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THE ALLIUM-TEST RESPONSE TO CYANAZINE

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The effects of cyanazine on mitosis in *Allium cepa* L. can be grouped as mitotic and chromosomal effects. Mitotic effects induced by delay in spindle formation were produced immediately after 3 hours treatments in all the concentrations.

The mitodepressive activities persisted in two highest concentrations (10^{-2} M and 10^{-3} M) only, whereas in solution of cyanazine intermediate concentrations (10^{-4} — 10^{-6} M) mitotic activity increased even more than in the control.

Chromosomal effects such as laggards, bridges, fragments, fragmentations and micronuclei were noticed after a 3-hours treatment but they were more intensively after 24- or 120-hours treatments in all the concentrations. Stickiness was very frequent and accompanied all the instabilities.

Comparison of these two effects shows that mitotic activity was parallel to the frequency of total changes in higher and intermediate concentrations.

Recovery from the cyanazine effects, after one day in water, caused normalization of mitotic activity, but a higher frequency of chromosome aberration continued in all the treated materials, depending on the concentration. We can conclude that there i no reversibility of cyanazine effects.

Introduction

Many herbicides with an s-triazine compound are widely used on corn (*Zea mays* L.) as selective herbicides for the control of most weeds. Why selective herbicides eliminate certain types of plants while producing no appreciable effect on others is still almost unknown. Critical stu-

dies of cytological effects can give some answers about the level and kind of cytotoxicity or genotoxicity because chromosome aberrations become a relevant bioassay (ICPEMC, 1983). This sort of investigations have already been done with some triazine herbicides; e.g. cytogenetic effects of atrazine on sorghum (Liang and Liang 1972), on barley (Wuu and Grant 1966) and simazine on *Vicia crassa* (Tomkins and Grant 1976) and on *Allium cepa* (Jagoda 1980).

The data on the cytogenetic response of plants to cyanazine, also a widely used triazine herbicide, are lacking. In addition to this fact, the reason for our investigation was the evidence of positive results of the cyanazine influence on two microbial test systems — bacteria *Salmonella typhimurium* and yeast *Saccharomyces cerevisiae* (Franekić et al. 1988) but negative results of atrazine on the same microbial test systems were obtained (Franekić et al. 1989).

On the other hand, genotoxic reaction of the cyanazine and atrazine herbicides proved positive in the *in vitro* and *in vivo* metabolic activation in plants and in rat (Franekić et al. 1988, 1989).

As neither of test-systems could compromise all types of changes, and because herbicides primarily affect plants, it was necessary to apply test on plant material in order to obtain an overal picture of cyanazine effects.

One of the main reasons for using Allium-test in cytogenetic investigation of cyanazine was to confirm the idea and the recommendation of the Royal Sedish Academy of Science (1973) and the GENE-TOX Program (Grant 1982a). They pointed out the high potential of plant genetic system for the first level of monitoring for chromosome abnormalities. In particular, this recommendation refers to Allium-test, which can be considered as a classical one, having been introduced by Levan in 1938. Since then, the Allium-test has became a routine one (Grant 1982, Fiskesjö 1885, 1988). It is a short-term test with many advantages: low cost, easy to handle, good chromosome conditions for the study of chromosome damage or disturbance of cell division, including the evaluation of risks of aneuploidy (Fiskesjö 1985).

Material and Methods

Equal-sized bulbs weighing about 4 g were chosen from a population of commercial variety of common onion *Allium cepa* L. (2n = 16). Bulbs were grown in water until the roots were about 1—1.5 cm long, and then their roots were immersed in a cyanazine-substance solution. The test substance solution was prepared from herbicide cyanazine (6-(1-cyano-1-methyl)amino-4-ethyl amino-2-chlor-s-triazine) as a halfliquid commercial product »BLADEX« by »Radonja«, Sisak — »Shell«, London, with a 50% active compound. As the cyanazine is insoluble in water, it was first dissolved in ethanol and the powder (concentration interval of 10^{-2}—10^{-8} M) was prepared. Normal tap water, with a neutral pH, was used for the controls and for diluting the test compound (Fiskesjö 1988). A series of 5 test bulbs of about 1.5—2.0 cm diameter fitted well into ordinary test tubes of 1.5 cm diameter and 10 cm length for each concentration and control.

