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In vitro study on the inhibition of enzymatic systems in Italian and Carniolan honey bees by piperonyl butoxide new derivatives

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Abstract

In this study we measured *in vitro* honey bee enzyme activities in presence of the synergist piperonyl butoxide (PBO) and some new benzodioxole and dihydrobenzofuran derivatives through absorbance and fluorescence spectrophotometric assays, to determine if and to what extent detoxification systems and acetylcholinesterases are affected by these compounds. Both Italian (Italy) and Carniolan (Czech Republic) honey bees were tested. In the case of Italian honey bees the esterase activity was partially but significantly inhibited by almost all the tested products. In Carniolan honey bees only the dihydrobenzofuran derivative EN16-41 significantly inhibited esterases. Other enzymatic systems, such as cytochrome P450 monooxygenases (P450s) and glutathione S-transferases (GSTs), often involved in xenobiotics detoxification, as well as acetylcholinesterases (AchEs) seem not to be targeted by the studied synergists. These data seem to suggest that some of the investigated PBO analogues could not be detrimental to honey bees.

Key words: *Apis mellifera*, piperonyl butoxide, synergists, esterases, glutathione S-transferases, acetylcholinesterases, mixed function oxidases.

Introduction

Insect evolved quite efficient mechanisms to escape the negative effects of xenobiotics. In many cases metabolic pathways that evolved in insects to detoxify or to sequester natural plant toxins proved to be very flexible to act also against insecticides (Panini *et al*., 2016). For this reason resistance to xenobiotics may include changes in detoxification enzyme activities compared with that of susceptible insects (Khot *et al*., 2008; Li *et al*., 2007b).

Metabolic detoxification mechanisms in insects are mediated by three main groups of enzymes: cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), and carboxylesterases (CEs) (Li *et al*., 2007b; Panini *et al*., 2016).

Several studies have demonstrated that the above described families of enzymes contribute significantly to pesticide and secondary metabolite detoxification in honey bees (Papadopoulos *et al*., 2004; Johnson *et al*., 2006; 2012; Mao *et al*., 2011).

Compared to other insects such as *Drosophila melanogaster* Meigen and *Anopheles gambiae* Giles, honey bees contain only about half of genes coding for P450s, GSTs, and CCEs (Claudianos *et al*., 2006; Honey bee Genome Sequencing Consortium, 2006), although this does not necessarily reflect a greater sensitivity to insecticides (Hardstone and Scott, 2010) and does not prevent bees from carrying out the functions associated with these genes (Yu *et al*., 1984).

Synergists, at the concentration used, are non-toxic compounds that successfully control resistant pests when combined with insecticides by temporarily inhibiting the metabolic detoxification systems which lead to resistance (Metcalf, 1967; Ishaaya, 1993; Moores *et al*., 2005). They have been used commercially for decades to enhance the efficacy of insecticides and have contributed significantly to improving household insect control (Cox, 2002). Piperonyl butoxide (PBO) was the first effective and commercially viable synergist to be developed (Wachs, 1947). Due to its low toxicity to humans, domestic mammals and birds, PBO is employed in mixture with insecticides for household, medical and veterinary use (Breathnach, 1998; Keane, 1998). There is a limited application in agriculture so far: PBO is registered to be used in mixture with pyrethroids in Australia and USA (Panini *et al*., 2017a). In Europe ready mixed formulations of PBO and natural pyrethrum are available.

Methylenedioxyphenyl (MDP) compounds such as PBO are thought to inhibit insecticide detoxification being known as specific inhibitors of P450 oxidases (Sun and Johnson, 1960; Casida, 1970; Hodgson and Philpot, 1974; Testa and Jenner, 1981; Hodgson and Levi, 1998) and esterases (Khot *et al*., 2008; Moores *et al*., 2009) in a wide range of important pests, especially where resistance mechanisms are present (Gunning *et al*., 1998; 1999; Moores *et al*., 1998; Young *et al*., 2005; 2006). On the other side it is also known that PBO can reduce insecticide efficacy by inhibiting the biotrasformation of thionates to oxons (Li *et al.*, 2007a; Mohamadia *et al.*, 2010). PBO also enhances the toxicity of xenobiotic compounds through the increase of cuticular penetration (Sun and Johnson, 1972; Gunning *et al*., 1991; Kennaugh *et al*., 1993; Bingham *et al*., 2011; Joffe *et al*., 2012).

