Biological Conservation 221 (2018) 114–117

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon

Short communication

Benzimidazole fungicides are detrimental to common farmland ants

Petr Heneberg[a,*], Jakub Svoboda[b], Pavel Pech[b]

[a] Charles University, Third Faculty of Medicine, Prague, Czech Republic
[b] University of Hradec Králové, Faculty of Science, Hradec Králové, Czech Republic

ARTICLE INFO

Keywords:
Carbendazim
Epoxiconazole
Flusilazole
Fuberidazole
Hymenoptera
Thiophanate

ABSTRACT

The impacts of pesticides on biodiversity of off-target organisms are neglected, and current conservation strategies pay little attention to these issues. Particularly the sublethal effects of chemical compounds used in agriculture and forestry are poorly understood, despite they may have detrimental effects on populations of the affected off-target organisms. Here, we tested the effects of 11 benzimidazole fungicides. Benzimidazole fungicides are still being widely used as broad-spectrum plant fungicides in agriculture since 1960s. We tested their effects on the fecundity and survival of queens of *Myrmica rubra*, a common farmland ant species, following the oral administration of the fungicides for a period of six weeks. We found that ethanolic solutions, as well as water suspensions of all but one tested benzimidazole fungicides, inhibited significantly the fecundity of *M. rubra* queens; in addition, the flusilazole was lethal when administered at 1.0 and 0.1 mg ml^{-1}. Combined data suggest that the whole class of benzimidazole fungicides has detrimental sublethal effects on the tested ant species and substantially decrease the fitness of the study species. The fact that common farmland species suffer from the effects the application of any of the whole class of fungicides questions their currently widespread use as agricultural fungicides. These findings bring into question the safety of agricultural benzimidazole fungicides and we recommend that their routine spraying should be re-assessed by regulatory agencies.

Three recent meta-analyses suggest that pesticide impacts on biodiversity have frequently been under-estimated, and current conservation strategies pay little attention to these issues (Dudley et al., 2017). Some studies that were published recently in this journal even provided surprising evidence for beneficiary effects of fungicides for the abundance of bumblebees and butterflies (Muratet and Fontaine, 2015). On the contrary, other studies argued against species-specific increases in abundance of most farmland butterflies (Rands and Sotherton, 1986).

In comparison to field observational data, experimental studies conducted under controlled laboratory conditions mostly focus on acute lethality of the tested compounds, which is also in agreement with the regulatory requirements. In contrast to acute lethal doses, sublethal effects of pesticides are poorly understood despite strong effects on populations of the affected off-target organisms (Desneux et al., 2007). Among the well-studied exceptions are neonicotinoid insecticides, which were recently shown to be behind a decline of the abundance of pollinators (Kessler et al., 2015; Rundlöf et al., 2015; Stanley et al., 2015; Straub et al., 2016) and insectivorous birds (Hallmann et al., 2014). In contrast to neonicotinoids, which received enormous attention in recent years, sublethal effects of other chemical compounds used in agriculture and forestry are heavily underreported. Previously, Pech and Heneberg (2015) have shown that a fungicide benomyl displays severe sublethal effects when applied to queens of common red ant *Myrmica rubra*, a common polydomous Palearctic species that lives in meadows and gardens. By a systematic extension of this rather accidental observation, here we aim to test, whether the off-target insecticidal effects of benomyl represent a common feature of the whole class of the benzimidazole fungicides, which are still being widely used in agriculture since the 1960s.

We used an experimental model that involved queens of *Myrmica rubra*, a common farmland ant species. We collected the queens in the Hradec Králové (50.19°N, 15.86°E, 243 m a.s.l.) and Jaroměř (50.34°N, 15.92°E, 253 m a.s.l.) environments. In total, we examined 220 *M. rubra* queens. Each queen was kept individually in a Petri dish and fed every three or five days by honey and *Acheta domestica* (Linnaeus, 1758) (Orthoptera: Gryllidae), and was supplemented with water ad libitum. We plugged the vials of water with cotton wool, which was in direct contact with the water beneath. The water was supplemented or not with indicated benzimidazole fungicides, which were provided either as a solution in ethanol (5% final concentration) or as a suspension in water. All tested fungicides were applied at a concentration 1.0 mg ml^{-1}. Flusilazole turned to be 100% lethal at this concentration, thus we performed the repeated experiments with flusilazole at a concentration 0.1 mg ml^{-1}, which, however, was lethal as well (see the

