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Cyto- and Genotoxic Activity of Pesticide Cypermex Plus 550 EC on *Allium cepa* L.

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Abstract: The impact of pesticides on crop production is undoubtedly profitable, but they constitute a common and widespread cause of soil, water and air pollution, especially in developing countries as Albania. Remaining available into the environment, pesticides can be locally and globally dangerous for ecosystems. Many studies using different bioassays have demonstrated their harmful toxic effects. The present study aimed to evaluate the short-term cyto- and genotoxic activity of the insecticide cypermex plus 550 EC (Chloropyrifos 50% + Cypermetrin 5% EC) on a crop plant and bioassay as *Allim cepa* L. The roots of onion bulbs were exposed for 48 h to three doses, representing ¼, ½ and EC_{50} concentrations of the pesticide. The following microscopic parameters: mitotic and phase indexes, micronuclei formation and chromosomal aberration frequency and types were evaluated and compared. The results showed obvious pesticide concentration-dependence. Mitotic index substantially decreased at the highest applied concentration in particular, while accumulation of dividing cells in prophase stage started being significant since at ½ EC_{50} of cypermex plus. The frequencies of abnormal dividing cells and intephase micronucleated cells were considerably increased, as well. Physiological and clastogenic types of chromosomal aberrations, as: bridges, multipolarity, laggard chromosomes and c-anaphase, were observed in all concentrations. The current data obviously demonstrated that the analyzed concentrations of cypermex plus insecticide (commonly used and applied for decades in Albanian agriculture) can potentially induce cyto/genotoxic effect (even mutagenic and clastogenic impact) on crops and non target organisms, ultimately damaging biota and human health.

***Key words**: insekticides, cypermex plus 550 EC,* Allium cepa *L. assay, cytotoxicity, genotoxicity*

Introduction

The impact of pesticides on crop production is undoubtedly profitable, but they constitute a common and widespread cause of soil, water and air pollution, especially in developing countries as Albania. A large specter of pesticides has been abundantly used all over the country during the last six decades, including mainly organochlorines, organophosphates and synthetic pyrethroids. The fate and the involved transport processes depend on their physic-chemical features, such as: solubility, degradation, adsorptivity and volatility; the aerial or ground application methods; the soil's characteristics and topography, weather and irrigation conditions of local area (Jeong & Forster, 2003; Bohmont, 2006). The management of agricultural production often implicates the misbalanced use of certain pesticides, not appropriate doses and incorrect time of application. Remaining available into the environment, pesticides can be locally and globally dangerous for ecosystems.

Cypermex plus 550 EC is a fosforganic and pyrethroid insecticide, characterized by the action of powerful contact and breathing. As a result of the content of two active substances with different action mechanisms (Chloropyrifos 50% + Cypermetrin 5% emulsified concentrations), it is displayed with a wide spectrum of operation and successful control of various pest groups having different ways of living mainly on corn, potatoes, vegetables, olive, vine and other fruit trees, etc. Chlorpyrifos in particular has a long residual action for the control of flies, mosquitoes, cockroaches, bedbugs and ants. It enters the environment being directly applied to crops, lawns, parks and private yards, golf courses, turf, green houses, etc. through volatilization, spills, and disposal of waste containing chlorpyrifos. Residues and metabolites of chlorpyrifos can cause contamination at many

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environmental sites because of long-persistence especially in the soil (Cox, 1995). In invertebrates, fish and mammals it inhibits the enzyme acetylcholinesterase (AChE), causing oxidative stress and metabolic disruption (PAN AP, 2013). On the other hand Cypermethrin is a synthetic pyrethroid insecticide of forth generation which exhibit good control on pests damaging vegetables, cereals, tree bark beetles, mosquitoes, ectoparasites of livestock and fish, etc. (Amir *et al.* 2015). After being environmentally released, it is quickly discharged into the closest water bodies mainly by runoff, producing adverse effects on nontarget species particularly on water invertebrates and fishes. Even reported data concerning their toxicity are often contrasting; EPA has currently restricted cypermethrin applications (1997) and lowered the rates of chlorpyrifos usage (2015).