Beneficial insects such as pollinators may also suffer from an increased efficacy of insecticides due to the synergistic action of PBO, although the acute oral toxicity of the synergist alone to adult bees is relatively low (LD_{50} > 25 μg/bee) (Moores *et al*., 2012).

Some studies (Hagler *et al*., 1989; Johnson *et al*. 2006; 2013; Alptekin *et al*., 2015; Rinkevich *et al*., 2015) showed that a pre-treatment with PBO increases pyrethroid and acaricide toxicity to honey bees, but the synergist rates applied were considerably higher than those encountered in the field and the bees may have been compromised such that a minimal insecticide exposure would give mortality: it has been demonstrated that field concentration of PBO (100 ppm) that enhanced tau-fluvalinate toxicity on *Meligethes aeneus* F. does not produce short-term detrimental effects against bees, neither increasing mortality nor altering behaviour (Moores *et al*., 2012).

PBO is also known to increase the toxicity of neonicotinoids thiacloprid and acetamiprid, whereas no significant difference was observed in the case of imidacloprid, suggesting that honey bee P450s are not involved in the detoxification of imidacloprid (Iwasa *et al*., 2004).

Before advocating the use of synergists, it is essential to characterise their effects against the defensive enzymes of honey bees, both in terms of potency and to identify which detoxification enzymes are inhibited (Alptekin *et al*., 2015). In recent literature some novel PBO derivatives with modifications in the methylenedioxyphenyl (MDP) moiety, alkyl and polyether side chains that have a more specific and higher efficacy against resistance-associated enzymes have been reported (Philippou *et al*., 2013; Panini *et al.*, 2017a; 2017b).

The activity of esterase E4, in *Myzus persicae* Sulzer, in presence of PBO and several benzodioxole and dihydrobenzofuran derivatives, was studied by Philippou *et al.* (2013) resulting that PBO binds with esterase E4 to accelerate small substrates to the active-site, while acting as a blockade to larger, insecticidal molecules.

Recently, Philippou *et al*. (2016) tested new benzodioxole derivatives of PBO, including EN1-126, synthesized according to Moores *et al*. (2011), and found that MDP compounds with an alkynyl ether side chain have greater synergistic effect than PBO against resistant *M. persicae* and *M. aeneus* populations. These findings support Pap *et al*. (2001) who previously reported that structures equivalent to EN1-126 were four-fold more potent than PBO against resistant houseflies (*Musca domestica* L.).

Novel benzodioxole derivatives EN1-126, EN1-213, EN1-215, EN1-216, EN1-218 and dihydrobenzofuran derivatives EN16-41 and EN16-55 were synthesised as reported in Panini *et al.* (2017a).

Panini *et al*. (2017a) reported that benzodioxole derivatives showed greater inhibition for P450 oxidases (CYP6CY3) while dihydrobenzofuran derivatives showed greater *in vitro* efficacy than PBO against *M. persicae* esterase FE4, a variant of carboxylesterase involved in insecticide resistance mainly in Mediterranean populations of the green peach aphid (Rivi *et al.*, 2013).

In this study we measured the *in vitro* activity of the enzymes involved in insecticide detoxification (esterases, P450s, GSTs, and CCEs) in presence of synergists in not purified protein extracts from Italian and Carniolan honey bees. Also the potential inhibition of acetylcholinesterase (AChE) has been investigated in both populations. Being AChE a key enzyme in neurotransmission in insect central nervous system and belonging to the serine hydrolase group like carboxylesterases, it could be of particular interest to evaluate, if any, its inhibition by synergists.

In particular PBO and some derivatives, with modifications in the methylenedioxyphenyl (MDP) moiety, alkyl and polyether side chains, were evaluated through absorbance and fluorescence spectrophotometric assays to study if these compounds are able to inhibit the bees detoxification enzymes as it happens in already studied pests. The goal of this study is to determine if PBO and other novel synergists when applied against resistant pests can be detrimental to bees.

This results could aid the design of specific synergists in order to successfully control resistant pests without compromising the safety of pollinators and other beneficial insects.

Materials and methods

Insects

Both Italian and Carniolan honey bees have been used for biochemical analyses in order to compare the enzyme activity and the possible inhibition by the synergists in the two populations.

The Italian honey bees (*Apis mellifera ligustica* Spinola) were collected by hand in the morning in an apiary, maintained according to local good beekeeping practices, in Piacenza (Northern Italy) and immediately stored at −80 °C. Young hive workers were chosen for the experiments, in order to exclude any former contact with treated crops or gardens.