* Corresponding author at: Charles University, Third Faculty of Medicine, Ruská 87, CZ-100 00 Prague, Czech Republic.
E-mail address: petr.heneberg@lf3.cuni.cz (P. Heneberg).

https://doi.org/10.1016/j.biocon.2018.03.004
Received 4 December 2017; Received in revised form 1 March 2018; Accepted 5 March 2018

Results chapter below). As we aimed to prevent any side effects that could be caused by other compounds admixed in commercially distributed fungicide formulations, we used only pure chemicals, mostly analytical standards that were provided by Sigma-Aldrich (St. Louis, MO). We used the following benzimidazole fungicides (catalogue numbers and the purity of the tested lots are indicated): 2-(2-chlorophenyl)benzimidazole (chlorfenazole) (632,600; 99.8%), albendazole (A4673; 99%), benomyl (381,586; 95%), carbendazim (378,674; > 99%), epoxiconazole (36,848; 98.996%), flusilazole (45,753; 99.804%), fuberidazole (45,515; 99.450%), prochloraz (45,631; 98.634%), thiabendazole (T5535; 99.8%), thiophanate-diethyl (Diethyl-4-4-*O*-Phenylene-bis[3-thioallophanate]) (PS223; 99.739%), and thiophanate-methyl (45,688; 99.317%).

Benzimidazoles are heterocyclic aromatic compounds that consist of the benzene ring and imidazole. They were introduced as broad-spectrum plant fungicides in the 1960s and early 1970s, and later they started to be used as veterinary and human antihelminthic, anticestodal and antiprotozoal agents. Their mechanism of action includes binding to β-tubulin in microtubules. Thus, they suppress cell proliferation and division, the assembly of spindle microtubules and chromosomal alignment at the metaphase plate, and leading to nondisjunction, chromosome and chromatid loss (De Brabander et al., 1976; Gupta et al., 2004; Koo et al., 2009; Rathinasamy and Panda, 2006; Venkatesan, 1998; Zhou et al., 2016). Despite the high evolutionary conservation of microtubules, it is believed that the previously reported preferential activity of benzimidazoles and their metabolites on fungi and parasites is mediated by their selectivity for the microtubules of these organisms over those of humans, other vertebrates or insects (Crawford and Franco, 1994). Here we tested a broad spectrum of benzimidazole fungicides, ranging from those that are currently widely used in agriculture (epoxiconazole, flusilazole, fuberidazole, prochloraz (not registered in the U.S.), thiophanate-diethyl and thiophanate-methyl) and human/veterinary medicine (thiabendazole) or in human/veterinary medicine only (albendazole), through those that were withdrawn from their use in agriculture in some countries (benomyl, carbendazim) to those that are not in use currently (2-(2-chlorophenyl) benzimidazole).

We performed three runs of the experiments. The first two, performed in July–August 2015, consisted of the treatments with fungicides dissolved in 5% ethanol, and differed only in a frequency of feeding (every three or five days). The follow-up experiments, performed in July–August 2016, consisted of the treatments with fungicides supplemented as a suspension in water; the frequency of feeding was every three days. During the experiments, we housed the queens in a shady area, with the temperatures varying from 10 to 30 °C according to the ambient weather conditions. We terminated the experiments after six weeks, when the first larvae hatched (because having the eggs-eating larvae in the experiment would compromise the resulting egg counts), and immediately counted the eggs and evaluated the fecundity of each queen. We also recorded the survival of the queens during the experiments. We provide the study flowchart of the study design in Fig. 1A. We tested every treatment on 7–15 *M. rubra* nests, each with a single macrogyne queen. Data are shown as the means ± SE unless stated otherwise. We used one-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks (the choice was made based on outcomes of the Shapiro-Wilk normality tests and Equal variance tests), which were followed by Student-Newman-Keul's post-tests in case of equal treatment group sizes, or Dunn's post-tests in case of unequal treatment group sizes. We used one-tailed Student's *t*-tests to analyze the differences in fecundity between the solvents used, and the Fisher exact tests to analyze the survival of the queens.

