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Genotoxic effects of fungicide Copper oxychloride on *Drosophila melanogaster*

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Abstract

The sex-linked recessive lethal test for mutagenicity in Drosophila melanogaster *was carried out with the fungicide Copper oxychloride. The test procedure provides the information on mutagenic effects on 800 loci on the X-chromosome at all germ line stages. The results presented here show that this fungicide induces recessive lethal mutations on X-chromosome of* Drosophila melanogaster *in I and II broods. Spermatids and spermatozoids are more sensitive stages of spermatogenesis than others, which indicates that the genotoxic effect of this chemical agent is exerted within 72 hours after application.*

INTRODUCTION

Fungicides are chemicals used for eradication of phytopathogenic and saprophytic fungi, bacteria and viruses which cause various plant diseases. These chemicals act in different ways. Fungistatics prevent development of fungi, while antisporulants prevent formation of reproductive organs. According to chemical composition, fungicides are classified into organic, more frequently used and inorganic iones. They can also be classified as systemic and unsystemic; systemic fungicides are present in all parts of plants after application, and in that way they offer reliable protection for a certain period. Although unsystemic fungicides offer shorter protection because they degrade faster under sunlight and rainfalls they are in wider use *(1, 2)*.

Active substances can be composed of organic compound of mercury, carbamates, copper compounds, inorganic and organic compounds of sulfur, derivatives of guanidine, etc. The presence of metallic ions in high quantity in cells has toxic effect and causes various disturbances. Eradication of funga diseases is based on consumption of metals *(2)*. The mechanism of action of fungicides is twofold: phenols, amines and ketones prevent mitosis, while carbamates, copper sulfates and other compounds act on the metabolism of fungi.

Frequently used soluble salts of copper are Copper sulfates and Copper oxychloride. Copper oxychloride has the widest application in protection of vegetables, fruit and hops. Synonyms of this preparation are: Coppesan, Coprex, Coprantol. Empirical formula is 3Cu(OH)2xCuCl2. Taking into consideration that they are in wide use, we decided to study the effect of one of these agents on the genetic material of eukaryotic model system of *Drosophila melanogaster*.

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Mutagenicity of various compounds widely used in agriculture can be tested through assays performed on different organisms, both *in vitro* and *in vivo (3)*. Among those, the sex-linked recessive lethal test (SLRL) with *Drosophila melanogaster* detects the occurrence of both point mutations and small deletions in the male germ line. This test is an *in vivo* assay able to screen for mutations at about 800 loci on the X-chromosome, which represents about 80% of all X-chromosomal loci and approximately one-fifth of the entire *D. melanogaster* genome.

MATERIAL AND METHODS

SLRL test was done with laboratory stocks of *Drosophila melanogaster* (obtained from Umea Stock center, Sweden). One was, Canton-S, whose individuals have normal phenotype (wild type), while B a s c line flies are characterized with individuals homozygous for a balancer X-chromosome which carries two genetic markers: Bar (B) which produces a narrow eye shape in homo- and hemizygous conditions and a kidney shaped eye when heterozygous in females. The eye restricted to a narrow vertical bar of about 80? facets appear in males and 70? facets in homozygous females. Heterozygous female has an intermediate number of facets (360?) between homozygous females (70?) and wild-type (780?). The character can be regarded as partially dominant; *white-apricot* (w^a) – changes the red eye color into light orange and is expressed only in homozygous females and hemizygous males; *scute* (sc) – recessive mutation that reduces the number of thoracic bristles. This mutation is linked with the long inversion on X-chromosome, which is necessary for suppression of the crossing-over that could change existing gene combinations on treated chromosome *(3, 4)*.

Copper as a metal is not toxic, therefore the fungicides that contain it belong to the so-called »pesticides with a low level of toxicity«, group III *(2)*. Toxicity for adult individuals of *Drosophila melanogaster* has not been observed for Copper oxychloride *(5, 6)*. For that reason, the 0.75% solution of investigated substance was used.

Three days old Canton-S males were starved in empty bottles for 5 hours prior to treatment, and than transferred and fed in bottles with filter paper soaked with solution of Copper oxychloride for 24 hours. After another 24 hours of recovery on standard medium, each male was mated individually to three Basc females, in 30 bottles, which made I brood. After two days, males were transferred to the new vials with three virgins of Basc line (II brood), and after three days males were transferred again to fresh vials with three Basc virgins (III brood). These males stayed with females for three days and were removed afterwards. Females were left for five days to lay eggs, and then they were removed. The solvent 1% Sucrose served as the negative control, while 2ppm Ethyl-methane sulfonate (EMS) was the positive control *(7, 8)*.