A batch of young Carniolan workers (*Apis mellifera carnica* Pollmann), collected in the same way as the Italian honey bees, was sampled in and apiary in Dol in the Central Bohemian region (Bee Research Dol Ltd., Czech Republic), and sent to Italy on dry ice. Bees were stored at −80 °C till use.

Materials

Piperonyl butoxide (PBO), its novel benzodioxole derivatives EN1-126, EN1-213, EN1-215, EN1-216, EN1-218 and the dihydrobenzofuran derivatives EN16-41 and EN16-55 were supplied by Endura SpA (Bologna, Italy) and synthesised according to protocols reported elsewhere (Moores *et al*., 2011; Panini *et al*., 2017a). All reagents used (pNO: 4-nitrophenyl octanoate; ATChI: acetylthiocholine iodide; DTNB: 5,5'-dithiobis(2-nitrobenzoic acid); PMSF: phenylmethanesulfonyl fluoride; EDTA: ethylenediaminetetraacetic acid; DTT: DL-dithiothreitol; GSH: L-glutathion reduced; CDNB: 1-chloro-2,4-dinitrobenzene; Triton™ X-100: t-octylphenoxypolyethoxyethanol; 7-EC: 7-ethoxycoumarin) were in analytical grade and purchased from Sigma.

Esterase, acetylcholinesterase and glutathione S-transferase activity assay

Esterase, acetylcholinesterase and glutathione S-transferase activity were measured in homogenized honey bee abdomens (in the case of esterase and glutathione S-transferase) or heads (in the case of acetylcholinesterase) according to the method by Badiou-Beneteau (2012) with some modifications.

One honey bee abdomen, with sting and venom gland removed, (esterase or glutathione S-transferase assays) or one head (acetylcholinesterase assay) was homogenized for three periods of 30 s with a Tissue Lyser LT homogenizer (Qiagen) in 200 µL of sodium phosphate buffer 50 mM pH 7.0 with 1 mM PMSF.

To solubilise the acetylcholinesterase from the membranes 1% (v/v) of Triton^{TM} X-100 was added to the extraction medium (Belzunces *et al*., 1988).

The homogenate was then centrifuged for 12 min at 9000 g at 4 °C, the supernatant was used as enzyme source and the protein concentration estimated with Bradford protein assay (Bradford, 1976) using a Biorad Protein Assay Kit with bovine serum albumin as standard and subsequently diluted to 0.6 $\mu g_{(protein)}\ \mu L^{-1}$ in the case of abdomen extracts, or 0.3 $\mu g_{(protein)}\ \mu L^{-1}$ in the case of head extract.

All enzyme assays were performed in triplicate in a transparent 96-well microtiter plate after pre-incubation of the homogenate for 10 min at 25 °C in the assay medium in the presence of synergists in acetone or acetone only (uninhibited control) and in the absence of the substrate. A blank was added to monitor the reaction in presence of substrate but in the absence of crude extract.

For esterase assay, the final volume of the well reaction was 250 µL: 125 µL of sodium phosphate buffer 50 mM pH 7.0; 25 µL of diluted homogenate (final concentration 0.06 $\mu g_{(protein)}\ \mu L^{-1}$); 50 µL of mixture of sodium phosphate buffer and PBO or derivatives or acetone (47.5 µL buffer + 2.5 µL PBO (stock 10 mM in acetone) or derivatives or acetone); 50 µL of 0.25 mM pNO.

For acetylcholinesterase assay, the final volume of the well reaction was 250 µL: 75 µL of sodium phosphate buffer 100 mM pH 7.0; 25 µL of diluted homogenate (final concentration 0.03 $\mu g_{(protein)}\ \mu L^{-1}$); 50 µL of mixture of sodium phosphate buffer and PBO (stock 50 mM in acetone) or derivatives or acetone (47.5 µL buffer + 2.5 µL PBO or derivatives or acetone); 50 µL of 0.075 mM DTNB (final concentration 0.015 mM); 50 µL of 2.5 mM ATChI (final concentration 0.5 mM).