The experiment was performed at a relatively constant room temperature of about +20°C and protected against direct sunlight. Test liquids and control water were changed every day.

For microscopic studies root tips were cut (five root tips for five slides) after 3, 24 and 120 hours (about 5 days). After that the treatment with cyanazine was stopped and all bulbs were transferred to tap water for 24 hours, for recovery studies.

Root tips from treatment and from controls, grown in water, were fixed in methanol-acetic acid (3 : 1) for 24 hours, and slides were prepared by using the Feulgen squash technique. Permanent slides were prepared by the use of liquid carbon dioxide (Sharma and Sharma 1972) and after one day room drying they were mounted in Euparal.

Microscopic parameters were counted and measured after Fiskesjö (1985), 1000—2000 cells were analysed per treatment and concentration.

Results

Results given in Fig. 1 and Table 1 could be discussed according to the effect under three main headings. As Rieger and Michaelis (1967) showed, the rate of spontaneous chromosome aberration was negligible in roots grown from *A. cepa* bulbs (0.12—0.73%). Our control proved to be close to this frequency (0.40—0.50%). The effects observed were considered as a direct result of the treatment.

Effects on mitotic activity

Taking into consideration that all the onion bulb roots were kept in tap water for two days before the treatment, the resuls — presented in Fig. 1 — revealed that the mitosis inhibition effect of cyanazine started immediately after 3-hour treatments. All the values concerning the mitotic activity of the treated material were lower than those for the control after 3 hours treatment. Mitotic activity increased at all the concentrations after a 24-hours treatment, except in the two highest ones. In the intermediate concentration solutions (10^{-4} — 10^{-6} M) mitotic activity was higher than in the control. Again the stimulation of mitosis was produced by 120-hour treatment with intermediate concentrations, and the inhibition with two high concentrations (10^{-2} and 10^{-3} M), while in the lowest concentrations (10^{-7} and 10^{-8} M) mitotic activity was almost identical to the one in the control. The cell division supression with high concentrations and the stimulation with middle concentrations was almost overcome after 24 hours in water.

Effects on spindle organisation

The pictures of particular mitotic stages in the material treated with cyanazine differ significantly from those observed in the control. Various deviations from the control have been noticed, especialy in the prophase after a 3 hour treatment at all the concentrations. While in the control there were 54.37% cells in prophase, the percentage of cells in prophase was proportional to the cyanazine concentrations (range from 82.66 — 56.86%). There was a greater number of nuclei in the late prophase, and chromosomes of metaphase dimension were scattered in the cytoplasm. This was only a delay in the formation of spindle, as almost normal spindles were present after 24 hours in water (Fig. 1). The most prominent spindle abnormalities were C-mitosis (Figs. 2 and 4) (Levan 1938) and disturbed anaphases which are connected with the delay in spindle for-

