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Impact of Air Pollution on Genetic Material of the Shallot (*Allium cepa* L. var. *ascalonicum*), Exposed in the Vicinity of Major Slovene Local Emission Sources in 1999

By

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With 1 figure

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Summary

GLASENČNIK E., RIBARIČ-LASNIK C., MÜLLER M., GRILL D. & BATIČ F. 2002. Impact of air pollution on genetic material of the shallot (*Allium cepa* L. var. *ascalonicum*), exposed in the vicinity of major Slovene local emission sources in 1999. – *Phyton* (Horn, Austria) 42 (2): 237–250, with 1 figure. – English with German summary.

The impact of air pollution on the genetic material of bioindicator plant species was assessed by cytogenetic analysis of shallots (*Allium cepa* L. var. *ascalonicum*), exposed at eight sampling plots in the vicinity of major Slovene local emission sources in 1999. At each sampling plot the frequency of chromosomal aberrations (CA) was determined under field conditions and clay pot experiments. No significant differences in the frequency of total CA could be found among localities in the clay pot experiment. However, in the field experiments the frequency of CA significantly

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differed among sampling plots. The relatively high frequency of CA indicates that the test plants were exposed to mutagenic or genotoxic substances; consequently, our results confirmed the impact of environmental pollution on the genetic material of the shallot.

Zusammenfassung

GLASENČNIK E., RIBARIČ-LASNIK C., MÜLLER M., GRILL D. & BATIČ F. 2002. Der Einfluss von Luftschadstoffen in unmittelbarer Umgebung slowenischer Schadstoffquellen auf das genetische Material der Schalotte (*Allium cepa* L. var. *ascalonicum*) im Jahr 1999. – *Phyton* (Horn, Austria) 42 (2): 237–250, mit 1 Abbildung. – Englisch mit deutscher Zusammenfassung.

Der Einfluss von Luftschadstoffen auf das genetische Material von Bioindikatorpflanzen wurde mit Hilfe von cytogenetischen Untersuchungen an Schalotten (*Allium cepa* L. var. *ascalonicum*) durchgeführt. 1999 wurden die Pflanzen an acht Probestandorten in der unmittelbaren Umgebung von slowenischen Hauptemissionsquellen ausgebracht und die Frequenz der Chromosomenaberrationen wurde unter Feldbedingungen und in Topf-Experimenten bestimmt. Während für die getopften Pflanzen keine signifikanten Unterschiede in der Anzahl der Chromosomenabnormalitäten zwischen den einzelnen Probestandorten festgestellt werden konnten, wurden im Feldexperiment hoch signifikante Unterschiede beobachtet. Die insgesamt gesehen hohe Anzahl von Störungen im genetischen Material der Testpflanzen bestätigt Hinweise auf mutagene und genotoxische Substanzen an den untersuchten Standorten.

Introduction

A large number of methods and testing systems, which might enable relatively fast, simple and cost-effective determination of alterations in organisms due to different environmental stressors, have been developed in recent decades. One of these methods is cytogenetic bioindication, i.e. the survey of genetic changes in material (chromosomes and mitosis), which is a very reliable method for determining the level and type of cytogenetic damage in plants (e.g. DRUŠKOVIČ 1988a, MÜLLER & BERMADINGER-STABENTHEINER 1996, PARADIŽ 1996). Indeed, any alterations in mitosis indicate the presence of some cytotoxic or genotoxic substances in the environment (GRANT 1982, FISKESJÖ 1985, 1988, 1993, DRUŠKOVIČ 1994, RANK & NIELSEN 1994, ZOLDOŠ & al. 1997, KOVALCHUK & al. 1998). The *Allium* test was introduced by LEVAN in 1938 and was later proposed as a standard method for testing exposure to different chemicals in the terrestrial environment (FISKESJÖ 1988, SOFRADŽIJA & al. 1989, PAVLICA & PAPEŠ 1997, ZOLDOŠ & al. 1997). Moreover, it became an important tool in environmental monitoring and toxicity screening of river water, wastewater and other waste fluids (FISKESJÖ 1985, 1988, 1993, VIDAČKOVIČ & al. 1993, RANK & NIELSEN 1994 and references therein, SMAKA-KINCL & al. 1996, PAVLICA & al. 2000). Worldwide, the onion (*Allium cepa* L.) has been most often used for indication of the cytotoxic effects of different industrial emissions, radionuclides, pes-

ticides and trace elements (GRANT 1982, SOFRADŽIJA & al. 1989, MA & al. 1992, SMAKA-KINCL 1993, FISKESJÖ 1995, PARADIŽ 1996, KRISTEN 1997).

