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Genetic Diversity Loss in *Chironomus sancticaroli* (Diptera: Chironomidae) Exposed to Pyrimethanil Fungicide: an Analysis Using RAPD Technique

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Abstract In the agricultural cultivation, the Pyrimethanil (4,6-dimethyl-N-phenyl-2-pyrimidinamine) fungicide is one of the most widely used compound in monocultures and has been detected in aquatic ecosystems. These genotoxic products increase the frequency of lesions in the DNA molecule, thereby increasing the risk of replication and transcription of altered DNA sequences. This study aimed to assess the loss of genetic diversity of *Chironomus sancticaroli*, a Brazilian native insect species, exposed to Pyrimethanil fungicide, using RAPD-PCR (random amplified polymorphic DNA) technique. The results showed that there was significant loss in the genetic diversity in the organisms exposed to high Pyrimethanil concentrations when compared with control site. Our findings reveal that RAPD-PCR is an effective method to access genetic loss derived to fungicide use and that the agriculture application may lead to a decrease in aquatic biota genetic diversity. This finding has important implications for conservation strategies and ecological management environments.

Keywords Stream · Chironomidae · Agriculture cultivation · Genetic diversity

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1 Introduction

In the context of agricultural land use, the Pyrimethanil (4,6-dimethyl-N-phenyl-2-pyrimidinamine) is one of the fungicides most widely used in monocultures and has been detected in aquatic ecosystems (Shinn et al. 2015). According to the EFSA (European Food Safety Authority), Pyrimethanil has no potential to bioaccumulate, has a low acute toxicity, is not teratogenic, and seems to have no neurotoxic effect; however, studies have shown toxicity in bioindicator species (Müller et al. 2012; Scherer et al. 2013; Seeland et al. 2013). These studies have used *Chironomus riparius* species, a common organism used in ecotoxicological tests in other countries. However, in Brazil, this species has been substituted by *Chironomus sancticaroli* in ecotoxicological tests because of the ease of collecting and cultivating in the laboratory conditions (Strixino and Strixino 1982), and for the reason that this species is native to the country.

After treatment with genotoxic compounds, the change in the RAPD (random amplified polymorphic DNA) profile is clearly identified by the variation of intensity of the bands as well as the gain or loss bands. These differences in the profiles of RAPD reaction of the bands may be related to DNA damage,

such as structural changes or rearrangements induced by genotoxins (Atienzar et al. 2002). The RAPD technique is very interesting in ecotoxicological analyses because it is simple, sensitive, relatively cheap, and effective for the identification of DNA damage (De Wolf et al. 2004).

2 Materials and Methods

Sensitivity tests in *C. sancticaroli* cultures, which consist of exposing the organisms to different concentrations of a reference substance, were performed. The ring test with the reference substance potassium-chloride (KCl) was carried out according to OECD recommendations (2011). The *C. sancticaroli* cultures were kept in plastic trays covered with nylon cages to retain the adult organisms. For the acute tests, six larvae of *C. sancticaroli* were added (IV instar – 7/8 d) to 240 mL of test solution with 60 g of sand sediment in three replicates. The sand sediment was composed of fine sterilized sand. The test lasted 96 h, after which the living organisms were counted. After 10 days, larvae were counted and the organisms were separated and fixed in isopropyl alcohol for the genetic analyses. The following solutions of Pyrimethanil were employed for acute toxicity tests: 25, 50, and 75 $mg.L^{-1}$ (nominal concentrations) using commercial Mythos® (Bayer S.A.). As the study wanted to cause damage to the genetic material of the larvae, it was purposely used solutions of high concentration of the fungicide.

The larvae were homogenized individually in 500 μL CTAB buffer (2% CTAB, 1.4 mol/L NaCl, 20 mmol/L EDTA, 100 mmol/L Tris-HCl) and 16 μL proteinase K (10 mg/mL) and incubated at 56 °C for 6 h, then was added the same volume of phenol/chloroform/isoamyl alcohol (25: 24: 1). The DNA was precipitated in 1 mL of ice cold 100% ethanol. After incubation for 2 h at − 20 °C, the DNA was washed with 70% ethanol. The pellet was resuspended and dissolved in 25 μl of TE buffer and stored at − 20 °C.