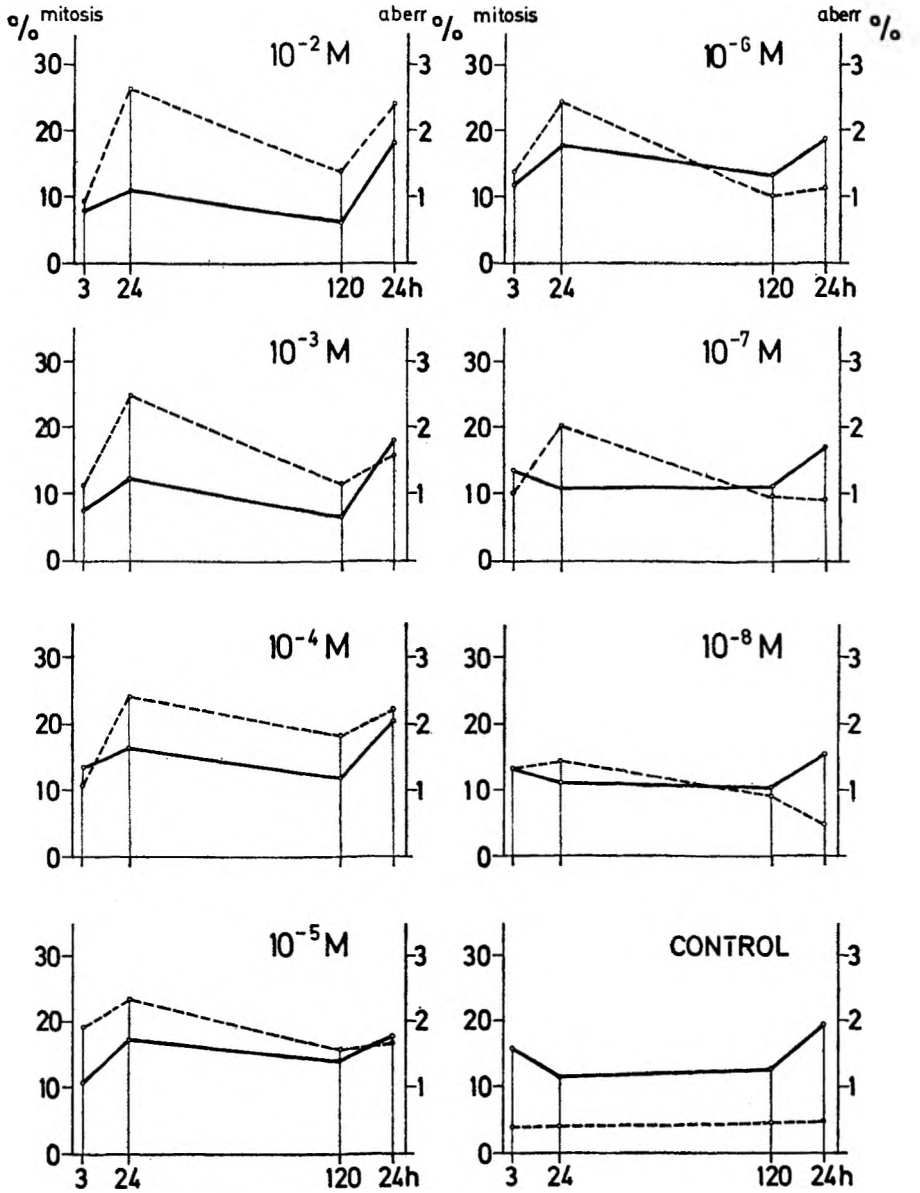
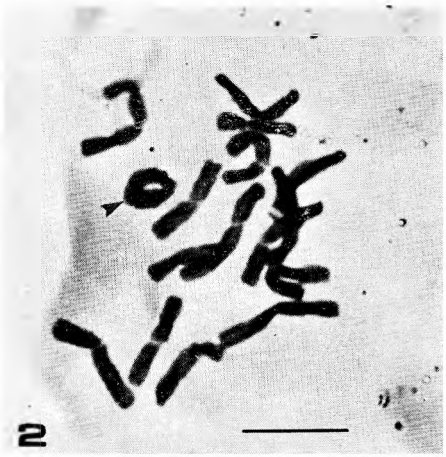
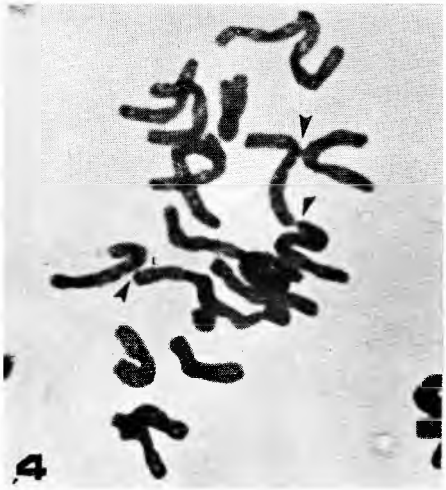
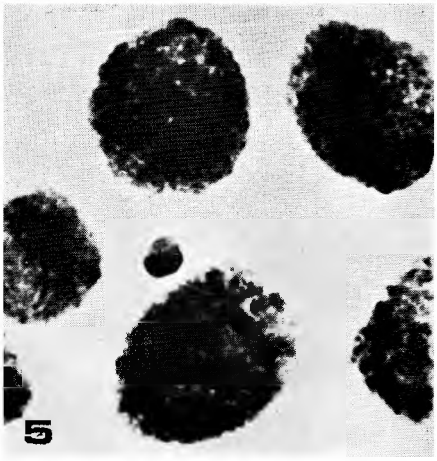