After F1 emerged, brother-sister matings were allowed for several days and 10 females from each vial were placed individually into new vials. Each vial was to give the progeny of one treated X-chromosome.

In F2, the phenotypes were scored according to the eye color and shape. Absence of the wild type males indicated the presence of recessive lethal induced by the test substance.

The stocks were maintained and all experiments were done under optimal conditions (t = 25 °C, relative humidity = 60%, 12/12 hours of light/dark regime) on a standard nutritive medium for Drosophila (corn flour, yeast, agar, sugar and nipagin to prevent mold and infection).

Total number of treated X-chromosomes is equal to the sum of lethal and nonlethal cultures, and frequency of sex-linked recessive lethal was calculated by the ratio of number of lethal to total number of treated X-chromosomes. Testing of the significance of difference in percentage of lethals was done by test for large independent samples (testing of difference between proportions – (9)).

RESULTS AND DISCUSSION

With accelerated development of industry and agriculture it became clear that toxicity and genotoxicity are not synonyms, so that numerous chemical compounds were tested *(10)*. With development of chemical industry and application of new scientific farming methods, the numerous groups of chemical materials of wide application have been used as pesticides that serve for eradication of weed, plant pests, and causes of plant diseases. They may contain active substances, such as alkylating agents, bromides and chlorides which have the ability of inducting of mutations in different organisms, including humans *(3)*. Chemical mutagens can be specific for sex, species, and cell cycle phase, so that extensive field investigations are necessary to determine at the field the chemical mutagenesis.

Since the preparations based on copper are the most frequently used protective agents, we chose to test one of them. *Copper oxychloride* is a preventive broad-spectrum fungicide for eradication of pathogens of fruit, vegetables and hops such as angular leaf spot and fruit scab, curly leaves, cavernous leaves of stone fruits and rust fungus. Depending on the type of plant, it is applied during the vegetation resting stage, in vegetation period until the beginning of inflorescence, only after inflorescence and before conditions for infections have occurred. Once they penetrate the cell, copper ions cause the coagulation of protoplasm, thus acting as anti-metabolites.

By short test for detection of mutagenicity in *Drosophila melanogaster in vivo* conditions, we found out the mutagenic effect of the investigated fungicide. The results are shown in Table 1. In concentration of 0.75% it induced sex-linked recessive lethal mutations on X-chromosome of *Drosophila melanogaster* males, which were treated acutely with this fungicide (I and II broods). The frequency of germinative mutations induced by this fungicide is significantly higher than the frequency of mutations induced by sucrose (negative control). The obtained results show that spermatid cell line (brood II) is especially sensitive to the influence of this fungicide.

TABLE 1

Frequencies of SLRL mutations after treatment of *Drosophila melanogaster* males with Copper oxychloride.

	EMS (positive control)	SUCROSE (negative control)	COPPER OXYCHLORIDE (test group)	$t_{co/s}$
I brood	77	300	261	
No of lethals	43	5	16	2.71
% of lethals	55.84	1.67	6.13	p<0.01**
II brood	140	269	254	
No of lethals	86	5	23	3.63
% of lethals	61.43	1.86	9.06	p<0.001***
III brood	59	252	243	
No of lethals	41	6	11	1.31
% of lethals	69.49	2.38	4.53	p>0.05
I+II+III	276	821	758	4.55
No of lethals	170	16	50	p<0.001***
% of lethals	61.59	1.95	6.60	

Statistically significant difference: p<0.01**; p<0.001***

The results of our experiments show that the tested fungicide *Copper oxychloride* used for protection of fruit, vegetables and hops in order to suppress phytopathogenic and saprophytic fungi induces recessive, lethal X-linked mutations in postmeiotic germ cell lines – spermatids and spermatozoids, while the premeiotic line – spermatocytes, is more resistant to genotoxic effects of the investigated agent. The results obtained are in accordance with those found in the literature *(11, 12, 13)* because they confirm that artificially synthesized compounds belonging to the group of pesticides, especially the ones containing chlorine, may have damaging effects on the genetic material of eukaryotic organisms as well as on their reproductive success *(14, 15)*.

Since the fungicide *Copper oxychloride*, applied in concentration of 0.75%, has genotoxic effect within 72 hours after the application and is often used in modern scientific farming, we point out the necessity to protect those who produce, distribute and apply it against the potential genotoxic effect. In conclusion, we showed that the application of lower concentration (0.25%) of this fungicide is recommended in European countries.

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