EVALUATION OF CYTOTOXIC AND GENOTOXIC POTENTIAL OF THE FUNGICIDE RIDOMIL IN ALLIUM CEPA L.

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Abstract: The cytogenetic effects exerted by the systemic fungicide mefenoxam and copper hydroxide (the active ingredients of Ridomil Gold Plus 42,5 WP fungicide) were studied in root tips of Allium cepa L. A progressive concentration- and time-related inhibition of the mitotic activity of meristematic cells was observed. The mitotic index was minimum (3.38%) at the highest concentration (1500 ppm) of the fungicide tested. The genotoxicity of the fungicide was measured by using the frequency of chromosomal aberrations. The highest percent of abnormal cells (8.39%) were determined for the lowest concentration of 100 ppm of Ridomil. The high frequency of sticky chromosomes, laggard and multipolarity indicated that the investigated fungicide caused abnormal DNA condensation, abnormal chromosome coiling and inactivation of the spindles, having an aneugenic potential.

Keywords: mefenoxam, copper hydroxide, mitotic index, chromosomal aberrations.

Introduction

Studying the effects of various pollutants released into the environment as a result of work done in different economic areas has become a priority in the last decades. Mode of action of various potential mutagenic factors in organisms, long-term implications of their presence on human health, represents numerous scientific research goals.

Pesticides used for modern farming represent a substantial input of toxic substances in the environment, and residues present in fruits and vegetables are important risk factors for consumers.

Mefenoxam is the R-enantiomer of metalaxyl and provides the same range and level of efficacy as metalaxyl (EPA, 1996). For many years, mefenoxam is a benzenoid fungicide used to control plant diseases caused by the Oomycetes or water-mold fungi. It is used on many food and feed crops, and also on non-food, residential and greenhouse crops such as tobacco, ornamental plants, trees, shrubs and vines, and lawns and turf. Mefenoxam inhibit mycelium growth and sporulation (Staub and Young, 1980), acting through affecting the activity of the RNA polymerase and leading to selective inhibition of ribosomal RNA synthesis (Davidse et al., 1983).

It has been reported that metalaxyl exposure leads to abnormal haematological and biochemical activities, induce oxidative stress and an observable toxicity (Al-Amoudi, 2012; Farag et al., 2012). Kaloyanova et al. (1991) reported that the oxidative stress was the principal manifestations of metalaxyl-induced toxicity. According to EPA (1988), in vitro exposure to metalaxyl did not induce gene mutation (in bacteria, yeast or mouse lymphoma cells), chromosomal aberrations (in yeast or Chinese hamster ovary), nucleus anomaly (in mice or hamsters) or DNA damage (in bacteria, human fibroblast cells or rat primary hepatocytes). In contrast, Hrelia et al. (1996) reported the in vitro genotoxic and

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carcinogenic potential of high dose of metalaxyl (300-1000 µg/ml), in cultured human peripheral blood lymphocytes.

Mefenoxam combined with other active ingredients, such as copper compounds, has been widely used in Romania. Copper hydroxide fungicide has multi-site activity. It is known that copper is a necessary element for the health of plants and animals, but it has toxic effects at high doses. Theophanides and Anastassopoulou (2002) reported that high copper doses increased the carcinogenic potential of other metal or non-metal agents. It is also recorded that various forms of cooper can negatively interfere with reproductive, developmental, and neurologic processes, or are mutagenic and/or teratogenic (Bodensteiner et al., 2004; Gerber et al., 2002; Klinefelter et al., 2004; Moser et al., 2004; Mitra et al., 2012).

Considering the negative effects of mefenoxam and copper hydroxide mentioned above, the present study was, therefore, conducted to investigate their effects on interphase stage of cell cycle, and mitotic index. A mixture of fungicide with trade name Ridomil Gold Plus 42.5 WP were tested for this purpose. It was also aimed to determine its potential to induce chromosome aberrations in the root meristem cells of Allium cepa L.

Materials and methods

The chemicals used in this study were provided commercially. The active ingredients of fungicide were mefenoxam in 5% w/w proportion and copper hydroxide in 60% w/w proportion. The plant used as test material was Allium cepa L. (2n= 16). Three clean and healthy bulbs of Allium cepa were chosen for each treatment group. After dry the scales of bulbs were removed, onions were grown in clean tap water, at room temperature.