Considering the adverse acute or chronic effects of complex pesticides and their residues (even at low concentrations) on biota and humans in particular, it has became indispensable to monitor their presence in the environment and toxic activity through bio-assays at biochemical, molecular or behavioral levels (Agrawal & Sharma, 2010; Bolognesi & Merlo; 2011**;** Hernández *et al*., 2013). Plants, which represent a crucial link in food chains, are widely utilized to evaluate the genotoxic and mutagenic effects of risk factors such as hazard chemicals, because of the strongly structural preservation of their genetic material (Steinkellner *et al*., 1998; Grant 1999; Ma *et al.*, 2005; Majer *et al*., 2005). The *Allium cepa* L., as a model system with multiple parameters, has gained notable recognition among plant-based bio-assays to detect environmental mutagens (Fiskesjö, 1997; Leme & Marin-Morales, 2009, Barberio, 2013), due to comparative simplicity, susceptibility even in very low concentrations of hazardous and mixed chemicals, low cost and good correlation, particularly to mammalian tests. The distinct sensitivity of the *A. cepa* roots was considered to be due probably to the great total length of the diploid complement and the high number of metacentric chromosomes of onion cells (Ma *et al.*, 1995).

The present study aimed to evaluate the short-term cyto- and genotoxic activity of the insecticide cypermex plus on *A. cepa*, an important crop plant for Albanian agriculture and bioassay.

Material and methods

Biological material and test procedure

Equal-sized bulbs (3-4 g) of common onion (*A. cepa* L.) untreated with plant growth regulators, were grown (rooted) and observed in the laboratory. All experiments were set up in a completely randomized design with twelve test tubes per sample. One bulb has been put on top of each test tube having the root primordia downward in the liquid.

Cypermex plus 550 EC (Chloropyrifos 50% + Cypermetrin 5% emulsified concentration) was purchased from albanian pesticide market. Root growth inhibition test was done second Fiskesjö (1997) and was used to determinate the corresponding EC_{50} value (effective concentration of chemicals, permitting 50% growth of the sample under study in relation to control) of analyzed pesticide. Preliminary range of toxic concentrations finding was conducted with concentration ranges between five times higher and lower the manufacturers recommended dose (% solution in water). Concentrations between the highest concentration that inhibited root growth and the lowest concentration that rarely inhibited the root growth were assessed. The respective EC_{50} value of Cypermex plus was statistically evaluated by plotting on graph root length values as percentage of control against treatment concentrations after four days of root growth (96 h). The best fit trendline equation was chosen for this evaluation and the EC_{50} value resulted 0.062%. After that the onion roots were exposed for 48 h to three doses, representing ¼(0.0155%), ½(0.031%) and EC_{50} (0.062%) concentrations of pesticide. Filtered drinking water was used as negative control sample (NC).

Cyto-genetic analysis of A. cepa roots

The assessment of cyto- and genotoxic activity on *A. cepa* roots of three Cypermex plus doses, representing corresponding ¼ EC_{50}, ½ EC_{50} and EC_{50} was done. Microscopic endpoints, such as: mitotic and phase indexes (MI and PI), frequencies of interphase cells with micronuclei (MNC) and aberrant mitotic cells (FAC) with chromosome aberrations (CA) in root meristematic tissue, were observed and evaluated. Root tips (10 mm) taken from 5 bulbs randomly chosen in each series, were placed on slides and the terminal root tips (1-2 mm) were cut off and used for further preparation of microscopy slides. Slides were prepared in accordance with the standard procedure for Feulgen staining of squashed material (Singh, 2000). The total number of dividing cells (NDC) was determined in 1000 examined cells in the field of view per each slide, than MI was scored as percent ratio of NDC. PI values of

prophase, metaphase and ana/telophase were scored as percentage to MI. The formation of micronuclei was examined in about 1000 cells at interphase per slide. Only cells with intact cellular and nuclear membranes were taken in account (Ma *et al*. 1995). 1500 dividing cells (300 cells per slide) have been observed for the characterization and classification of chromosome aberrations (CA). The frequencies of micronuclei (MNC), aberrant cells (FAC) and CA types were expressed as percent ratio. All values of parameters have been calculated as means of 5 slides per sample.

Statistical analysis

Analysis of Variance (One-way ANOVA) test was used to test for significant differences of all evaluated parameters in *A. cepa* roots exposed to three Cypermex plus doses. All the results were expressed as the mean of three replicates per sample ± standard deviation (SD). Parameter differences between exposure treatments and NC were considered statistically significant at level 5%.

Results and Discussion

Graphs in Figure 1 and 2 and Table 1 represent all data related to the analyzed cyto-genetic endpoints, as: mitotic and phase indexes, frequencies of interphase cells with micronuclei and aberrant mitotic cells with chromosome aberrations and types on *A. cepa* roots exposed to 0.0155% (¼ EC_{50}), 0.031% (½EC_{50}) and 0.062% (EC_{50}) concentrations of Cypermex plus. The results generally showed obvious pesticide concentration-dependence of all parameters (using One-way ANOVA test).