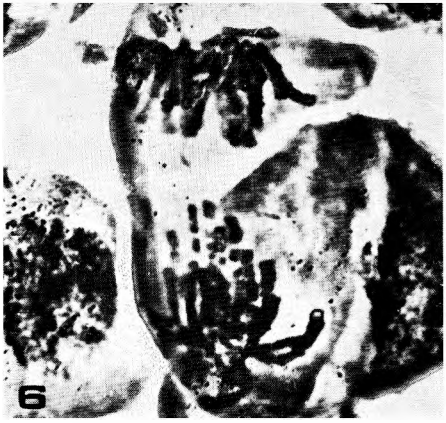
Fig. 1. Mitotic activity (——) and chromosome aberration (- - - -) frequency during root tips growth in all investigated intervals and at all cyanazine concentrations.

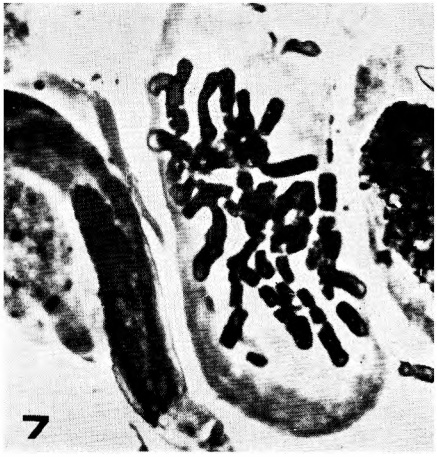
Figs. 2-7. Cytogenetic effects of cyanazine on onion (*Allium cepa*, 2n = 16) root tip cells: 2. C- metaphase with a ring chromosome (arrowed). 3. Telophase bridge connected with stickiness, 4. Aneuploid C- metaphase 2n = 20 (16+4) sticky chromosome connections (arrowed), 5. Micronucleus among interphase nuclei, 6. Anaphase and 7. metaphase with fragmented chromosomes. Bar = 10 μm. ▶











Figs. 2—7.

Table 1. Type and percentage of aberrant onion root-tip cells induced by cyanazine.