When the roots reached 1.5-2 cm in length, they were treated with different concentrations of aqueous solution of Ridomil Gold Plus containing 100, 500 and 1500 ppm of active ingredients, for 3 and 6 hours period. The concentrations were chosen to be lower than those doses used in agricultural field to control different diseases. The control was prepared by exposing the bulbs to water only. After time went by, the roots were collected and fixed in Carnoy 1:3 acetic acid-ethyl alcohol mixture for overnight, and then preserved in 70% alcohol at 4ºC for cytological studies. The root tips were hydrolyzed in 1 N HCl at 60ºC for 12 minutes, followed by staining with 2% aceto-orcein at 60ºC for 12 minutes. After proper fixation and staining, appropriate squash preparations were made for each of the treatments and control. Effects of chemical treatment and control on different chromosome plates were observed under light microscope. All observations were made from temporarily prepared slides. To determine the effects of this fungicide on mitotic index, 3000 cells were scored in control group and in each treated sample.

Mitotic index was computed by determining the mitotic cell frequency at the root tip cells as:

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\text{Mitotic index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells observed}} \times 100
\]

Cytological abnormalities were also observed and scored. Photomicrographs of cells showing chromosomal aberrations, as well as showing normal mitosis, were taken using Olympus CX31 microscope.
Percentage of cells showing chromosomal abnormalities, such as sticky chromosomes, laggard chromosomes, multipolar anaphases, as well as aberrant interphases (vacuolated nuclei and binucleated cells), were recorded at the appropriate mitotic stages.

Results and discussions

Analysis of mitotic index variations, types of chromosomal aberrations and their frequency, respectively, were performed in order to establish the cytotoxic and genotoxic effects of mefenoxam and copper hydroxide fungicide mixture. The mitodepressive effect of the Ridomil fungicide was obvious, regardless of the concentration of aqueous solutions used for imersion of adventitious roots formed by onion bulbs. Moreover, a decrease of mitotic index value was associated with the increase of aqueous solution concentrations. Thus, if 15.6% of the cells analyzed in control was found in different stages of mitosis, dipping the roots in 100 ppm, 500 ppm to 1500 ppm solutions of fungicides for 3 hours, the frequency of dividing cells decreased to 3.60%, 3.45% and 3.38% respectively. In this context, it should be noted that the immersion of roots in the same concentrations of fungicide solutions tested, but for a longer time (6 hours), completely inhibited mitotic activity, all the cells observed in the prepared slides being in interphase. Arresting cells at G1 or G2 periods of cell cycle have been mentioned by a number of studies, as a consequence of cyclin-dependend kinases (CDKs) synthesis inhibition (Polit et al., 2003; Cvikrova et al., 2003; Mitra et al., 2012). According with De Veylder et al. (2003), an important regulatory point among the different checkpoints in cell cycle was represented by the G1/S phase transition. In this transition, plant cells decided to divide (Stals and Inze, 2001), differentiate, or be inactive (Francis, 2007). The importance of this phase was considered by essential gene expression for DNA replication in the S phase of cell cycle. Therefore, G1/S checkpoint was sensitive in response to various exogenous stimuli (Nejad et al., 2012).

Generally, the used concentration of fungicide tested induced a dose-dependent inhibition of mitotic index, which could be due to intracellular stress, including the DNA damage, preventing the cells from entering mitosis. Mitodepressive action may be due to a negative interference of the active substances contained by the fungicide tested with specific proteins and enzymes which mediate DNA polymerase (Hidalgo et al., 1989), DNA synthesis, microtubule formation, impaired nucleoprotein synthesis and reduced level of ATP to provide energy for spindle elongation, microtubule dynamics and chromosomal movement (Majewska et al., 2003; Türkoğlu, 2012). Inhibition of mitosis by several other pesticides have been reported (Yıldız and Arıkan, 2008; Pandey, 2008; Srivastava et al., 2008; Aydemir et al., 2008; Liman et al., 2011; Popescu et al., 2013). Therefore, these processes explained the inhibitory effect of mefenoxam and copper hydroxide induced in Allium plant-system in the present study.

While no chromosomal aberrations were recorded in the chromosomes of Allium cepa meristematic root cells from control bulbs, exposure to different concentrations of fungicide (100, 500 and 1500 ppm) induced chromosomal aberrations in root tip cells of Allium cepa, with high frequency of metaphases with sticky chromosomes, and multipolar anaphases. In addition, interphase aberrations, such as vacuolated nuclei and binucleated cells, were observed with high frequency (Fig. 1), suggesting the cytotoxic/genotoxic effect of active substances in Ridomil.
The highest frequency of chromosomal aberrations (8.39%) was corresponding to the lowest concentration tested, 100 ppm respectively. Decrease of percentage of chromosomal aberrations in root tip cells of *Allium cepa* exposed to higher concentrations (500 and 1500 ppm) may be the consequence of very small fraction of the cells which were dividing, and the inhibition of mitosis (Table 1). The fungicide mixture tested acted mainly in interphase, as confirmed by high frequency of vacuolated nuclei (7.45%) and binucleated cells (6.52%). Induction of nuclear vacuoles may be attributed the presence of a nuclear poison. The presence of nuclear lesions and nuclear dissolution show cytological evidence for the inhibitory action on DNA biosynthesis during S phase of mitotic cell cycle (Mercykutty and Stephen, 1980; Akaneme and Iyioke, 2008).