In contrast to the onion and shallot (*Allium* test), which have both been scarcely used for bioindication of air pollution in Slovenia (but see DRUŠOVIČ 1987, BAVCON & al. 1993), cytogenetic biomonitoring on spruce (*Picea abies* (L.) Karsten.) is also very common (e.g. DRUŠOVIČ 1988a,b, 1996), as well as in neighbouring Austria (TAUSZ & al. 1994, MÜLLER & GRILL 1994, MÜLLER & BERMADINGER-STABENTHEINER 1996).

In this study, *Allium* test was performed with shallot root tips in both field and clay pot experiments in four regions of Slovenia (see Material and Methods for a short description of the areas). But great advantage of the shallot is its life and vegetation propagations. Each plant forms several lateral bulbs, which are genetically identical and represent clones (VIDAKOVIČ & al. 1993, PAVLICA & PAPEŠ 1997, ZOLDOŠ & al. 1997, PAVLICA & al. 2000). In field experiments in which many disturbing factors might mask the influence of the principal component, a genetically uniform material is a prerequisite for reliable cytogenetic assays. The aims of the study were as follows: (a) to make a comparison among the sampling plots as regards the frequency of chromosomal aberrations (CA) as a measure of genotoxic effects arising from environmental pollution and (b) to establish a possible correlation between the frequency and type of CA and the concentrations of air pollutants. In comparison with the clay pot experiments (where plants were exposed primarily to air pollution) we expected a higher frequency of CA in plants grown in indigenous soils, since these shallots were exposed to stress due to both air and soil pollution.

Material and Methods

Study areas

In total, eight sampling sites were selected in four areas of Slovenia (the Šalek valley, the Upper Meža valley, the Zasavje region and the Ljubljana basin), where larger sources of air pollution such as power plants, smelters and traffic are located.

The Šalek Valley is a small subalpine region, where the major Slovene thermal power plant of Šoštanj is located (a detailed description of environmental conditions in the valley was given in POKORNY & RIBARIČ-LASNIK 2002). There, two sampling sites were selected in the surrounding hills (*Veliki vrh* – 570 m a.s.l.; *Zavodnje* – 537 m a.s.l.) which are known to be highly polluted area due to their climatic conditions (both sites situated in the direction of the prevailing winds above the power plant, relatively high precipitation) and topography (both sites situated in the upper layer of the frequent thermal inversion). In addition, three sampling sites were chosen in the bottom of the valley. Site *Arnače* (360 m a.s.l.) represents a site situated in a sheltered position in relation to prevailing winds, and thus relatively clean air can be found there. On the contrary, the last two sampling sites were selected in the highly polluted environment caused by the power plant *ash dump* (405 m a.s.l.) and in the village of *Škale* (420 m a.s.l.) where huge amounts of ash from dump had been deposited before 1995.

The *Zasavje region* is one of the most polluted areas of Slovenia, where the large thermal power plant of Trbovlje as well as a cement factory are located. Shallots were exposed at site *Kovk* (659 m a.s.l.), a rural area that is situated at the plateaux very close to the power plant chimney.

The *Upper Meža Valley* is a region with a centuries-old tradition of mining (the beginning of excavation dates to 1424) and smelting of lead and zinc ore. There, the sampling site was chosen in the very close vicinity of the lead smelter in *Žerjav* (540 m a.s.l.). Although the smelter ceased primary manufacture of lead from ore in 1990, the environment is still drastically polluted with heavy metals, especially with lead, cadmium and zinc (e.g. KUGONIČ & KOPUŠAR 2000, POKORNY & RIBARIČ-LASNIK 2000).

The Ljubljana basin is an area around the Slovene capital of Ljubljana, where the heating plant represents one of the most important local emission sources apart from traffic and minor industry activities. Air is polluted throughout the basin, but concentrations of air pollutants generally do not exceed critical levels. Shallots were exposed at site *Vnajnjarje* (792 m a.s.l.), a village located on hills above Ljubljana.