For glutathione S-transferase assay, the final volume of the well reaction was 250 µL: 75 µL of sodium phosphate buffer 100 mM pH 7.0, EDTA 1 mM, DTT 1 mM, NaCl 10 mM; 25 µL of diluted homogenate (final concentration 0.06 $\mu g_{(protein)}\ \mu L^{-1}$); 50 µL of mixture of sodium phosphate buffer and PBO or derivatives or acetone (47.5 µL buffer + 2.5 µL PBO (stock 50 mM in acetone) or derivatives or acetone); 50 µL of 10 mM CDNB (final concentration 2 mM); 50 µL of 10 mM GSH (final concentration 2 mM).

The concentrations of PBO and derivatives in the esterase, acetylcholinesterase and glutathione S-transferase assays were selected as they resulted the minimum enzyme inhibiting doses in preliminary assays.

The kinetic of the reaction was read with a Thermo Scientific Multiskan™ GO Microplate Spectrophotometer every 5 s in absorbance at 405 nm in the case of esterases, 412 nm for acetylcholinesterases and 340 nm for glutathione S-transferases for 10 min at 25 °C.

All the analyses have been performed on at least 15 young honey bee workers of each subspecies belonging to the same apiary.

Oxidase activity assay protocol

Oxidase activity was measured by the deethylation of ethoxycoumarin in intact honey bee abdomens according to the method developed by Ullrich and Weber (1972) and adapted to microplate format by De Sousa (1995) with some modifications. Three honey bee abdomens, with sting and venom gland removed, were placed in a 2 mL Eppendorf tube containing 588 µL sodium phosphate buffer 100 mM pH 7.6 with a final concentration of 0.05 mM synergist (buffer only for the untreated control) and 12 µL of 20 mM 7-EC (final concentration 0.4 mM) and incubated with constant shaking for 1 hour at 30 °C. Abdomens floated in buffer only and buffer with 7-EC were used as untreated controls. The protein content of the reaction mix in which the abdomens were floated was quantified with the method developed by Bradford (1976) as for the previous assays.

An aliquot of the reaction mix (100 µL) was added to 100 µL of mixture of 100 mM glycine buffer pH 10.4 and ethanol (1:1 v/v) to stop the reaction.

Fluorescence endpoint was measured in three replicates in a 96-well microtiter plate (Cellstar, white) with a Tecan GENios Pro Multifunction Microplate Reader using 390 nm excitation and 490 emission filters. Oxidase activity was expressed as fluorescence units (FU) $\mu g^{-1}_{protein}$.

This analysis has been replicated in 10 assays for Italian honey bees and 11 assays for Carniolan ones.

Statistical analysis

All statistical analyses were performed with SPSS Statistics 21.0 (IBM).

Linear regressions were fitted to absorbance and time data to evaluate enzyme activities. Enzymatic activities for each treatment were compared between the two populations with Mann-Whitney non parametric test for independent samples. Kruskal-Wallis non-parametric test for independent samples was applied to enzymatic activity data in order to investigate any significant difference among different treatments.

Results and discussion

Esterase activity

The esterase activity (mOD min^{-1} μg^{-1} of protein) in Italian and Carniolan honey bees is shown in figure 1.

The esterase activities measured after pre-incubation with synergists or with acetone only in Italian and Carniolan honey bees were compared with Mann-Whitney non-parametric test for independent samples.

No significant differences have been detected between the two populations for any treatment (figure 1).

Significant differences between the activities measured after pre-incubation with synergists in acetone or with acetone only were present (Kruskal Wallis test for independent samples) in Italian honey bees ($\chi^2 = 71.75$; df = 6; $p < 0.000$) as well as in Carniolan ones ($\chi^2 = 25.55$; df = 6; $p < 0.000$). Complete pairwise comparisons among treatments are reported in tables 1 and 2. In Italian honey bees (table 1), significant differences in esterase activity were detected between the untreated control and all the products, except for EN1-213. Moreover the activity measured after treatment with EN16-41 was significantly different from each treatment, except for EN16-55. No evidence of statistically significant differences were present among the other synergists. In Carniolan honey bees (table 2), only EN16-41 produced significant differences from all treatments except for EN1-216.

