Pesticide Biochemistry and Physiology 103 (2012) 224–230

Contents lists available at SciVerse ScienceDirect

Pesticide Biochemistry and Physiology

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

Determination of genotoxic effects of chlorfenvinphos and fenbuconazole in *Allium cepa* root cells by mitotic activity, chromosome aberration, DNA content, and comet assay

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ARTICLE INFO

Article history:
Received 21 March 2012
Accepted 2 June 2012
Available online 15 June 2012

Keywords:
Chlorfenvinphos
Fenbuconazole
Plant assay
Genotoxic effect
Mutagenic effect
Cytotoxic effect
DNA content
Comet assay

ABSTRACT

Genotoxic effects of Chlorfenvinphos and fenbuconazole were examined by using mitotic index, mitotic phase, chromosomal abnormalities, 2C DNA content and Comet assay on the root meristem cells of *Allium cepa.* The roots were treated with 10, 20, 40, 60, 80, and 100 ppm concentrations for 24 and 48 h. The results indicated that Chlorfenvinphos and fenbuconazole significantly decreased the mitotic index in all treatments when compared with their controls. The percentages of mitotic phases have changed. Chlorfenvinphos and fenbuconazole significantly increased the abnormal cell frequency at all concentrations and treatment periods when compared with their controls. Different abnormal mitotic figures were observed in all mitotic phases. Among these abnormalities were stickiness, anaphase bridges, c-mitosis, laggards, and micronucleus. These pesticides remarkably depressed the 2C DNA content in the root meristems of *A. cepa*. The genotoxicity of chlorfenvinphos and fenbuconazole in *A. cepa* root cells was analyzed using comet assay, which allows the detection of single strand breaks. In all concentrations, chlorfenvinphos and fenbuconazole induced a significant increase in DNA damage. Additionally, it was also researched to determine if there is a relation between the amount of DNA and the DNA damage and a regression analyses was conducted. When the data that was accumulated via comet analysis from *A. cepa* root tip cells that are treated with type chlorfenvinphos and fenbuconazole, was compared to the data that was acquired as the result of the measurement of 2C DNA amount, a relation with negative correlation was found, (respectively, $r = -0.80$ and $r = -0.82$). This relation factor is statistically important and strong ($p < 0.05$).

1. Introduction

Pesticides used in the modern agricultural practices represent a very large input of toxic chemicals in our environment. Pesticide residues can be present in fruit and vegetables and present a risk for human exposure. Children in particular may be exposed to pesticides by dietary ingestion because they eat more food from those of adults. The children's diet is often rich in foods containing higher levels of pesticide residue, such as fruits, juices and vegetables.

The pesticides chlorfenvinphos and fenbuconazole are widely used in the Turkey. Chlorfenvinphos is an organophosphorus (OP) insecticide and an acaricide. Chlorfenvinphos is used to control household pests such as flies, fleas, and mice. Organophosphate insecticides inhibit acetylcholinesterase. The neurotoxicity of these chemicals has been documented in accidental human poisoning cases, epidemiological and animal model studies [1]. Organophosphorus pesticides might affect the immune response and induce apoptosis of immune cells [2]. Fenbuconazole is a triazole fungicide intended for use as an agricultural and horticultural fungicide spray for the control of leaf spot, yellow and brown rust, powdery mildew and net blotch on wheat and barley. The triazoles are very specific in their mode of action – they inhibit the biosynthesis of sterol, a critical component for the integrity of fungal cell membranes. Sterols, such as ergosterol, are needed for membrane structure and function, making them essential for the development of functional cell walls. Therefore, these fungicides result in abnormal fungal growth and eventually death [3]. Since fenbuconazole is used as an agricultural fungicide, there is concern regarding potential human exposure, as well as wildlife exposure, from its residues in the environment. This fungicide has been associated with an increase in the incidence of liver adenomas in female mice following long-term dietary exposure [4]. In addition, fenbuconazole was identified as a new inhibitor of human aromatase activity [5]. Previous studies also demonstrated that fenbuconazole is a phenobarbital-type inducer of mouse liver adenomas [6]. Such data reflect both the biological and tumor response for fenbuconazole and have been suggested for consideration when evaluating

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http://dx.doi.org/10.1016/j.pestbp.2012.06.001

cancer risks [6,7]. Chlorfenvinphos was tested for mutagenic effects on human lymphocytes *in vitro*. The chlorfenvinphos concentrations used the mitotic index was reduced to approximately 60% of controls at 250 μg/ml [8].

Pesticide use raises a number of environmental concerns. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, including non-target species, air, water, and soil [9]. During the literature reviews, no research that covers the genotoxic, mutagenic or cytotoxic effects of those two indicated substances has been detected. In addition to being a frequently consumed food in our daily life, *Allium cepa* is also a test material to determine the genotoxic effects of many different chemical substances. The use of plant as test organism has been indicated and valicated by several environmental agencies, like the United Nations Environmental Program (UNEP), World Health Organization (WHO) and US Environmental Protection Agency (USEPA) [10–12]. Among the plants used in studies of bio-monitoring, some species present more adequate responses. The *Allium* genus, specialy *A. cepa* species has showed to be an efficient species for such studies, mainly when it is used for assays of chromosome aberrations and for genetic tests [12–15].