Test organisms and sampling procedure

For every sampling site we used approximately equal-sized shallot bulbs (*Allium cepa* L. var *ascalonicum* 'Escalote de Jersey', $2n=16$).

Plants were exposed in 15-litre self-watering pots (five per site, three bulbs per pot) and directly in field soil (six bulbs per site) in the end of May 1999. Growth and development of exposed plants as well as the presence of typical visible ozone injuries on shallot leaves were observed bimonthly. After maturation all plant material was removed at the end of August. Bulbs were dried in the storehouse and weighed for determination of biomass. Bulbs were stored under dry conditions (4 °C) for two months or until the genotoxicity test procedure started.

Sample treatment

Allium test was performed as described by FISKESJÖ (1993, 1994) with slight modification. At each sampling site five bulbs were used which represent a clone. The experiment was performed at a relatively constant temperature (20 °C) and the shallots were protected against direct sunlight. The root tips were cut off after 5 days at the same time from 7 to 9 a.m., when the cycle of division is maximal. The root tips of the bulbs were immediately put to Clarke fixative. After fixation, the root tips were hydrolysed in 1N HCl at 60 °C for 10 minutes and stained according to the standard Feulgen technique. After squashing, cytogenetic analyses were performed. For each microscope slide we used two root tips. Microscope slides were examined in Axiophot Photomicroscope (Opton) light microscope. Types of CA were determined by immersion with 1000-fold magnification (Imersionel 518C, Opton).

Microscopic parameters and statistical methods

Determination of the frequency and types of CA was used for estimating genotoxicity in the experiment. In metaphase-anaphase analysis, the types of CA in at least 200 metaphase cells per slide and 50–100 anaphase cells per slide were recorded. For each sampling site five slides were made. The cells in metaphase and anaphase were classified into the following categories: metaphase, early anaphase, anaphase, fragments and breaks, bridges, vagrant chromosomes, C-metaphase and stickiness.

The last type includes metaphase cells with connection, clumping chromosomes and chromosomes with amorphous chromatin mass.

All statistical treatments were performed using the Statistica for Windows 5.5 software package (STATSOFT 1999). Differences among sampling sites were tested using Kruskal-Wallis ANOVA. Cross comparisons between means were performed using the Mann-Whitney U test. The application of non-parametric statistical methods was necessary due to the small sample size (3 to 6 per site) and due to non-homogeneity of variances among samples.

Results and Discussion

Weather conditions and air pollution during the experiment

The mean daily temperature during the exposure period (June–August 1999) for the sampling sites was 19.5 °C, which is slightly higher than the mean temperature of 18.4 °C in the last thirty years. Similarly, the mean rainfall during the exposure period (170.2 mm/m²) was higher than the long-term average of 134.1 mm/m² (HMZ SLOVENIA 2001). Therefore, the weather during the experiment was both warmer and more humid in comparison with average climatic conditions in last 30 years.

Mean daily concentrations of SO₂ and NO₂ during the exposure period did not exceed the permitted values defined by Slovene legislation (125 µg/m³ and 150 µg/m³, respectively; OFFICIAL GAZETTE of RS 73/94) at the sampling site Kovk, Vnajnarje, Veliki Vrh and Zavodnje. Therefore, data on these air pollutants are not presented here. However, the permitted daily value for the vegetation period for ozone (60 µg/m³) was exceeded at several times at Kovk, Vnajnarje and Zavodnje (ČUHALEV & al. 2000). The AOT 40 values during the exposure period were calculated according to MILLS & al. 2000. The highest cumulative concentration of ozone was measured at Kovk (70948 ppb.h), where the critical threshold for decreased crop production (3000 ppb.h) was exceeded 23.7-fold. (This threshold represents a cumulative value for three months (June–August), considering the intensity of global solar radiation of 50 W/m²). The ozone concentrations during the exposure period were considerably lower both at Zavodnje (6996 ppb.h) and Vnajnarje (4472 ppb.h); there, the critical threshold was exceeded by 2.3 and 1.5-fold, respectively. No data on ozone concentrations and other air pollutants are available for the other sampling sites.

Although the mechanism of ozone induced injuries in plants is still unknown, the experimental data revealed that plants exposed to high concentrations of ozone (80 to 100 ppb for one to three months) show disturbances in the genetic material of the meristematic tissues in root tips (MÜLLER & GRILL 1994, MÜLLER & al. 1994, 1995), which are (in the case of trees) evident even two years after exposure. Kovk is the sampling site, where the highest cumulative concentration of ozone was measured in the vegetation period of 1999.

