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Evaluation of the toxicity of fungicides to flight muscle mitochondria of bumblebee (*Bombus terrestris* L.)

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ABSTRACT

Insects pollinate 75% of crops used for human consumption. Over the last decade, a substantial reduction in the abundance of pollinating insects has been recorded and recognized as a severe matter for food supply security. Many of the important food crops destined for human consumption are grown in greenhouses. A unique feature of greenhouse agriculture is the extensive use of fungicides to curb multiple fungal infections. The most widely used pollinating insects in greenhouses are commercially reared bumblebees. However, there is no data regarding the toxicity of fungicides to bumblebee mitochondria. To fill this gap in knowledge, we examined the effects of 16 widely used fungicides on the energetics of the flight muscles mitochondria of *Bombus terrestris*. We found that diniconazole and fludioxonil uncoupled the respiration of mitochondria; dithianon and difenoconazole inhibited it. By analyzing the action of these inhibitors on mitochondrial respiration and generation of reactive oxygen species, we concluded that difenoconazole inhibited electron transport at the level of Complex I and glycerol-3-phosphate dehydrogenase. Dithianon strongly inhibited succinate dehydrogenase and glycerol-3-phosphate dehydrogenase. It also strongly inhibited mitochondrial oxidation of NAD-linked substrates or glycerol 3-phosphate, but it had no effect on the enzymatic activity of Complex I. It may be suggested that dithianon inhibits electron transport downstream of Complex I, likely at multiply sites.

1. Introduction

Insects pollinate 75% of crops used for human consumption [1]. Over the last decade, a substantial reduction in the abundance of pollinating insects [2,3] has been recorded and recognized as a severe matter for food supply security. The toxic effects of novel pesticides are considered among the major factors contributing to the decline of pollinators [4–6]. The flight of pollinating insects requires enormous energy expenditure that is primarily served by the mitochondria oxidative metabolism [7,8]. Thus, it might be expected that even subtle impair of mitochondrial energetics of pollinating insects could reduce pollinators' flight activities, thereby decreasing the efficiency of pollination, or even contribute to pollinators' premature death by exhaustion. Indeed, the damage to mitochondrial bioenergetics was proposed to be a major mechanism of pesticides toxicity to insects [9].

Bumblebees are extensively used in greenhouses for pollinating tomatoes and other crops [10]; commercial rearing of bumblebees is one of the most dynamically developing sectors of agriculture [11]. A prominent feature of greenhouses is the abundant use of various fungicides to curb a large number of fungal infections. Outside of greenhouses, fungicides are widely used on fruit trees that are pollinated by bees. However, there is no data on the effect of fungicides on functioning of mitochondria in pollinating insects. The aim of this study is to fill this gap by investigating the effects of widely used fungicides on the energetics of bumblebee flight muscle mitochondria.

2. Materials and methods

2.1. Preparation of mitochondria

Isolation of flight muscle mitochondria from male bumblebees *Bombus terrestris* L. was performed exactly as described earlier [12]. Mouse liver mitochondria were isolated as described in [13]. The concentration of mitochondrial protein was determined using BCA assay ("Thermo Scientific," USA).

2.2. Mitochondrial respiration

The rate of oxygen consumption by isolated mitochondria was recorded using an "Oxygraph" system ("Hansatech Instruments", UK). All measurements were performed at 24 °C in 1 ml of incubation medium comprising 220 mM mannitol, 100 mM sucrose, 1 mM EGTA, 4 mM KH_2PO_4, 20 mM HEPES (pH 7.3), and respiratory substrates. Rotenone

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and antimycin A were added at 2 μM for the final concentration. Experiments were carried out in a range of oxygen concentration from about 100 nmol/ml to 250 nmol/ml to avoid oxygen depletion in the chamber, according to the recommendations given earlier [14,15].

The fungicides (chromatography-grade, 98.1–99.8% purity) were obtained from "RespektLab", Russia. The compounds were dissolved in DMSO; the effects of DMSO on flight muscle mitochondria were examined in separate experiments and found negligible (data not presented).

2.3. Mitochondrial membrane potential

The membrane potential of isolated mitochondria was evaluated by following the changes in fluorescence of membrane potential probe Safranin O with the use of spectrofluorometer Hitachi F-7000 (Hitachi, Japan) [16]. The excitation wavelength was 495 nm, emission 586 nm. Incubation medium (1 ml) comprised 220 mM mannitol, 100 mM sucrose, 1 mM EGTA, 4 mM KH_2PO_4, 0.2 mg/ml bovine serum albumin fatty acid free, 20 mM HEPES (pH 7.4), 0.1–0.12 mg of mitochondria protein, 2–4 nmol Safranin O, and 10 mM glycerol 3-phosphate (racemic mixture).

2.4. Mitochondrial ROS production

Measurement of H_2O_2 emission rate was performed with fluorescent dye Amplex Red Ultra (Sigma, USA) as described in [17]; incubation medium (1 ml) comprised 220 mM mannitol, 100 mM sucrose, 1 mM EGTA, 4 mM KH_2PO_4, 20 mM HEPES (pH 7.4), 2 μM Amplex Red, 0.1–0.2 mg of mitochondria protein and 1 mg/ml horseradish peroxidase; excitation was set at 568 nm and the emission was registered at 581 nm.