Comet assay evaluates damage to genomic DNA in individual cells, caused by genotoxic agents. This assay enables sensitive detection of DNA lesions including single strand breaks during the exposure of cells to potent mutagens. Because of the simple procedures, high sensitivity, short response time and the requirement of relatively small number of cells and test sustances, it has been a powerful tool for the determination of genotoxicity. Studies on plants reporting DNA damage through comet assay are very few despite the fact that they are the primary recipients of several polluting agents, including pesticides and heavy metals [16–19].

The purpose of this study was to investigate the effects of chlorfenvinphos and fenbuconazole pesticides on mitotic index (MI), mitotic phases, chromosome aberrations and nuclear DNA content in the root meristem cells of *A. cepa*. Besides, the effects of two chemicals on the DNA strands are studied by making use of comet assay and we present the results of our study whether there is a correlation between the use of two chemicals and nuclear DNA content.

2. Material and methods

2.1. Test material

A.cepa ($2n = 16$) onion bulbs, 25–30 mm diameter, without any treatment, and they are purchased from a local supermarket.

2.2. Pesticides

Chlorfenvinphos (2-chloro-1-(2,4-dichlorophenyl) vinyl diethyl phosphate; Cas No:470-90-6 74) and Fenbuconazole (4-(4-chlorophenyl)-2-phenyl-2-(1,2,4-triazol-1-ylmethyl) butanenitrile; Cas No: 114369-43-6).

2.3. EC50 determination

To determine appropriate concentrations for the genotoxicity assay, an *Allium* root inhibition test was carried out. The procedure of the root inhibition test as described by Fiskesjö [20] was followed with few modifications [21]. The onions were grown in freshly made distilled water for the first 24 h and afterwards exposed for 4 day to the chemical solutions (10, 20, 40, 60, 80, and 100 ppm, respectively). In order to assess the effective concentration (EC_{50}) values, the roots from each bundle were cut off on the fourth day and length of each root was measured. EC_{50} value was considered as the concentration which retards the growth of root 50% less when compared to the control group. The EC_{50} values for chlorfenvinphos and fenbuconazole are approximately 100 and 100 ppm respectively. In order to demostrate possible concentration-dependet effects of these pesticides, the root tips were treated with 10 ppm ($EC_{50/5}$), 40 ppm ($EC_{50/2.5}$), 60 ppm ($EC_{50/1.6}$), 80 ppm ($EC_{50/1.25}$), and 100 ppm (EC_{50}) concentrations of chlorfenvinphos and fenbuconazole.

2.4. Experimental design, mitotic activity, aberrations, nuclear DNA content

The preparation of slide, analysis of mitotic index and chromosomal abnormalities, nuclear DNA content were investigated by the methods described in the author's previous works [22].

2.5. Comet assay (Single Cell Gel Electrophoresis)

We exposed the root meristem cells of *A. cepa* to the concentrations similar to the ones used for cytogenetic analysis. The method used for comet assay was carried out as described by Gichner et al. [23]. In brief, the root tips of *A. cepa* exposed to pesticides (10–100 ppm) were placed in a watch glass which is kept in an ice base and gently sliced using a sharp razor blade to isolate the nuclei in Tris buffer pH 7.5. The microscope slides are pre-treated by 40 ml of 0.3% NMP agarose prepared in phosphatebuffered saline (PBS) evenly spread were air-dried. The suspension of nuclei (15 ml) mixed with 150 ml of low melting point agarose in PBS kept at 37 °C was pipetted over the slides. Slides were covered and left in a metal tray kept on ice. Nuclei were left for 1 h and slides were rinsed in TAE buffer (40 mM Tris–acetate buffer, 1 mM EDTA, pH 8) to remove salt. All operations were conducted under dimmed with yellow light. The slides were placed in an horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 4,13). The nuclei were incubated 10 min to facilitate DNA unwinding prior to the electrophoresis at 0.72Vcm_1 (26 V, 300 mA) for 25 min at 4 °C. Electrophoresed slides were stained with 80 ml ethidium bromide (20 mgml_1) for 5 min, dipped in ice-cold water to remove the excess ethidium bromide, and covered with a cover slip. For each slide, 25 randomly chosen nuclei were analyzed using a fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. Three slides were evaluated for treatment and each treatment was repeated at least twice. Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of 0, 1, 2, 3, or 4 so that the total scores of slide could be between 0 and 400 arbitrary units (AU migrogel-1110) [24].

2.6. Statistics

The SPSS computer program is used to analyze the variance of the data, The one-way analysis of variance (ANOVA) and Duncan mean range test (DMR) were used for the statistical analyses.

3. Results

Tables 1 and 2 summarize the effect of chlorfenvinphos and fenbuconazole on mitotic index and mitotic phases in the root cells of *A. cepa* which were treated for 24 and 48 h. At all concentrations used in the incubations of roots decreased the MI compared to control at each exposure time. The mitotic activity was reduced as the concentration increased and the period of treatment prolonged. However, significant ($p < 0.05$) levels of inhibition were observed