We also noticed some visual ozone-like injuries on the leaf surface of shallots, which likely originated in exposure to high ozone concentrations, it should be emphasised that similar types of chlorosis and necrosis might also appear due to presence of other stressors (e.g. diseases, pests, unsuitable ratio of nutrients, alternation of dry and moist periods).

Chromosomal aberrations (CA)

We examined the influence of genotoxic substances in metaphase and anaphase cells of the root tips of exposed shallots. The frequency of CA was between 6 and 10% in our experiment (Table 1), which is considerably higher than the frequency of 3%, which represents a control environment without appreciable presence of stress inducing factors (VIDAKOVIČ & al. 1993). Genotoxic substances may induce the appearance of fragments in the metaphase and anaphase, structural alterations of chromosomes (stickiness and decay of chromosomes or chromosomal mass, appearance of bridges in the anaphase) or disturbances in activity of the mitotic spindle (C-mitosis or chromosomes outside the mitotic spindle) (SMAKA-KINCL 1993).

We found significant differences in the frequency of CA among the sampling sites for shallots grown in field soils (Kruskal-Wallis ANOVA: $H_{(5, 32)} = 17.71$, $p=0.003$; Fig. 1a), while for plants grown in pots no significant differences could be found ($H_{(6, 36)} = 4.98$, $p=0.55$; Fig. 1b). Detailed statistics of pair-wise comparisons are presented in Table 2.

We could not find significant differences in the frequency of CA among different sampling sites for the clay pot experiments except a slight increase of CA in Arnače, Kovk and Veliki Vrh (Fig. 1b). We used the same soil mixture for all clay pots, and therefore plants grown in pots were exposed only to air pollutants. Nevertheless, the high frequency of CA, which was at least two-fold higher in comparison with published data for a control environment (3%; e.g. VIDAKOVIČ & al. 1993) together with the presence of different types of CA doubtless indicates the presence of some mutagenic substances at the sampling sites. The influence of genotoxic substances in clay pots was the most pronounced at the sampling sites Arnače, Kovk and particularly Veliki Vrh in clay pots and field conditions (Fig. 1b). Veliki Vrh is known to be one of the most polluted areas of the Šalek valley due to its position just facing power plants chimneys and in the wind direction in high concentration of air pollutants directly from chimneys. The Šoštanj power plant had emitted huge amounts of gaseous pollutants and heavy metals before the cleaning device was built in 1995. Since then, emissions are drastically reduced, which has been reflected in an improvement of ecosystem conditions (RIBARIČ-LASNIK & al. 1999a). Therefore, the sampling site of Veliki Vrh used to be highly exposed to pollution in the past and in recent times the situation has not completely changed

Table 1.
Frequency of different types of CA in root tips of shallots, exposed on eight sampling sites in 1999.

Sampling site		Number of cells	Frequency of CA (%)		Fragments (%)	Bridges (%)		Stickiness (%)	Vagrant chromosomes (%)		C-mitosis (%)				
			±SD	±SD		±SD	±SD		±SD	±SD					
Arnače	soil														
	pots	805	134,6	8,7	2,4	/	/	6,4	1,5	0,8	1,1	0,6	0,4	0,5	
	soil	1298	131,3	6,9	3,2	0,7	0,9	2,5	2,3	0,7	0,8	2,6	1,3	0,4	0,5
	pots	1220	85,1	7,8	3,7	0,4	0,4	3,7	2	0,9	0,7	1,5	1,1	1,2	0,9
	soil														
	pots	1361	51,1	6,2	3,1	0,3	0,5	2,5	1,7	1,4	0,8	1,3	0,4	0,8	0,7
	soil	1562	24,1	6,1	0,7	0,2	0,3	3,7	1,0	0,8	0,4	0,9	0,5	0,5	0,6
	pots	1533	67,5	6,1	0,7	0,6	0,5	3,0	1,6	0,5	0,5	1,2	0,6	0,8	0,5
	soil	330	55,2	12,2	0,7	0,2	0,3	9,4	0,1	0,	0,3	1,9	0,6	0,4	0,6
	pots	1000	46,1	7,0	3,5	0,2	0,5	4,1	2,2	0,5	0,5	1,4	0,6	0,8	1,1
Vnajnarje	soil	1318	117,8	9,9	1,3	0,1	0,1	6,2	2,4	0,6	0,7	1,9	0,7	1,2	1,1
	pots	1550	60,6	6,4	1,3	0,3	0,3	3,5	0,6	0,5	0,3	1,5	0,6	0,6	0,4
	soil	1843	36,2	8,8	2,1	0,2	0,2	5,0	1,4	1,7	0,8	1,4	0,4	0,5	0,3
	pots														
Žerjav	soil	1850	52,9	7,8	1,1	0,4	0,4	3,1	0,6	0,8	0,4	2,5	0,4	1,0	0,6
	pots	1377	56,8	6,0	1,6	0,2	0,5	3,1	1,3	0,6	0,6	1,6	0,4	0,5	0,3