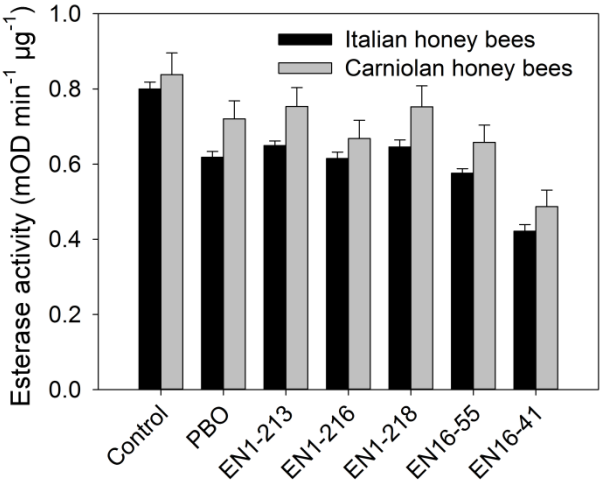

Figure 1. Comparison of esterase activity (mOD min^{-1} $\mu g^{-1}_{protein}$) in Italian and Carniolan honey bees.

Table 1. Statistics and significances of esterase activity pairwise comparison following incubation with different synergists in acetone or with acetone only (control) in Italian honey bees (significance level = 0.05; n.s.: not significant).

	Control	PBO	EN1-213	EN1-216	EN1-218	EN16-41	EN16-55
Control	-	3.925 / 0.002	2.874 / n.s.	3.941 / 0.002	3.163 / 0.033	8.013 / 0.000	5.367 / 0.000.
PBO		-	-1.051 / n.s.	0.016 / n.s.	-0.762 / n.s.	4.088 / 0.001	1.442 / n.s.
EN1-213			-	1.067 / n.s.	0.288 / n.s.	5.139 / 0.000	2.493 / n.s.
EN1-216				-	-0.778 / n.s.	4.072 / 0.001	1.426 / n.s.
EN1-218					-	4.850 / 0.000	2.205 / n.s.
EN16-41						-	2.645 / n.s.
EN16-55							-

Table 2. Statistics and significances of esterase activity pairwise comparison following incubation with different synergists in acetone or with acetone only (control) in Carniolan honey bees (significance level = 0.05; n.s.: not significant).

	Control	PBO	EN1-213	EN1-216	EN1-218	EN16-41	EN16-55
Control	-	1.524 / n.s.	1.056 / n.s.	2.210 / n.s.	1.116 / n.s.	4.616 / 0.000	2.395 / n.s.
PBO		-	-0.468 / n.s.	0.686 / n.s.	-0.408 / n.s.	3.092 / 0.042	0.871 / n.s.
EN1-213			-	1.154 / n.ss	0.060 / n.s.	3.560 / 0.008	1.339 / n.s.
EN1-216				-	-1.094 / n.s.	2.406 / n.s.	0.185 / n.s.
EN1-218					-	3.560 / 0.010	1.279 / n.s.
EN16-41						-	2.221 / n.s.
EN16-55							-

Residual activity (%) calculated as the ratio between activity in samples pre-incubated with synergist and those treated with acetone only (control) is plotted in figure 2. In Carniolan honey bees residual activities are always higher than in Italian ones. The maximum esterase inhibition was produced by dihydrobenzofuran derivative EN16-41 in both populations: 53% and 57% residual activity in Italian and Carniolan honey bees respectively.

Acetylcholinesterase activity

The acetylcholinesterase (AChE) activity (mOD min^{-1} μg^{-1} of protein) in Italian and Carniolan honey bees is shown in figure 3, and the residual activity (%) is shown in figure 4.

The AChE activity of Italian and Carniolan honey bees in the untreated control and after incubation with different synergists was compared with Mann-Whitney non-parametric test for independent samples: no significant difference between the two populations was detected.

Both populations showed no significant differences among the activities measured following a pre-incubation with the different synergists or with acetone only (Kruskal-Wallis non-parametric test for independent samples; Italian: $\chi^2 = 0.772$, df = 6, n.s.; Carniolan: $\chi^2 = 1.964$, df = 6, n.s.). AChE residual activity is always near 100% in Italian honey bees while a small, not statistically significant, reduction (10%) was observed in Carniolan honey bees after pre-incubation with both dihydrobenzofuran derivatives (EN16-41 and EN16-55) (figure 4).