Table 1. Mitotic index and different types of chromosomal aberrations in root tip cells of *Allium cepa*.

<table>
<thead>
<tr>
<th>Concentrations of Ridomil (ppm)</th>
<th>Mitotic index (%)</th>
<th>Vacuolated nuclei</th>
<th>Sticky chromosomes</th>
<th>Laggards</th>
<th>Multipolar anaphase</th>
<th>Binucleate cells</th>
<th>Total aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.39</td>
</tr>
<tr>
<td>100</td>
<td>3.60</td>
<td>2.93</td>
<td>0.91</td>
<td>0.76</td>
<td>1.66</td>
<td>2.13</td>
<td>7.80</td>
</tr>
<tr>
<td>500</td>
<td>3.45</td>
<td>2.46</td>
<td>0.66</td>
<td>0.92</td>
<td>1.23</td>
<td>2.53</td>
<td>7.80</td>
</tr>
<tr>
<td>1500</td>
<td>3.38</td>
<td>2.06</td>
<td>0.53</td>
<td>0.34</td>
<td>1.20</td>
<td>1.86</td>
<td>5.99</td>
</tr>
<tr>
<td>Total</td>
<td>26.03</td>
<td>7.45</td>
<td>2.10</td>
<td>2.02</td>
<td>4.09</td>
<td>6.52</td>
<td>22.18</td>
</tr>
</tbody>
</table>

Chromosome stickiness as physiological aberration (Ping et al., 2012) was also observed in this study (Fig. 1). Stephen (1979) mentioned that stickiness was a type of physical adhesion that involves mainly the proteinaceous matrix of the chromatin material. Mercykutty and Stephen (1980) reported that this stickiness might be interpreted as a result of depolymerisation of DNA, partial dissolution of nucleoproteins, breakage and exchanges of the basic folded fiber units of chromatids, and stripping of the protein covering DNA in chromosomes. As it was reported, sticky chromosomes indicated the presence of a highly toxic substance, inducing irreversible effects in the physical state of the chromatin (Östergren, 1944; Fiskesjö, 1985).

The low percentage of different types of abnormalities (sticky chromosomes, laggards, multipolar anaphase) can be attributed to the short time of exposure to fungicide action.

It is likely that many of chromosomal aberrations induced by the action of various types of mutagenic agents might be due to the dysfunction of nuclear spindle. Under the action of mefenoxam and copper hydroxide, the disturbance of mitotic spindle may occur, which could cause C-metaphases and multipolar anaphase. The abnormal C-metaphases were formed as a result of the complete inactivation of division of the spindle (Fiskesjö, 1993; Fernandes et al., 2009). Consequently arrest of cells in metaphase stage might be one of the causes of mitotic inhibition. In this study, occurrence of C-mitosis, lagging chromosomes and multipolar anaphases, clearly showed the accumulated effect of Ridomil on spindle function.

Inhibition of cytokinesis following telophase was responsible for binucleated cell formation visible in the next interphase of a new cell cycle (Fig. 1). In *Allium cepa*, some authors suggested that phragmoplast inhibition at the early stage of telophase is the
responsible disturbance for binucleated cell formation (Fiskesjo, 1997; Rank et al., 2002; Badr and Ibrahim, 1987; Majewska et al., 2003).

Regardless of the concentration tested, exposure of root tip cells to mefenoxam and copper hydroxide fungicide mixture induced an imbalance in the osmoregulation of the cells resulting in plasmolysation, and shifting the nucleus aside, to the polar position beside the cell wall (Fig. 2).

Conclusions

The present study proved that the mefenoxam and copper hydroxide, which have been frequently used in the field, cause decreases of the mitotic index in Allium cepa, and chromosomal aberrations, which suggest an aneugenic potential of Ridomil Gold Plus fungicide. Our findings suggest that the low concentrations tested were toxic for root tip cells of Allium cepa. The concentrations used in the field are higher, and could be more harmful for the end-receptors by food-chain. For this reason, is preferable to use as low as possible concentrations of this pesticide, to prevent mutagenic and genotoxic effects in target and non-target organisms.

REFERENCES


Figure 1. Different type of aberrations induced by the mefenoxam and copper hydroxide in *Allium cepa* L. (a) sticky chromosomes; (b) laggard chromosomes; (c) binucleate cells; (d) multipolar anaphase.
Figure 2. Plasmolysed cells induced by the mefenoxam and copper hydroxide fungicide mixture in *Allium cepa* L.