Table 2.
Significance of differences in frequency of CA between sampling sites (Mann-Whitney U-test).

	SOIL									
	ARNAČE	KOVK	ASH	ŠKALE	VNAJNARJE	VELIKI VRH	ZAVODNJE	ŽERJAV		
CLAYPOTS	ARNAČE	/	/	/	/	/	/	/		
	KOVK	NS	/	NS	*	*	NS	NS		
	ASH	NS		/	/	/	/	/		
	ŠKALE	NS			**	*	*	*		
	VNAJNARJE	NS	NS	NS		NS	NS	*		
	VELIKI VRH	NS	NS	NS	NS			*		
	ZAVODNJE	/	/	/	/	/		NS		
	ŽERJAV	NS	NS	NS	NS	NS	NS	NS		

***: p < 0.001; **: p < 0.01, *: p < 0.05; NS: not significant.

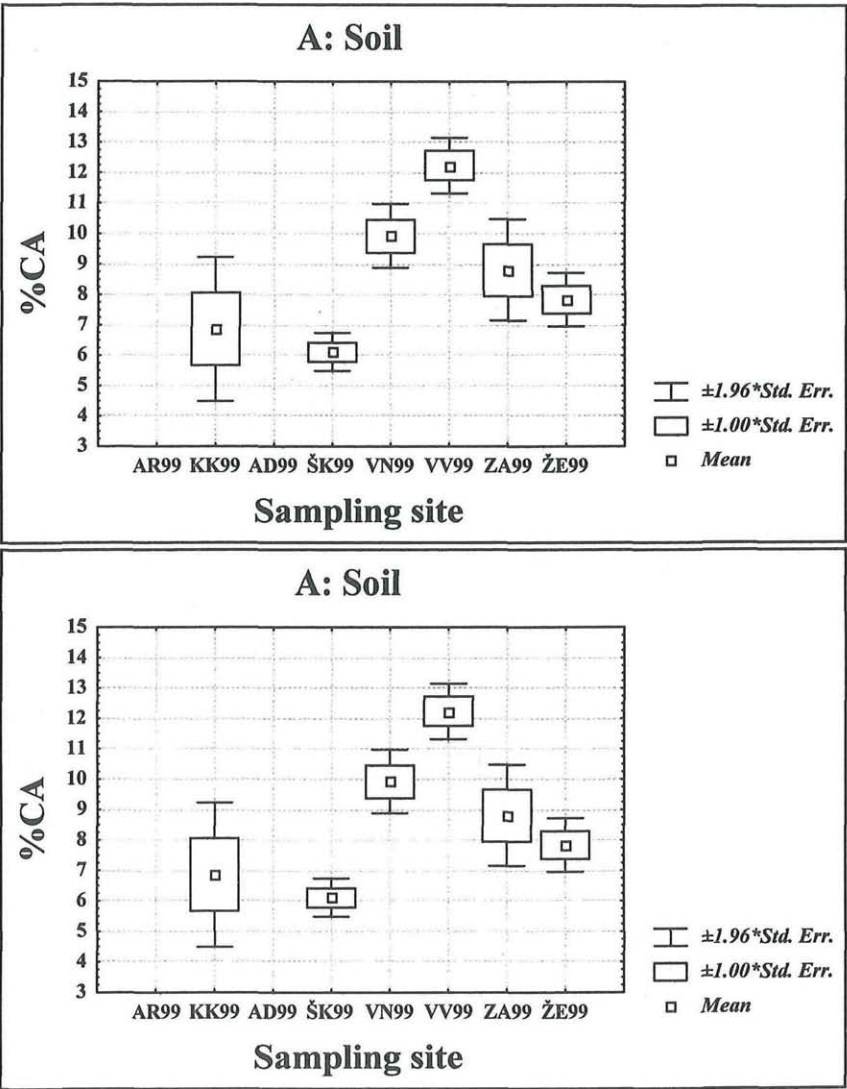


Fig. 1a, b. Frequency of CA in root tip cells of shallot, exposed in soil (A) or in clay pots (B) at different localities in Slovenia in 1999 (AR: Arnače, KK: Kovk, AD: ash dump, ŠK: Škale, VN: Vnajarje, VV: Veliki Vrh, ZA: Zavodnje, ŽE: Žerjav).

due to its position facing power plants chimneys. This side gets most acid precipitation ($\text{pH} < 5.6$) and contains the highest concentrations of sulphates and nitrates in the whole area of the Šalek valley (SVETINA-GROS 1994, SEVŠEK & al. 2000). Moreover, the concentration of trace elements is

very high at Veliki Vrh (e.g. concentration factors of 78 and 8 for Cd and U, respectively; SVETINA 1994, 1999), therefore it is not surprisingly that high levels of Cd were determined in soils, vegetables (KUGONIČ & STROPNIK 2001) and in higher fungi, in which very high levels of As and Hg were also determined as well (AL SAYEGH-PETKOVŠEK & al. 2002).

Nevertheless, the lowest frequency of CA was found for both (soil and pot) experiments at the Škale sampling site, where environmental pollution has rapidly decreased since the ash dump was grassed over in the late nineties. Thereafter, the maximal monthly airborne deposition at the dump decreased from 116.07 mg/m².day to 48.73 mg/m².day in 1997 and 1999, respectively (SEVŠEK & al. 2000).

In field experiments plants are not only exposed to air pollution but also to other toxic or mutagenic substances (e.g. heavy metals), which occur in the soil. This is probably one reason for statistical differences in the results of soil impacted plants (Fig. 1b). The experimental data from ozone fumigation by research group from Graz (see MÜLLER & BERMADINGER-STABENTHEINER 1996 and references therein) revealed that plants exposed to high concentrations of ozone show disturbances in the genetic material. The two-to three-fold higher frequency of CA in comparison with published data in soil experiments at the sites