Treatment		Effects on spindle %					Effects on chromosomes %			Total aberrations	Total number of cells
Time in hours	Concentration in M	C-mitosis	Disturbed anaphase	Polyploidy	Two nuclei cells	Aneuploidy	Bridges	Fragments and laggards	Micronuclei		
3 h	10^{-2}	0.12		0.25			0.06		0.47	0.90	1700
	10^{-3}		0.12		0.12	0.06	0.06		0.73	1.09	1600
	10^{-4}	0.12	0.24	0.06	0.06		0.12		0.43	1.03	1600
	10^{-5}		0.18	0.06	0.12		0.06	0.18	1.35	1.95	1700
	10^{-6}	0.14		0.07			0.07		1.10	1.36	1500
	10^{-7}	0.07	0.08	0.07	0.07		0.13		0.58	1.00	1500
	10^{-8}	0.13	0.07	0.07	0.07		0.20		1.00	1.47	1500
	Total	0.58	0.76	0.51	0.44	0.06	0.70	0.18	5.66	8.89	11100
	Control		0.20	0.10			0.10			0.40	1000
24 h	10^{-2}	0.07	0.20	0.13	0.14		0.20	0.07	1.86	2.67	1500
	10^{-3}		0.05	0.20		0.05	0.50	0.05	1.65	2.50	2000
	10^{-4}	0.06	0.20	0.13			0.27	0.06	1.60	2.32	2000
	10^{-5}		0.07		0.07		0.60	0.53	1.10	2.37	1500
	10^{-6}		0.35				0.30	0.45	1.15	2.25	2000
	10^{-7}	0.33		0.12			0.53	0.20	1.00	2.18	1500
	10^{-8}	0.06		0.20			0.46		0.73	1.45	1500
	Total	0.52	0.87	0.78	0.21	0.05	2.86	1.36	9.09	15.74	12000
	Control						0.10		0.30	0.40	1000
120 h	10^{-2}	0.18	0.06		0.31		0.06	0.31	0.31	1.23	1600
	10^{-3}	0.12	0.23		0.23		0.17	0.18	0.23	1.16	1700
	10^{-4}	0.27	0.27	0.18			0.36	0.64	0.27	1.99	1100
	10^{-5}	0.83	0.08				0.16	0.08	0.20	1.35	2400
	10^{-6}	0.27	0.14				0.30	0.06	0.34	1.11	1500
	10^{-7}	0.10	0.40				0.10	0.20	0.20	1.00	1000
	10^{-8}	0.50	0.10	0.10					0.20	0.90	1000
	Total	2.27	1.28	0.28	0.54		1.15	1.47	1.75	8.74	10300
	Control	0.10					0.20		0.20	0.50	1000
24 h in water	10^{-2}	0.30	0.80	0.10			0.60		0.60	2.40	1000
	10^{-3}	0.20	0.30	0.10			0.90		0.20	1.70	1000
	10^{-4}	0.20	0.70		0.20		0.50		0.60	2.20	1000
	10^{-5}	0.10	0.50	0.10		0.20	0.40	0.30	0.20	1.80	1000
	10^{-6}	0.20	0.30		0.10		0.40		0.20	1.20	1000
	10^{-7}	0.20	0.40				0.20	0.10		0.90	1000
	10^{-8}		0.40				0.10			0.50	1000
	Total	1.20	3.40	0.30	0.30	0.20	3.10	0.40	1.80	10.70	8000
	Control		0.20				0.30			0.50	1000
Σ Total		4.57	6.31	1.87	1.49	0.31	7.81	3.41	18.30	44.07	30650

mation. Both phenomena cause polyploidy and the formation of cells with two nuclei. The most frequent appearance of C-mitosis was induced after 120 hours and intermediate concentration (10^{-5} M). The frequency of ditsurbed anaphases increased with the duration of treatment, so that they were still present even after 24 hours in water. Finally, both phenomena caused the formations of polyploidy and of cells with two nuclei, which were also noticed. Cyanazine may also induce the inhibition of cytokinesis.

Effects on chromosomes

Results presented in Table 1 show that cyanazine affected chromosome change in number and structure. All types of change were detected in meta-, ana- and telophase, except micronuclei.

Direct changes in number — aneuploidy (2n = 15, and 17—20) (Fig. 4) were seen in 10^{-3} M treatment after 3 and 24 hours and in 10^{-4} M after 24 hours in water. Structurally changed chromosomes were usually present in aneuploid cells, in the shape of centric rings (Fig. 2), fragments and duplication.

