Acknowledgements

Many thanks to all the contract labs (BioChem agrar, Eurofins, ibacon, IES and RIFCON) and companies (Adama, BASF SE, Bayer AG, Dow AgroSciences, E. I. duPont de Nemours and Company, Sparta Research and Syngenta) for providing their data on honey bee brood testing. Thanks also belong to Dr. O. Jakoby (RIFCON) who performed the statistical analysis and M. Metz (RIFCON) for his remarks on the manuscript.

References

BECKER, R., C. VERGNET, C. MAUS, J. PISTORIUS, I. TORNIER, S. WILKINS, 2009: Proposal of the ICPBR Bee Brood Group for testing and assessing potential side effects from the use of plant protection products on honey bee brood. In: Hazards of pesticides to bees, 10th Internat. Symp. ICP-BR, Bucharest, Romania 2008, ed. by OOMEN, P.A., H. THOMPSON, Julius-Kühn-Archiv **423,** 43-44.

BECKER, R., J. LÜCKMANN, J. PISTORIUS, 2015: Effectiveness of method improvements of OECD GD 75 – Evaluation of the ICP-PR Bee Brood Working Group. In Hazards of pesticides to bees, 12th Internat. Symp. ICP-PR, Ghent (Belgium) 2014, ed. by OOMEN, P. A., J. PISTORIUS, Julius-Kühn-Archiv **450**, 83-92.

EFSA, 2013: EFSA Guidance Document on the risk assessment of plant production products on bees (Apis mellifera, Bombus spp. and solitary bees) (published on July 04, 2013, updated on 04 July 2014). - EFSA Journal **11(7)**: 3295, 268 p.

EPPO, 2010: EPPO STANDARDS PP1/170(4), Efficacy evaluation of plant protection products: Side-effects on honeybees. Bulletin OEPP/EPPO Bulletin **40**, 313-319.

LÜCKMANN, J., R. BECKER, 2016: Honeybee brood studies under field conditions: Is there a difference of the brood termination rate compared to semi-field studies? – Poster on the 26th Annual Meeting of SETAC Europe, 22-26 May 2015, Nantes, France.

LÜCKMANN, J., S. SCHMITZER, 2019: The Oomen bee brood feeding test – revision of the method top current needs and developments. Bulletin OEPP/EPPO Bulletin **49(1)**, 137-146.

OECD, 2007: Guidance document on the honey bee (*Apis mellifera* L.) brood test under semi-field conditions. Series of testing and assessment, Number 75, ENV/JM/MONO(2007)22, 27 p.

OOMEN, P. A., A. DE RUIJTER, J. VAN DER STEEN, 1992: Method for honeybee brood feeding tests with insect growth-regulating insecticides. Bulletin OEPP/EPPO Bulletin **22(4)**, 613–616.

SZCZESNIAK, B., E. PILLING, S. BOCKSCH, R. BECKER, J. LÜCKMANN, 2018: ICP-PR Bee Brood Working Group – Variability of brood termination rates in reference to validity criteria and limited effectiveness of method improvement in honeybee semi-field studies (OECD GD 75). In: Hazards of pesticides to bees, 13th Internat. Symp. ICP-PR, Valencia (Spain) 2017, ed. by OOMEN, P.A., J. PISTORIUS, Julius-Kühn-Archiv **462**, 111-115.

VON DER OHE, W., M. JAHNKE, D. LÜKEN, 2015: Referenzwertprojekt 2014 – 2015. Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), Institut für Bienenkunde Celle. Abschlußbericht. 21 S.

2.2.P Toxicity of oxalic acid on *in vitro* reared honeybee larvae

Lucia Sabová[1], Martin Staroň[2], Anna Sobeková[1], Dana Staroňová[2], Jaroslav Legáth[1], Rastislav Sabo[1]

[1]University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovakia

[2]Institute of Apiculture Liptovský Hrádok, Gašperíkova 599, 033 80 Liptovský Hrádok, Slovakia

Correspondence: rastislav.sabo@uvlf.sk

DOI 10.5073/jka.2020.465.044

Abstract

Varroa destructor is considered as a serious pest of honeybees (*Apis mellifera*) and its resistance to acaricides has been reported since the early 1990s. Because large colony loses are yearly reported from over the world, new methods of treatment for *Varroa* mites are still in focus of many scientists. In our bioassay, we determined the lethal concentration 72 h LC_{50} of 2.425% oxalic acid solution following single spray exposure of honeybee larvae under laboratory conditions (Guideline OECD 237, 2013).