Vnajnjarje and Zavodnje is probably the consequence of very huge concentrations of ozone. However, the results of analyses might be modified due to extreme weather conditions (especially the synergistic effect of drought and heat may mask the influence of genotoxic pollutants) or due to individual ecology, which doubtless influences the expression of injuries in the genetic material of an individual.

In comparison between the data from clay pots and the soil-impacted plants (Fig 1a,b) we can find nearly no significant differences between soil and clay pots in the sites Kovk, Škale and Žerjav, but big differences between sites Vnajnjarje and Veliki Vrh. However, we are not able to explain the relatively low frequencies of total CA in the area Kovk in spite the highest cumulative concentration of ozone in the vegetation period of 1999.

Considering the extreme pollution with heavy metals in the Upper Meža valley (see RIBARIČ-LASNIK & al. 1999b and references therein), the low frequency of CA (in both experiments in comparison with other sampling sites) in Žerjav is also surprising. Nevertheless, also at this sampling site the frequency of CA is two-fold higher in comparison with the control figure of 3%, indicating the presence of negative influences on the genetic material in the Upper Meža valley. However, it should be noted that the result does not depend just on the amount of heavy metals in the soil. It depends on the availability of the metals for the plants (pH of the soil, humidity, etc., see KUGONIČ & KOPUŠAR 2000 and references therein).

The results obtained in our study were not completely in accordance either with our hypothesis or with the existing data on the stage of environmental pollution (e.g. the Žerjav sampling site), we confirm the influence of mutagenic factors on the meristematic tissues of shallot root tips. Since an elevated frequency of CA in shallot root tips may result from the effect of a combination of different pollutants and their synergistic effects, the identification of stress-inducing factors on the basis of CA assessment is extremely difficult. Therefore, further studies e.g. assessment of the influence of soil pollution on the appearance of CA, soil condition and CA, as well as further microscopic and biochemical data are needed for precise determination of the behaviour of plants at the investigated sites.

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