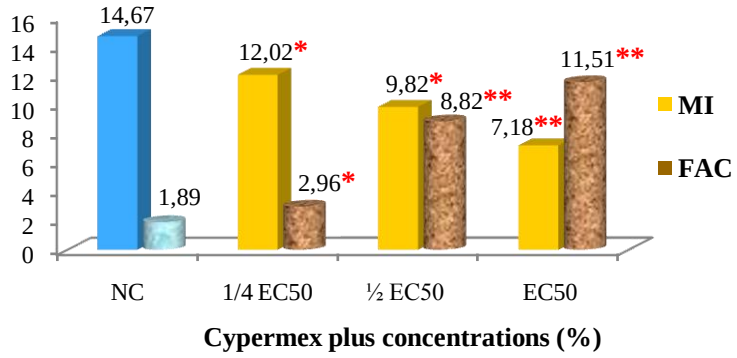

Figure 1. Cyto- and genotoxic activity induced by selected effective concentrations (1/4 EC_{50}, ½ EC_{50} and EC_{50}) of Cypermex plus insecticide on *A. cepa* root tip meristem.

(Notes: Means labeled with asterisks are significantly different from control according to One-Way ANOVA test (P<0.05; ** P<0.01); NC-negative control; MI-mitotic index (in % of NDC); FAC-frequency of aberrant cells with chromosome aberrations (in % of MI).*

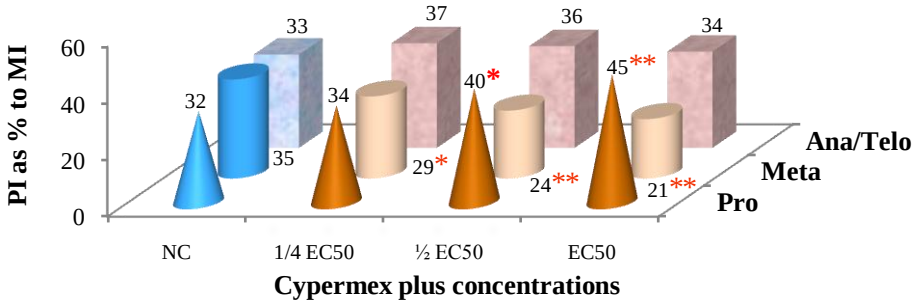

Figure 2. Variation of phase index values in *A. cepa* L. root meristematic cells exposed to different concentrations of Cypermex plus pesticide (¼ EC_{50}, ½ EC_{50} and EC_{50})

(Notes: Means labeled with asterisks are significantly different from control according to One-Way ANOVA test (P<0.05; ** P<0.01); NC-negative control; PI- phase index; MI-mitotic index; Pro-prophase; Meta-metaphase; Ana/Telo-Ana/Telophase).*

The exposure of living beings and crops in particular to mixtures of different pesticide classes may induce effects that exert difficulties to be predicted. Threshold toxicity tests are often used to

establish the point at which pesticides and other chemical pollutants cause significant growth decreases (Fiskesjö, 1997; Mesi & Kopliku, 2013b; Nowell et al., 2014). In the present experiments the EC_{50} value used as a phytotoxicity threshold indicated that Cypermex plus 550 (emulsifiable concentration) strongly affected onion root growth at 0.062%. This pesticide concentration is commonly included in cypermex plus agricultural applications. Reduction in root length could be due to the over accumulation of the insecticide into root cells, which adversely modifies the metabolic activities and restricts the cell wall elasticity. On the other hand this paper data confirmed that EC_{50} is an appropriate toxicity endpoint to select the appropriate concentrations for genotoxicity screening according to pesticide deleterious effects, because there were present enough mitotic cells to permit the microscopic observation of chromosomal abnormalities.

Table 1. Genotoxic activity of different concentrations (1/4 EC_{50}, ½ EC_{50} and EC_{50}) of Cypermex plus insecticide on *A. cepa* root tip meristem

Cypermex plus cc (%)	MNC	Spectrum of CA types (% of FAC)					
		Stc.	C-M	C-A	Br/Fr	Vg/Lg	Others
0 (NC)	0.11±0.01	0.24	0.13	0	0.12	0	0
1/4 EC_{50}	0.30±0.05	1.01*	0.71	0.42	0.17	0.51	0.14
½ EC_{50}	0.67±0.09**	1.48*	2.34**	1.03*	1.28*	2.23**	0.46
EC_{50}	1.24±0.23**	2.19**	2.80**	1.33*	1.61*	3.07**	0.72