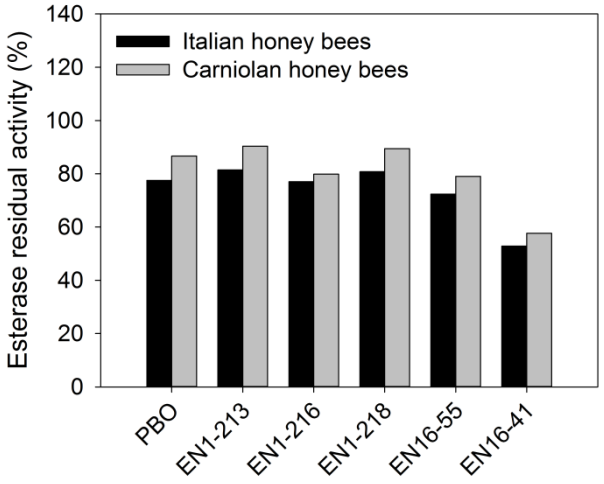

Figure 2. Residual total esterase activity (%) in Italian and Carniolan honey bees after pre-incubation with different synergists.

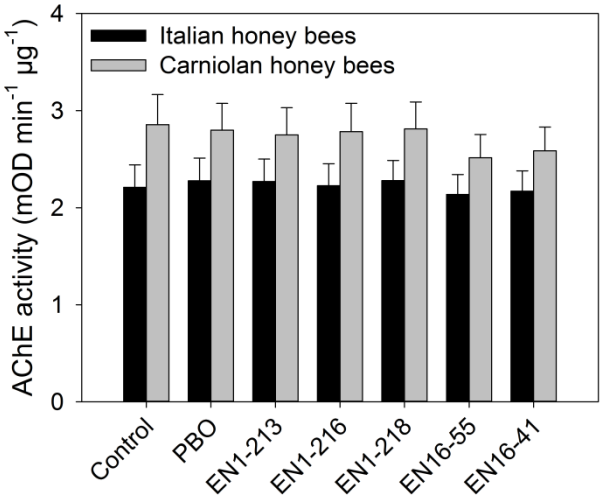

Figure 3. Comparison of acetylcholinesterase activity (mOD min^{-1} $\mu g^{-1}_{protein}$) in Italian and Carniolan honey bees.

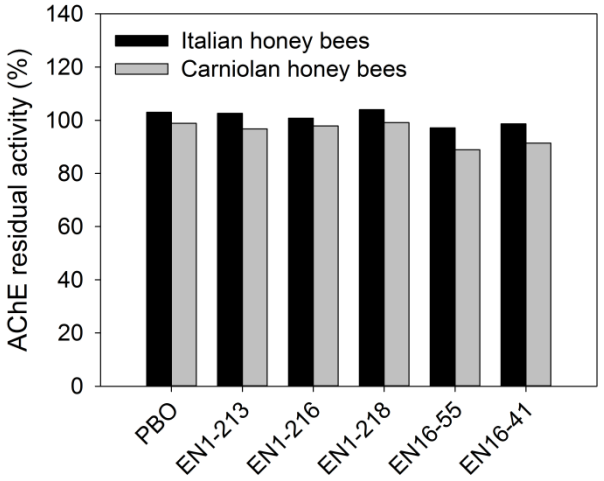

Figure 4. Residual acetylcholinesterase activity (%) in Italian and Carniolan honey bees after pre-incubation with tested synergists.

Glutathione S-transferase activity

The glutathione S-transferase (GST) activity (mOD min^{-1} μg^{-1} of protein) in Italian and Carniolan honey bees is shown in figure 5, and the residual activity (%) is shown in figure 6.

The comparison with Mann-Whitney non parametric tests of GST activity in Italian and Carniolan honey bees after treatment with synergists or with acetone only showed no significant difference between any of the activities in the two populations.

As observed with AChE, both populations showed no significant difference among the activities measured following pre-incubation with the different synergists or with acetone only (Kruskal-Wallis non-parametric test for independent samples; χ^2 = 5.133, df = 6, n.s.; Carniolan: χ^2 = 5.859, df = 6, n.s.). A limited and not statistically significant reduction of GST activity was observed in EN16-41 treated samples.

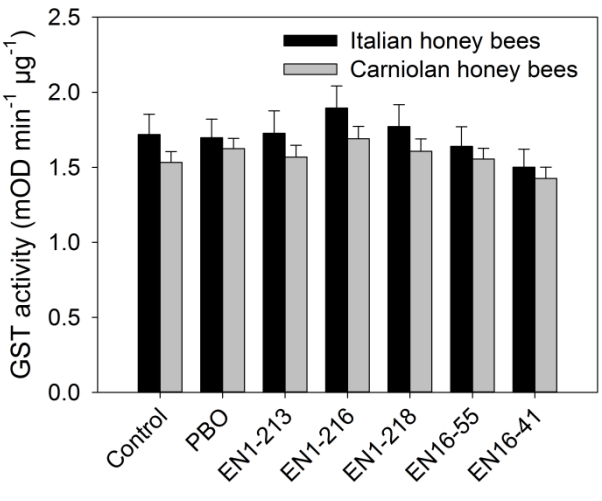

Figure 5. Comparison of glutathione S-transferase activity (mOD min^{-1} $\mu g^{-1}_{protein}$) in Italian and Carniolan honey bees.