2.5. Enzyme activity

Succinate dehydrogenase (SDH) activity was measured in mitochondria isolation media supplemented with 10 mM succinate, 0.2 mM KCN, 100 μM 2,6-dichlorophenolindophenol (DCPIP), 100 μM decylubiquinone and 0.2 mg mitochondria protein. The enzyme activity was recorded with a Hitachi U-2900 spectrophotometer (Hitachi, Japan) and a decrease in absorbance of DCPIP at 600 nm for 1 min was observed. The activity was expressed in nmol DCPIP/min/mg protein. Mitochondrial glycerol 3-phosphate dehydrogenase (GPDH) was measured similarly except that the succinate was substituted with 10 mM glycerol 3-phosphate.

NADH dehydrogenase (NADH-DH) activity was measured in a mitochondria isolation media supplemented with 100 μM NADH, 100 μM decylubiquinone and 0.2 mg mitochondria. A decrease in NADH absorbance was recorded with spectrophotometer Hitachi U-2900 set on 340 nm, for 1 min reaction time. Activity was expressed in nmol NADH/min/mg protein.

Table 1
Effect of fungicides on the rate of flight muscle mitochondrial respiration *B. terrestris* ($n = 6$).

Fungicides	Respiratory substrate – glycerol 3-phosphate (10 mM)		Respiratory substrate – pyruvate (5 mM) + glutamate (5 mM)	
	Inhibition	Activation	Inhibition	Activation
Diniconazole	−	+	−	+
Dithianon	+	−	+	−
Difenoconazole	+	−	+	−
Fludioxonil	−	+	−	+
Hymexazol, iprodione, carbendazim, penconazole, procymidone, thiabendazole, thiram, triadimefon, fluatriafol, chlorothalonil, cyproconazole, epoxiconazole.	No effect			

2.6. Statistical analysis

Statistical analysis was performed with MS Excel statistics package; the data are presented as means with standard errors. The significance

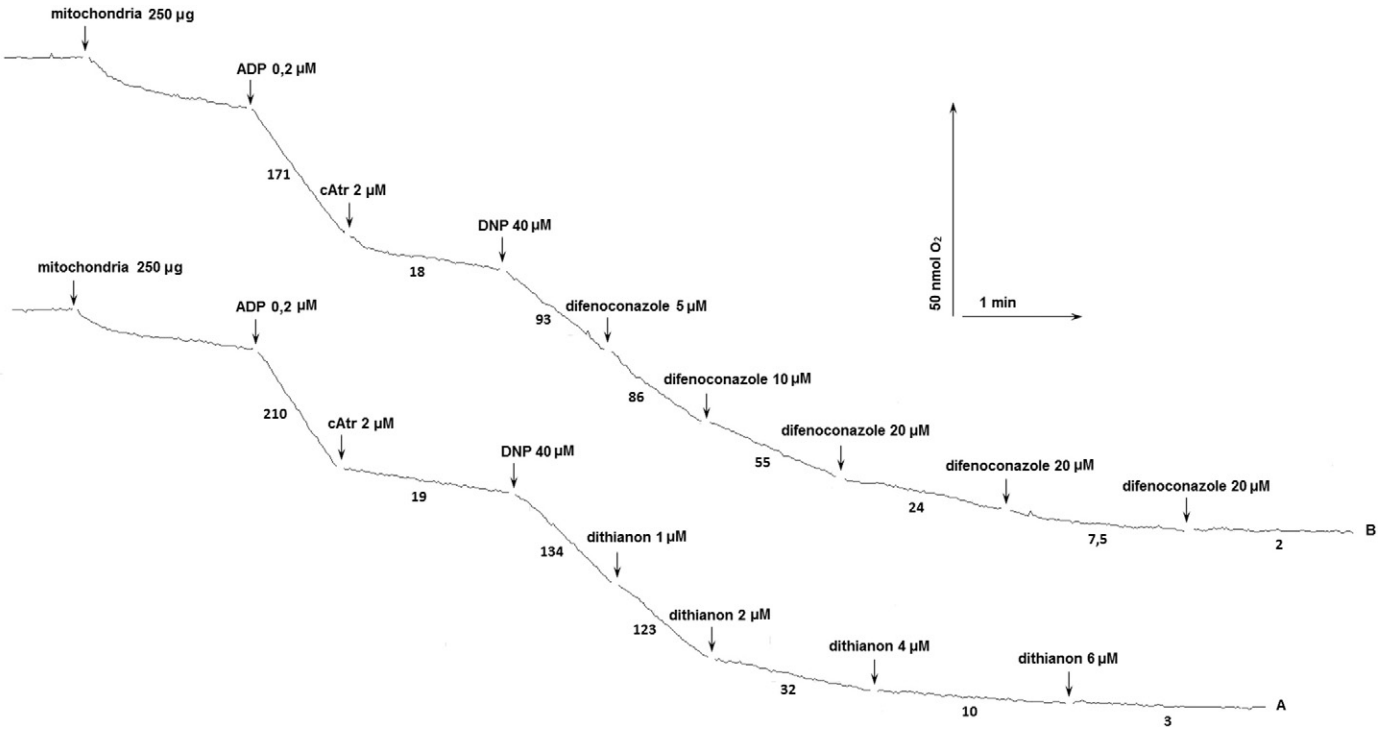

Fig. 1. The rates of respiration of flight muscle mitochondria oxidizing 10 mM glycerol 3-phosphate. The values under the curves are the rates of oxygen consumption expressed in nmol O_2/min/mg protein. Abbreviations: kAtr, carboxyatractylate; DNP, 2,4-dinitrophenol.