Chromosome bridges which are usually an indirect consequence of structural changes were noticed at all the treatment intervals and concentrations with a relatively high frequency appearance (Fig. 3). The highest percentages of bridge appearance (3.10%) were observed after 24 hours of recovery in water. The occurence of acentric fragments, which are the result of breakage and laggards, which caused aneuploidy, were detected in all the treatments, except at the lowest concentrations.

Finally, the most frequent kind of changes were micronuclei, obtained in interphases, prophases and telophases (Fig. 5).

Stickiness, a phenomenon which was not specially taken into account, was very frequent and accompanied all the instabilities of spindle and chromosomes (Fig. 4).

Chromosome fragmentation resulting from multiple breaks of the chromosomes in which there was less chromosome integrity (Grant 1982) was seen at $10^{-2} - 10^{-5}$ M concentration after 120-hour treatment (Figs. 6 and 7).

In Fig. 1 where we presented effects on spindle organisation and effect on chromosomes as total chromosomal aberrations, the comparison of total chromosomal aberrations to the mitotic activity shows that these two effects were parallel in higher and intermediate concentrations. After one day in water, recovery of the cyanazine effects caused normalization of mitosis frequency in all concentrations but not in chromosomal aberrations frequency. In all treated material chromosomal aberrations were higher than in control.

Discussion

Results presented by Allium-test show that herbicide cyanazine certainly caused damages of genetic material. A part of cytogenetic changes can overcome the selection of cell elimination, since they were built in the DNA molecules of the test organism — onion (*Allium cepa* L.).

Damages in the genetic material, counted by chromosomal aberrations, appeared at all the time intervals (3, 24 and 120 hours) and at all the concentrations ($10^{-2} - 10^{-8}$ M) compared with the control. Depending on the concentration, their appearances counted in the recovery period of 24 hours in tap water.

Comparing our results with the previously detected, somatic chromosome aberrations in different plants which were affected by other triazine herbicides such as atrazine (Wuu and Grant 1966, Liang and Liang 1972) and simazine (Tomkins and Grant 1976, Jagoda 1980), it is obvious that cyanazine caused a similar type of aberrations in onion root tip cells chromosomes. Appearances such as polyploidy and aneuploidy, which can both be connected with the delay in spindle formation, have also been reported as an effect of simazine (Jagoda 1980) and atrazine (Liang and Liang 1972). Acentric fragments which were seen in the cells of all our treatments, and the cells with total fragmentation which were noticed only at the highest concentration (10^{-2} M) of the longest (120 hours) treatment were found to be the effect of cyanazine. The same appearances were described as simazine effect in plant *Vicia crassa* (Tomkins and Grant 1976) and in onion (*Jagoda* 1980).

Along with the above mentioned chromosomal changes, we noticed that cyanazine induced chromosome stickiness which was noticed among chromosome ends and along chromosome arms (Fig. 4), and some bridges were also connected by stickiness. Stickiness is a very common change induced by all triazine herbicides (Grant 1982). Finally, the most frequent aberration was the occurrence of micronuclei, which could be the result of many changes already mentioned and numerous others such as disturbed anaphases, aneuploidy, acentric fragments, fragmentation, etc. Apparently, similar changes were reported by Jagoda (1980), too. Yet, structural changes such as duplications and ring chromosomes (Fig. 2) which we noticed as a cyanazine effect, have not been discribed yet. With regards to our results of mitotic activity frequences, cyanazine could be characterised as a mitodepressive and mitostimulating substance. The results obtained in all the treatments were not uniform. After a short treatment mitodepressive reaction appeared at all concentrations, in longer treatments (24 and 120 hours) the depression of mitosis persisted at the highest concentrations only, whereas in solutions of other concentrations mitotic activity increased even more than in the control.