We found that ethanolic solutions of all tested benzimidazole fungicides, except prochloraz, inhibited significantly the fecundity of *M. rubra* queens when provided at 1 mg ml^{-1} (Fig. 1B–C; feeding every three days: Shapiro-Wilk normality test $p < 0.05$; Equal variance test $p > 0.05$; one-way ANOVA $F = 6.3$, $d_f = 5$, $p < 0.001$, Student-Newman-Keul's post-tests vs. solvent-treated group $p < 0.05$ each except prochloraz; feeding every five days: Shapiro-Wilk normality test $p < 0.05$; Kruskal-Wallis one-way ANOVA on ranks $H = 22.0$, $d_f = 6$, $p = 0.001$, Student-Newman-Keul's post-tests vs. solvent-treated group $p < 0.05$ each). Prochloraz displayed a similar trend (6.75 ± 1.30 eggs per queen in the treated group, $n = 12$, and 9.10 ± 1.14 eggs per queen in the control group, $n = 10$) but had the weakest effect. Due to the variability among the queens, the difference between the prochloraz- and solvent-treated groups was not significant (Student-Newman-Keul's post-test $p > 0.05$) as the experiment was not sufficiently powered to examine these rather minor effects ($n = 29$ would be needed to have 95% power under these settings).

We found that suspensions of all tested benzimidazole fungicides in water inhibited significantly the fecundity of *M. rubra* queens when provided at 1 mg ml^{-1} (Fig. 1D; Shapiro-Wilk normality test $p < 0.05$; Kruskal-Wallis one-way ANOVA on ranks $H = 46.4$, $d_f = 6$, $p < 0.001$, Dunn's post-tests vs. solvent-treated group $p < 0.05$ each). As the previous tests revealed very strong effects of flusilazole, in this experiment, we decreased the flusilazole concentration by one order of magnitude to 0.1 mg ml^{-1}, but still, flusilazole-treated queens under these settings produced no eggs (Fig. 1D). Thus, when provided in water suspension, all tested compounds (we did not test prochloraz under these settings) displayed very strong effects. In all cases, the mean numbers of eggs produced per queen decreased below one egg produced per queen during the whole treatment period, whereas the control solvent-treated queens produced 26.7 ± 2.0 eggs per queen (Fig. 1D).

In contrast to fecundity, the survival of the queens was only marginally affected by any of the treatments, except flusilazole. Control groups displayed 100% (water) or 97% (5% ethanol) survival. Five of the 17 treated cohorts (29%) also displayed 100% survival, other nine cohorts displayed 8–43% mortality, for testing of which the experiments were not sufficiently powered. The treatment with thiophanate-methyl in water suspension was associated with 50% mortality ($n = 12$), which was significantly higher than that in the control solvent-treated cohort ($n = 15$; Fisher exact test $p = 0.01$). And both cohorts treated with flusilazole displayed 100% mortality; the first flusilazole-treated cohort received 1 mg ml^{-1} of flusilazole in 5% ethanol (Fisher exact test $p < 0.001$), and the second flusilazole treated cohort received 0.1 mg ml^{-1} of flusilazole suspension in water (Fisher exact test $p < 0.001$; Fig. 1C–D).

During the first year of the experiments, we noticed that the overall fecundity was lower than expected and that the control solvent-treated cohorts produced only 9.1 ± 1.1 and 3.1 ± 0.7 eggs per queen when fed and treated every three and five days, respectively. Thus, assuming that the solvent itself may be associated with a decrease in the fecundity of examined queens, the next year, we used suspensions of the fungicides in water instead of their ethanolic solutions. That year, we tested two control cohorts, the water-treated cohort, and 5% ethanol-treated cohort. The results have clearly shown that 5% ethanol itself decreases the fecundity of *M. rubra* queens, as their fecundity was six-times lower when treated with 5% ethanol (4.5 ± 0.8 eggs per queen) compared to water only (26.7 ± 2.0 eggs per queen; *t*-test $p < 0.001$; Fig. 1D).

The study species is the most ecologically tolerant of all European *Myrmica* spp., and often occupies anthropogenic habitats, including those covered with anthropogenic waste, which they preferentially use as nest sites (Michlewicz and Tryjanowski, 2017). Despite the broad ecological valency of *M. rubra*, combined data suggest that the whole class of benzimidazole fungicides has detrimental sublethal effects on this ant species (Fig. 1A, bottom part). The evidence in this paper strongly indicates that benzimidazole fungicides decrease the fecundity of *M. rubra* queens, and thus substantially decrease their fitness. The finding of sublethal effects of the whole class of fungicides questions their currently widespread use as agricultural fungicides. The effects of chronic exposure to low concentrations of these compounds, as well as the possible existence of thresholds for acute intoxication, remain to be