Keywords: honeybee larvae, oxalic acid, spray exposure, OECD 237

Introduction

Oxalic acid (OA) is a naturally occurring carboxylic acid used worldwide in apiculture to control *Varroa destructor*. It´s mode of action of OA is unknown, but the direct contact between them is required (Aliano *et al*. 2006). Some authors attributed its acaricidal action partly to a sensitivity of this species to acid pH (Maggi et al. 2016; Nanetti 2017). The instructions for administration of the authorised veterinary medicinal products with OA as an active ingredient recommend spraying,

trickling and evaporation as three main application methods (EMEA 2003). Results from several studies showed the efficacy greater than 90% in honeybee colonies when broodless or almost broodless colonies have been treated with the tricking method (Gregorc and Planinc 2001; Charrière and Imdorf 2002; Nanetti *et al*. 2003). Observed efficacy in broodright colonies was only around 60% (Hatjina and Haristos 2005; Gregorc and Planinc 2004). When the oxalic acid treatment occurs in broodright colonies, honeybee larvae may be exposed to OA *via* diet and, potentially, *per* cuticula (by evaporation and/or spraying). Rapid and consistent distribution of oxalic acid dihydrate within a colony was shown by macro-computed tomography (Rademacher *et al.* 2017). Two *in vivo* studies showed adverse effect of oxalic acid on bee brood following direct spray application (Higes et al. 1999; Gregorc et al. 2004), but so far, toxicological data on individual *in vitro* reared bee larvae have not been available.

Materials and Methods

The honeybee larvae were reared *in vitro* using the methodology described by Aupinel et al. (2007) and Guideline OECD 237 (2013). Authorised veterinary medicinal products containing oxalic acid dihydrate with the recommended dosage of 0.3 ml of 3% (w/v)/dm2 comb have been licenced in many countries worldwide over recent years (EMA 2017, 2018). In our bioassay, we tested nominal concentrations of 0% (control), 0.87%, 1.75%, 3.5% and 7.0% of oxalic acid (VWR BDH Prolabo® Chemicals) in spraying form (recommended dosage of 0.3 ml of 3% (w/v)/dm2 comb is covered). Respective tested doses are 0 (control) µg OA/ larva, 16.1 µg OA/larva, 32.3 µg OA/larva, 64.6 µg OA/larva and 129.2 µg OA/larva. Twelve larvae from each of three colonies (12 larvae × 3 per tested group; n = 3), allocated on 48-well culture plate, were homogeneously sprayed with a manual sprayer (Lenz; NS 19/26) before feeding on day 4 with respective solution prewarmed on 37.5 °C from a 25 cm distance from the plate at right angle (90°). Control was sprayed with distilled water. Larval mortalities were checked and recorded at the time of feeding on days 5 and 6 and at the termination of the test on day 7 and are expressed in the number of dead larvae and in a corrected percentage according to Guideline OECD 237 (2013).

Results

The effects of spray exposure of oxalic acid on honeybee larvae reared *in vitro* were assessed according to Guideline OECD 237 (2013). The results (Tab. 1) showed the highest observed corrected mortality of 97% on day 6 in the tested nominal concentration of 7.0% and the lowest corrected mortality of 3.1% at the lowest tested concentration of 0.87% on day 7. Only the mortality observed in the lowest tested concentration of 0.87% showed no statistical significance compared to control. The established 72-h lethal concentration which kills 50% of tested individuals (LC50) following the single spray exposure of oxalic acid for *A. mellifera* larvae was 2.425% with a 95% confidence interval of 2.073–2.835 ($\chi 2 = 0.03753$; df = 2; slope = 4.19590; intercept = – 1.61392; $P(F) < 0.001$). The no-observed-effect concentration (NOEC) was estimated to be 0.87%.

Tab. 1 Mortality of honeybee larvae (*Apis mellifera carnica*) after single spray exposure of oxalic acid (dose-response test)

		Rearing day	5	6	7	Statistic	
Test concentration (nominal) [%]	**n**					**P (exact)**	**Significance***
0 Oxalic Acid	36	Mortality (larvae)	2	1	0	- - -	- - -
0.87	36	Mortality (larvae)	0	2	2	0.128	-
	-	Corrected mortality [%]**	0	0	3.1	- - -	- - -
1.75	36	Mortality (larvae)	3	7	2	<0.001	+
	36	Corrected mortality [%]**	2.9	21.2	27.3	- - -	- - -
3.50	36	Mortality (larvae)	5	22	1	<0.001	+
	-	Corrected mortality [%]**	8.8	72.7	75.8	- - -	- - -
7.00	36	Mortality (larvae)	27	8	0	<0.001	+
	-	Corrected mortality [%]**	73.5	97.0	97. 0	- - -	- - -

* Fisher`s Exact Binomial Test with mortality at 7 d: Two-sample comparisons between sample and control (Alpha is 0.050; one-sided greater); Ho (no effect) is accepted, if the probability p(exact) > Alpha;' p(exact) is the probability that the increase in category "Dead" observed in the treatment(s) is due to chance.
+ significant; - non-significant
- - - not relevant
** treatment response compensated using Abbott´s formula
n number of tested larvae

Conclusion

Oxalic acid is the active ingredient of several authorised veterinary medicinal products and is becoming more prevalent as a Varroa control method in apiculture around the world. According to the instructions for administration, they can be used on colonies with and without brood. In our study performed according to Guideline OECD 237 with a spray way of exposure on Day 4, we demonstrated a dose-response adverse effect of oxalic acid on honeybee larvae under the laboratory conditions. Despite the recommended spray application with 2.1% OA solution is slightly lower than LC_{50} observed in our study, it may be harmful to bee brood when present during application.

