicity of AMZ is characterized by a rapid shock effect. However, the data recorded within 24 h do not reflect the potential efficacy that can be reached after several days. This was not the aim of our work and it should be pointed out, moreover, that AMZ becomes toxic to bees during prolonged treatments (Cascino *et al.* 1989).

The general conclusion drawn from the chemiluminesence results is that AMZ can react with $CuSO_4$, forming a complex. This conclusion is based on the fact that we were able to isolate the $(AMZ)_2Cu^{2+}$ complex and proposed its chemical structure (Kuriata *et al.* 1992). Additionally, complex formation in low amounts is strongly supported by the observation that the chemiluminescence is decreased by $CuSO_4$ with a high efficiency during autoxidation and peroxidation of AMZ. In addition, the efficiency of the treatment of AMZ associated with $CuSO_4$, being much higher than the effect resulting from the sum of the efficiencies of separate compounds, confirms the conclusion that such complexes can be formed and then exhibit different physiological properties. This complex may be responsible for the observed enhancement of the parasites killed by AMZ (Table 1 and 2) in the presence of Cu^{2+} ions according to the Haber–Weiss reaction catalyzed by the cations (Czapski & Ilan 1978):

$$(AMZ)_2Cu(II) + O_2^{\cdot -} \longrightarrow (AMZ)_2Cu(I) + {}^1O_2$$

$$(AMZ)_2Cu(I) + H_2O_2 \longrightarrow (AMZ)_2Cu(II) + HO^{\cdot} + HO^-$$

In this reaction, it is assumed that $O_2^{\cdot -}$ reduces the Cu(II) ion. The reduced ion may react with H_2O_2 to yield the $HO^{\cdot}$ radical. The reaction can generate both $HO^{\cdot}$ radicals and $^1O_2$, which are known as initiators of lipid peroxidation in membranes.

Hydroxyl radicals react with organic compounds forming secondary free radicals that, in the presence of oxygen, yield peroxy radicals ($ROO^{\cdot}$) and hydroperoxides (ROOH). Therefore, they are expected to damage cell constituents (Singh 1982). This hypothesis is consistent with the literature data (Aronovitch *et al.* 1984) showing that Cu^{2+} enhances the killing of bacterial cells caused by ascorbate in the presence of H_2O_2. It has also been shown that copper complexes enhance DNA damage (Czapski & Goldstein 1987) either by addition to the biological target or the formation of oxygen species such as $^1O_2$ or $HO^{\cdot}$ in a cycle of oxidation–reduction reactions. Additionally, the coordination environment of the metal can strongly alter its effectiveness as a catalyst by increasing the valency state of the metal.

Since correlations between the increase of the light emission and H_2O_2 concentration (Figure 2) and the decrease of the light emission to 2% in the presence of 80 μg/ml catalase (which converts H_2O_2 to H_2O and O_2; data not given) are observed, reaction (2) may be responsible for the quenching effect exerted by copper ions on the quantum yield of the chemiluminesence (Figures 1 and 3). As feeding honeybees with cupric salts results in a long-lasting increase of copper concentration in their hemolymph (Navone-Nectoux 1990), it may also be suggested that the protective effect of cupric ions on the AMZ molecule might play at least one part in the observed synergistic interaction between these compounds.

In our experiments Cu^{2+} ions exert two independent effects on the generation of electronically excited light emitting molecules. Firstly, they can form a complex with AMZ protecting this compound from oxidative degradation and in this way decrease the intensity of the observed chemiluminesence.

Secondly, Cu^{2+} ions play a key role in the formation of highly reactive oxygen species such as $^1O_2$, $O_2^{\cdot -}$ and $HO^{\cdot}$. The participation of $^1O_2$ and the above mentioned oxygen radicals during oxidation of AMZ in the presence of Cu^{2+} ions has been confirmed by a study of the influence of $^1O_2$ quenchers as well as $HO^{\cdot}$ and $O_2^{\cdot -}$ inhibitors on the chemiluminesence (Kruk & Bounias 1992). In this previous paper we have also documented the formation of $^1O_2$ and $HO^{\cdot}$ by the spin-trapping method.

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