>Amplification was performed in a volume of 20 μl containing buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl2, dNTP mix at 200 mM each, 50 nM of primers (OPB 4 GGACTGGAGT, OPB 5 TGCGCCCTTC, OPB 6 TGCTCTGCCC, OPB 7 GGTGACGCAG, OPB 17 AGGGAACGAG, OPB 18 CCACAGCAGT), 50 ng of genomic DNA, and 2.5 U of Taq DNA polymerase (Gibco-BRL). Amplification was in a thermal cycler (Perkin Elmer, Norwalk, CT) programmed for 5 min at 94 °C, followed by 42 cycles of 94 °C for 1 min, 35 °C for 2 min, and 72 °C for 2 min. After the final cycle, an additional extension step was performed at 72 °C for 7 min. PCR products were loaded on a 1.5% agarose gel at 120 V in TAE (40 mM Tris acetate, 1 mM EDTA) and quantified after staining with ethidium bromide (0.5 μg/mL) under UV light (Costa et al. 2000).

The difference observed in RAPD profiles (disappearance and appearance of bands in comparison with control RAPD profiles) was scored using a binary matrix by Sequentix – GELQuest 3.2.1 Version. The statistical difference on banding pattern obtained from Pyrimethanil concentrations was tested with ANOVA ($\alpha = 0.05$). In addition, to determine the phenetic diversity in larvae exposed was used the Shannon-Wiener Index (H′) (De Wolf et al. 2004).

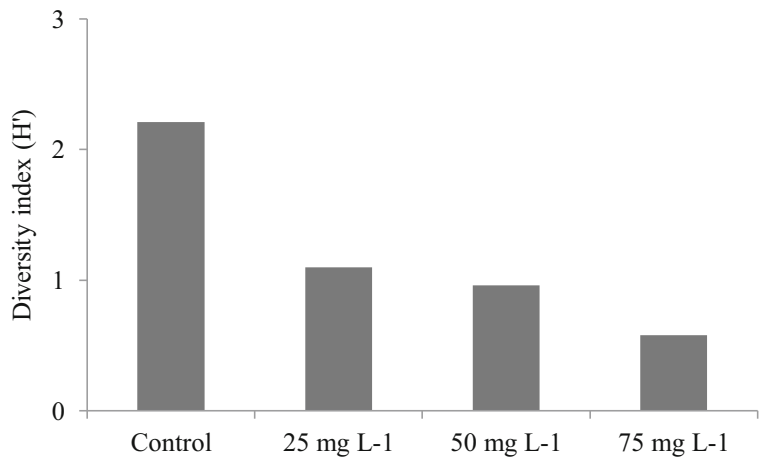

Fig. 1 Pyrimethanil solutions and phenetic diversity (Shannon-Wiener Index - H′) of exposed larvae to 25, 50, and 75 $mg.L^{-1}$ of Pyrimethanil

3 Results

The genetic diversity was estimated using six primers (OPB4, OPB5, OPB6, OPB7, OPB17, and OPB18) 10 bases. The phenetic diversity was presented in Fig. 1. RAPD profiles revealed substantial differences between the control and exposed organisms. In the organisms without treatment, they were found 58 bands between 2500-250pb. In the organisms treated with fungicides there was a decrease in the number of bands in all tested concentrations. We found 24 bands in the 25 $mg.L^{-1}$ Pyrimethanil concentration, 21 bands in the 50 $mg.L^{-1}$ concentration, and 11 bands in the 75 $mg.L^{-1}$ concentration.

4 Discussion

The use of markers to evaluate the genetic variability among individuals and populations is promising because many polymorphic loci can be obtained easily in a relatively short time and at low cost (Nybom 2004). Changes in the pattern of DNA bands may reflect alterations in the genome from a single base variation (point mutations) or chromosomal rearrangements (Atienzar et al. 2002). Considering these data, we can infer that the difference in the profile of the bands obtained in RAPD-PCR reaction of *C. sancticaroli* larvae, exposed to contaminated sediments can refers to toxicity Müller et al. (2012) and Seeland et al. (2013).

5 Conclusions

Our findings reveal that RAPD-PCR is an effective method to access genetic loss derived to fungicide use and that Pyrimethanil applications in agriculture cultivation may lead to a decrease in aquatic biota genetic diversity in adjacent areas. This conclusion has decisive consequences for conservation plans and ecological management.

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