Funding information

Funding was provided by the Slovak Grant Agency VEGA (Grant Nos. 1/0858/16 and 1/0176/16) and by the National Reference Laboratory for Pesticides of University of Veterinary Medicine and Pharmacy in Košice, Slovakia.

References

ALIANO NP, ELLIS MD, SIEGFRIED BD (2006) Acute contact toxicity of oxalic acid to Varroa destructor (Acari: Varroidae) and their Apis mellifera (Hymenoptera: Apidae) hosts in laboratory bioassays. J Econ Entomol 99:1579–1582

AUPINEL P, FORTINI D, MICHAUD B, MAROLLEAU F, TASEI JN, ODOUX JF (2007) Toxicity of dimethoate and fenoxycarb to honey bee brood (Apis mellifera), using a new in vitro standardized feeding method. Pest Manag Sci 63:1090–1094

CHARRIÈRE JD, IMDORF A (2002) Oxalic acid treatment by trickling against Varroa destructor: recommendations for use in central Europe and under temperate climate conditions. Bee World 83:51–60

EMA (2017) EPAR summary for the public, EMA/567879/2017. https://www.ema.europa.eu/en/documents/overview/oxybee-eparsummarypublic_lv.pdf. Accessed 26 Sept 2018

EMA (2018) EPAR summary for the public, EMA/221765/2018. https://www.ema.europa.eu/en/documents/overview/danysbienenwohlepar-summary-public_en.pdf. Accessed 26 Sept 2018

EMEA (2003) Committee for veterinary medicinal products. Oxalic acid summary report, EMEA/MRL/891/03-FINAL. https://www.ema.europa.eu/en/documents/mrl-report/oxalic-acid-summary-reportcommitteeveterinary-medicinal-products_en.pdf. Accessed 26 Sept 2018

Abstracts: Poster

HATJINA F, HARISTOS L (2005) Indirect effects of oxalic acid administration by trickling method on bee brood. J Apic Res 44:172–174

HIGES M, MEANA A, SUÁREZ M, LLORENTE J (1999) Negative long-term effects on bee colonies treated with oxalic acid against Varroa jacobsoni Oud. Apidologie 30:289–292

GREGORC A, PLANINC I (2001) Acaricidal effect of oxalic acid in honeybee (Apis mellifera) colonies. Apidologie 32:333–340

GREGORC A, PLANINC I (2004) Dynamics of falling Varroa mites in honeybee (Apis mellifera) colonies following oxalic acid treatments. Acta Vet Brno 73:385–391

GREGORC A, POGAÈNIK A, BOWEN ID (2004) Cell death in honeybee (Apis mellifera) larvae treated with oxalic acid. Apidologie 35:453–460

MAGGI M, TOURN E, NEGRI P, SZAWARSKI N,MARCONI A, GALLEZ L, MEDICI S, RUFFINENGO S, BRASESCO C, DE FEUDIS L, QUINTANA S, SAMMATARO D, EGUARAS M (2016) A new formulation of oxalic acid for Varroa destructor control applied in Apis mellifera colonies in the presence of brood. Apidologie 47(4):596–605. https://doi.org/10.1007/s13592-015-0405-7

NANETTI A, BÜCHLER R, CHARRIERE J-D, FRIES I, HELLAND S, IMDORF A, KORPELA S, KRISTIANSEN P (2003) Oxalic acid treatments for Varroa control (review). Apiacta 38:81–87

NANETTI A (2017) Back to some basic knowledge on oxalic acid. COLOSS workshop: "Assessment of alternative methods for Varroa control" Bologna (Italy), 21–22 March 2017: 16.https://coloss.org/proceedings/COLOSS_Varroa_Workshop_2017.pdf. Accessed 09 Mar 2019

OECD 237 (2013) Guideline for the testing of chemicals no.237: honey bees (Apismellifera) larval toxicity test, single exposure. Section 2: effects on biotic systems. https://doi.org/10.1787/9789264203723-en.

RADEMACHER E, HARZ M, SCHNEIDER S (2017) Effects of oxalic acid on Apis mellifera (Hymenoptera: Apidae). Insects 8:84. https://doi.org/10.3390/insects8030084