Notes: Means labeled with asterisks are significantly different from control according to One-Way ANOVA test (P<0.05; ** P<0.01); NC-negative control; SD-standard deviation; MNC-frequency of micronucleated interphase cells; CA-chromosome aberrations; Stc-stickiness; c-M-c-mitosis; c-A-c-Anaphases, Br/Fr-bridges/fragments; Vg/Lg-vagrant/lagging chromosomes; Others-multipolarity, chromosome losses, binucleated cells, pyknosis.*

Into the same context, the mitotic index is a widely used endpoint to reliably identify the influence of cytotoxic pollutants in the environment (Grover & Kaur, 1999). MI reduction of 50% is considered a threeshold value for sublethal and lethal effects on test organism (Panda & Sahu, 1985; Antonise-Wiez, 1990). Additional data from literature have shown that the decreased mitotic activity in various test organisms might be due to the environmental presence of pesticides and trace metals (Amin, 2002). Concerning cytological analysis of treated *A. cepa* roots with Cypermex plus (Fig. 1), the mitotic activity of meristematic cells markedly changed across tested concentrations: the detected inhibition came progressively increasing with addition of pesticide quantity. Since at the lowest concentration (1/4 EC_{50}) mitotic index started being significantly lower than corresponding NC (79%, P<0.05). Meanwhile it substantially decreased at 1/2 EC_{50} treatment (67% of respective NC) demonstrating even obvious sublethal effect of highest assessed concentration (EC_{50}) on onion roots (49% of NC P<0.01). The reduction of mitotic index was probably due to the blocking of G_1 (suppressing cells from DNA synthesis) or of G_2 phases (preventing cells from entering mitosis). It might occur through the uncoupling of respiration processes and carbohydrate metabolism leading to low ATP content, which is essential for spindle elongation, microtubule dynamics and chromosomal movement and for balance installation between promoters and inhibitors of growth regulators (Türkoğlu, 2012).

Root growth inhibition and reduced penetration of roots into the soil due to pesticide toxicity (particularly of chlorpyrifos ingredient, which has a long persistence period in soil) could be due to the suppression of root cell division/root elongation or to the extension of cell cycle. As shown in Figure 2, PI characterization of *A. cepa* root tip meristem showed remarcable irregularities in cell cycles. The results revealed a progressive and significant increase of prophase rate through concentrations to corresponding NC (34-45% of MI respectively, P<0.05 and 0.01). On the other hand the frequency of metaphase generally decreased, resulting significant ((P<0.05) since at the lowest cc applied and reaching the minimum value of 21% at EC_{50}, compared to corresponding NC of 35% (P<0.01). Ana/telophase rates altered, tending mostly to decrease with addition of pesticide. The decrease of M/P ratios demonstrated an obvious obstruction of dividing cells entering metaphase and probably an inhibition of mitosis or extension of cell cycles. This may be attributed to the blocking of cell division by Cypermex plus components at the end of the prophase. The depression may be a reason of MI reduction previously discussed or indicate that after the mitotic suppression, the surviving cells start to divide again, but cannot obviously pass the metaphase stage. Similar chlorpyrifos and cypermethrin-

induced variation of mitotic and phase indexes have been reported in other studies as well (Chauhan *et al*. 1999; Rao *et al*. 2005; Asita & Matobole, 2010; Yekeen *et al*. 2013).

The suppression of mitotic activity in root meristem is usually accompanied by the increase of chromosome aberration and micronucleus frequencies. Meanwhile genotoxicological effect often occurs in concentrations low order than that for phytotoxicity anomalies (Kopliku & Mesi, 2012). That is the reason why eco-toxicological studies should include the assessment of genotoxicity, because toxic effects of chemical mixtures as Cypermex plus pesticide, mainly during chronic exposure, are not immediately detectable. Root meristematic tissue is widely used as an effective indicator of genotoxic and clastogenic potency of environmental pollutants, especially pesticides. In the present investigation the Cypermex plus-induced genotoxic effect was assessed evaluating the frequencies of abnormal cells with chromosomal aberrations (FAC) and micronuclei (MNC) in root meristematic cells of *A. cepa*. The data showed a considerable presence of chromosomal aberrations and micronuclei, positively correlated with tested insecticide concentrations (Fig. 1 and Tab. 1). FAC values started being significant since at the lowest tested concentration (1/4 EC_{50}) 164% of respective NC ($P<0.05$). At the other concentrations they distinctly increased, varying 4.7 (at ½ EC_{50}) and 6.1 (at EC_{50}) folds higher than corresponding NC ($P<0.01$). These results indicated strong and cumulative genotoxic activity of Cypermex plus (especially at ½ EC_{50}) on onion root. Second Wielgomas & Krechniak, (2007) the co-exposure to CPF and CM inhibits the carboxylesterase mediated hydrolysis of CM, leading to an increased tissue concentration of this compound.