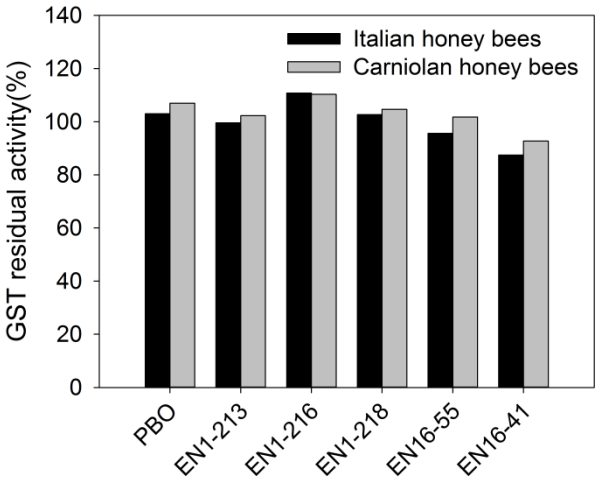

Figure 6. Residual glutathione S-transferase activity (%) in Italian and Carniolan honey bees after pre-incubation with tested synergists.

P450 oxidase activity

Oxidase activity was measured by the deethylation of ethoxycoumarin in intact honey bee abdomens due to the fact that the homogenization process releases midgut proteases that destroy the P450 activity before it can be measured (Alptekin *et al*., 2015). The deethylation of ethoxycoumarin reported as fluorescence units (FU) $\mu g^{-1}_{protein}$ in Italian and Carniolan honey bees is shown in figure 7. The residual activities (%) as the ratios between the activity measured in samples incubated with the synergist and the corresponding sample treated with acetone only are plotted in figure 8.

The oxidase activity was compared between Italian and Carniolan honey bees for each treatment with Mann-Whitney non-parametric test for independent samples, which showed no significant difference between the two populations.

The fluorescence units measured under different treatments within the two populations were analyzed with Kruskal-Wallis non-parametric test for independent samples. Both populations showed no significant difference among the different treatments (Italian: $\chi^2 = 6.945$, df = 7, n.s.; Carniolan: $\chi^2 = 4.116$, df = 7, n.s.).

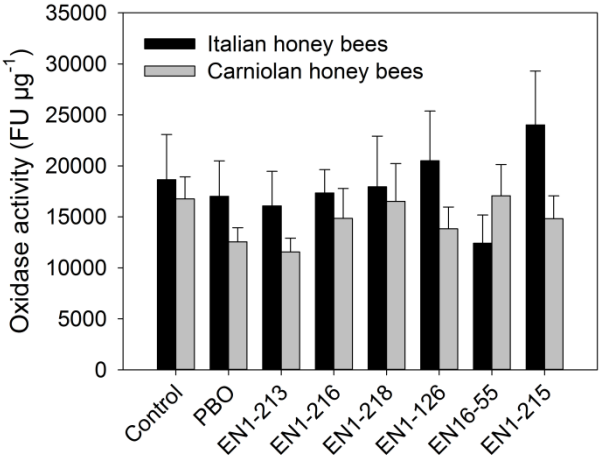

Figure 7. Deethylation of 7-ethoxycoumarin FU $\mu g^{-1}_{protein}$ in Italian and Carniolan honey bees.

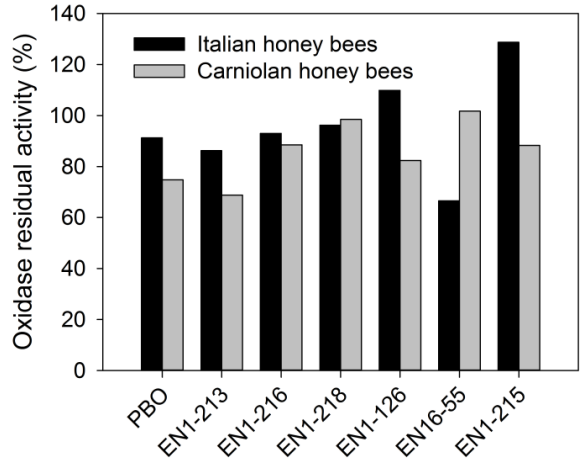

Figure 8. Residual oxidase activity (%) in Italian and Carniolan honey bees after pre-incubation with tested synergists.