A similar effect has been observed (Krsnik-Rasol and Rendić 1977) in duckweeds. The authors had found that only high solutions of triazine compound concentrations (atrazine and newly synthesised di (s-triazinyl) sulphides by Mildner et al. 1973) had an inhibitory effect on the growth of duckweeds, but low concentrations of the same triazine compounds stimulated their growth. Other authors mention only the mitodepressive character of triazine derivates (Wuu and Grant 1966, Liang and Liang 1973, Tomkins and Grant 1976, Jagoda 1980).

Triazine herbicides belong to rare chemical agents, with a consistent effect on chiasma frequency decrease, which is a decrease of genetic recombination (Murty et al. 1983). A direct triazine effect on DNA molecules (Temperli et al. 1966, Penner and Early 1972, Carere et al. 1978) was also proved.

The results of Allium-test confirm the mutagenic potential of cyanazine, a herbicide primarily used for the protection of maize cultures from weeds. Resistance in maize and barley was proved to be under the control of a single recessive gene, but polygenes may also play a role (Grant 1972). The fact that maize is resistant to triazine herbicides does not mean that mutagenic effect does not exist. While resistance to herbicides such as 2, 4-D and 2, 4, 5-T may be less serious for the cultivated species, the triazine resistant species would appear more critical for the cultivated crops of agricultural importance (Grant 1982).

The mutagenicity was proved by the plant activation of triazine (Gentile and Plewa 1976) and cyanazine (Franekić et al. 1988) into mutagens, which metabolized in both maize leaves (Franekić et al. 1988) and leaves and kernels as well (Gentile and Plewa 1976).

Our results are yet another contribution to the multiple mechanisms of cyanazine action. There is a clear correlation (Grant 1978) between chromosome aberrations found in the root tip system (Liang and Liang 1972) and mammalian cell cultures made from lymphocyte chromosomes of agricultural workers during extensive occupational exposure to atrazine (Yoder et al. 1973). Our results also prove the high potential of the plant genetic system, especially in the Allium-test system which could serve as a first monitoring for chromosomal abnormalities and give us the first answers about the level and kind of genotoxicity.

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SAŽETAK

ALLIUM-TEST — ODGOVOR NA DJELOVANJE CIJANAZINA

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Upotrebom Allium-testa ispitan je citogenetski odgovor na djelovanje triazinskog herbicida cijanazina u raznim vremenskim intervalima (3, 24 i 120 sati) kao i u otopinama raznih koncentracija (od 10^{-2} do 10^{-8} M), te nakon 24 sata oporavka u vodovodnoj vodi. U svrhu dobivanja traženog odgovora u tretiranom i kontrolnom materijalu, tj. u stanicama meristema korijena isklijalih lukovica crvenog luka (*Allium cepa* L.) vršena je analiza različitih tipova citogenetskih promjena, kao i komparacija mitotske aktivnosti i učestalosti sveukupnih kromosomskih aberacija. Utvrđeno je da u višim (10^{-2}, 10^{-3} i 10^{-4}) i srednjim (10^{-5} i 10^{-6} M) koncentracijama mitotsku aktivnost paralelno prati učestalost sveukupnih kromosomskih poremećaja, dok je u nižim koncentracijama (10^{-7} i 10^{-8} M) mitotska aktivnost bila slična kontroli. Mitodepresivno djelovanje cijanazina zadržalo se samo u najvišim koncentracijama, a u otopinama srednjih koncentracija njegovo djelovanje bilo je stimulativno i mitoza je bila učestalija od kontrole. Oštećenja genetičkog materijala, evidentirana kromosomskim aberacijama, javljala su se u otopinama svih koncentracija, kao i u svim vremenskim periodima, a također su se ovisno o koncentracijama održala i nakon 24 sata oporavka u vodovodnoj vodi.

Rezultati provedenog Allium-testa pokazuju da cijanazin nesumljivo inducira oštećenja genetičkog materijala te da dio citogenetičkog učinka može nadživjeti eliminacijsku staničnu selekciju pošto se oštećenje vjerojatno ugradilo u molekulu DNA test organizma luka (*Allium cepa* L.).

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