Table 1 represents the data related to the spectrum of observed chromosomal aberrations induced by analyzed Cypermex plus concentrations. The most detected CA types were due to chromatin dysfunction (stickiness, bridges and fragments) or spindle failure (c-mitosis, vagrant and lagging chromosomes). Since at 1/4EC_{50} it could be noted a notable increase of physiological aberrations as sticky chromosomes (significant compared to corresponding NC, $P<0.05$). Stickiness which raised its pick at EC_{50}, is considered a common sign of highly toxic effects on chromosomes, an irreversible type eventually leading to cell death (Fiskesjö, 1997). It is caused probably through immediate reaction of chemicals with DNA during its inhibition period, inducing DNA-DNA or DNA-protein cross-linking or through altering the physicochemical properties of nucleic acids and/or nucleoproteins (Amin, 2002). High, but insignificant percentages of c-mitosis, vagrant/laggard chromosomes and c-anaphases were observed during the same treatment, as well. As compared to the lowest concentration, their presence drastically increased at the medium insecticide treatment (1/2 EC_{50}), resulting 3.3 and 4.3 higher for c-mitosis and vagrant/laggard chromosomes, respectively (significant to NC at $P<0.01$). At the highest concentration both these chromosomal abnormalities consisted in the 51% of respective FAC, significantly differing from NC ($P<0.01$). C-mitosis, c-anaphases, vagrant chromosomes and lagging are common spindle disorders which might be induced by the cypermethrin as part of cypermex plus insecticide, demonstrating its turbagenicity on onion roots, a phenomenon emphasized even by other studies (Rao *et al*., 2005, Yekeen et al., 2013). Meanwhile the chromosome bridges and breaks, increased gradually, predominating at the highest concentrations. They are true indicators of a clastogenic action (Kopliku & Mesi, 2015), potentially incurred by harmful effect of the pesticide. Additionally multipolarity, chromosome losses, binucleated cells and pyknosis were detected and quantified in lower and insignificant percentages thorough all treatment concentrations of *A. cepa* roots.

Micronuclei formation (MNC) is considered an important cytogenetic endpoint of observed toxicity by indicating the level of accumulated genetic damage during the cells cycle. MNC often results from the acentric fragments or laggard chromosomes that fail to incorporate into the daughter nuclei during telophase stage of dividing cells and can cause cellular death due to the deletion of primary genes (Ma *et al*., 1995). As summarized in table 1, it was noticed a notably high MNC frequency in root cells treated with 1/2 EC_{50} and EC_{50} cypermex plus samples (significant at $P<0.01$), being 6 and 11.3 folds greater than corresponding NC, respectively.

Pesticide trading is going to be more and more interested on insecticide mixtures. One of the most popular insecticide combinations is OP and PYR. The trend toward increased marketing of OP-PYR mixtures is likely to result in the creation of new patterns of mixed toxicity (Badrane *et al*., 2014). The use of plant test systems for the evaluation of potential genotoxicity and mutagenicity of pesticides is explicitly important, because plants are direct recipients of agrotoxics and then they abundantly enter food chains in general and the human food chain in particular. In this context many

reports using *A. cepa* bio-assay have demonstrated the toxic effects of different pesticides (Barberio, 2013). To our knowledge no previous research work according the toxic activity of cypermex plus on plants was found. The current data showed that cypermex plus insecticide (commonly used for decades in Albanian agriculture) can potentially induce cyto/genotoxic effect (even mutagenic and clastogenic impact) on crops and non target organisms, ultimately damaging biota and human health. Moreover the indices obtained in this investigation confirmed the statement that cypermex plus insecticide must be considered a potentially human carcinogen and at the same time fulfilled the rare (Nuro & Marku, 2011, Mesi & Kopliku 2013a & b), and insufficient eco-toxicological studies according to environmental pesticide toxicity assumed in Albania.

Conclusions

The detected growth inhibition, reduction of mitotic activity and induction of chromosomal aberrations by the insecticide cypermex plus 550 EC on *Allium cepa* root tip meristem clearly demonstrated its cyto-genotoxic activity. The results of the present study provided valuable information on the cypermex plus substantial concentration of which exposure may constitute health risk to non-target organisms as crops and they could be considered an indicator that the anthropogenic pesticides enrichment may be a potential risk to the environment. We consider the data as preliminary, which should be followed by further and periodic investigations of toxic impact caused by different pesticides in Albania.

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