High variability was observed among experiments; this could be explained by the fact that, as already reported in former studies, abdomen homogenization destroys P450s activity, therefore it was not possible to perform the test on a single bee (as done in the absorbance assays) while several different individuals were involved in a single assay, causing an increased variability in the results. The highest reduction of oxidase activity was observed with EN16-55 in Italian honey bees (residual activity = 67%) while this product did not affect oxidases of Carniolan honey bees. All the other products generally showed higher activity against Carniolan bees than against Italian ones.

Conclusions

The synergist PBO has been proved to successfully control resistant pests when combined with insecticides by temporarily inhibiting the detoxification systems which lead to resistance.

New synergists, starting from the molecular structure of PBO, have been developed to control resistant pests such as aphids and whiteflies, but before their practical application it is important to verify if pollinators could suffer from an increased efficacy of insecticides produced by inhibition of enzymatic detoxification systems.

The purpose of this study was to evaluate if some key enzymatic systems of the honey bee are affected by PBO and novel synergists that have been selected considering literature data on the inhibition of semi-purified and recombinant enzymes from the green peach aphid (Panini *et al*., 2017a) and the tobacco whitefly (Panini *et al*., 2017b).

The statistical comparison between the enzyme activities, uninhibited and after incubation with synergists, of Italian and Carniolan honey bees gave no significant difference for all the studied enzyme systems, which leads to the conclusion that the two populations are biochemically similar and comparable, although Rinkevich *et al*. (2015) found that Italian bees are more sensitive than Carniolan bees to some classes of insecticides. It is possible that small differences in the sequences of the genes coding for the target enzyme systems could account for the not statistically significant but constantly observed differences, *e.g.* in AChE.

In the case of Italian honey bees the esterase activity after incubation with synergists PBO, EN1-216, EN1-218, EN16-55 and EN16-41 was significantly different from the untreated control. The activity was reduced of 19-22% by PBO and EN1-nnn products and from 28% to 47% by EN16-nnn products. In the case of Carniolan honey bees the observed reduction was in the range 10-20% but only the dihydrobenzofuran derivative EN16-41 was significantly different from the untreated control with a 42% reduction of activity. The difference in esterase inhibition between the two populations could be ascribed to a different exposure of the colonies to pesticides, environment or genetic: however, it was slight and not significant.

Panini *et al*. (2017a) reported that dihydrobenzofuran derivatives EN16-55 and EN16-41 could partially in-

hibit *M. persicae* FE4 esterase as well as benzodioxole derivatives EN1-213, EN1-216 and EN1-218, although the latter are more efficient as P450s inhibitors.

Other enzymatic systems, such as P450s and glutathione S-transferases, directly implicated in the detoxification of xenobiotics, and acetylcholinesterases seem not to be targeted by the studied synergists at the applied concentrations.

On the contrary, benzodioxole derivatives EN1-126, EN1-213, EN1-216 and EN1-218 revealed potent inhibition of P450 enzyme coded by CYP6CY3 gene in *M. persicae* (Panini *et al*., 2017a).

The apparent contrast with results of former studies (Hagler *et al*., 1989; Johnson *et al*., 2006; 2013; Alptekin *et al*., 2015; Rinkevich *et al*., 2015) which showed that PBO increased pesticide toxicity to honey bees may be explained by different synergist rates applied and by the intrinsic variability of the oxidase assay.

This data could lead to the preliminary conclusion that the use of PBO and some of these novel derivatives may not be detrimental to the studied honey bee enzymatic systems but further investigations will be necessary for dihydrobenzofuran derivatives that proved to have quite high effects against honey bee esterases and in some extent also against monoxygenases.

Laboratory acute oral and contact toxicity tests carried out at the Bee Research Institute in Dol (Czech Republic), on honey bee workers belonging to the same breed of Carniolan honey bees tested for enzyme inhibition in the present work, showed no significant difference between the mortality after exposure to the insecticides imidacloprid and alpha-cypermethrin alone and combined with PBO and benzodioxole derivatives, and a very low toxicity of the synergists alone (Titěra *et al*., unpublished data/personal observation).

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