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Mitotic index and pollen viability of *Helianthus annuus* l., after treatment with $Pb(NO_3)_2$

Митотски индекс и фертилност на полен кај *Helianthus annuus* l., по третмани со $Pb(NO_3)_2$

Gordana Dimeska*, **Gordana Bilbiloska**

Institute of Biology, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University, Skopje, Macedonia

Earlier research indicates that the presence of the lead in the soil demonstrates inhibitory influence on mitosis, meiosis, fertility and the complete growth and development of the plant.

Thus this experiment aimed to investigate the influence of known concentrations of lead applied as $Pb(NO_3)_2$, on the mitotic cycle and fertility of the sunflower (*Helianthus annuus* L.). In the process, this cytotoxin was added in various proportionally increasing concentrations (40, 80, 160, 320 mg/kg) in two separate growth phases, in elongation phase and in flowering phase. It has been analysed what was the influence of various concentrations of lead over sunflower production of fertile and sterile pollen grains in correlation with the miotic index. Changes of the mitoitic index (MI) of the treated plants were detected, which was the main indicator for proliferation status of the cell population, and proportionally the increase of lead concentration in the soil resulted in decreasing of fertility and increasing of sterility.

The obtained results on the influnce of lead on the cytogenic parameters, and the influence on development of this important agricultural crop, indicate the importance of undertaking activities, greater responsibilities and caution of mankind, being the main factor for waste water release and agricultural activities, i.e. inappropriate use of agricultural chemicals.

Key words: sunflower, lead, mitosis, mitotic index, pollen analysis, fertility

Досегашните истражувањата укажуваат дека присуството на олово во почвата има инхибиторно влијание врз митозата, мејозата, фертилноста, и конечно врз целокупниот раст и развој на растението.

Целта на овој експеримент беше да се истражи влијанието на познати концентрации на оловото, аплицирано во форма на $Pb(NO_3)_2$, врз митотскиот циклус и фертилноста кај сончогледот (*Helianthus annuus* L.). Притоа овој цитотоксин е додаден во различни, пропорционално растечки концентрации (40, 80, 160, 320 mg/kg), во две посебни фази од растот, и тоа во фаза на елонгација и во фаза на цветање. Анализирано е влијанието на различните концентрации на олово кај сончогледот врз неговата продукција на фертилни и стерилни поленови зрна во корелација со митотскиот индекс. При тоа се констатирани промени на митотскиот индекс (МИ) кај третираните растенија кој е основен показател за пролиферациский статус на клеточната популација, а пропорционално со зголемување на концентрацијата на олово во почвата доаѓа и до намалување на фертилноста и зголемување на стерилноста.

Добиените резултати за влијанието на оловото врз цитогенетските параметри, а со тоа и на самиот развој на ова значајна индустриска култура, се прилог во активностите за преземањето на поголема одговорност и претпазливост од страна на човекот како главен фактор при испуштањето на отпадните води и агрикултурни активности, т.е. (не)соодветното манипулирање со аграрните хемикалии.

Клучни зборови: сончоглед, олово, митоза, митотски индекс, полен анализа, фертилност

Introduction

Different chemicals in nature may have negative influence on plants, especially the heavy metals (Påhlsson 1989; Barceló & Poschenrieder 1990; Nagajyot et al. 2010; de Vries 2013). Heavy metals can cause DNA damage and various chromosome abnormalities, and their cancerous effects on animals and humans are connected with their mutagenic activities (Aitken & Krausz 2001; Ercal et al. 2001; Bal & Kasprzak 2002; Hengstler et al. 2003; Gichner et al. 2006).

Some metals like cadmium, mercury, lead and arsenic precipitate on the upper horizontal soil profile. In most cases, heavy metals are accumulated on the upper layer of the soil, due to the fact that pedogenic processes of soil formation were not effective long enough after pollution to cause distribution of the metals in the soil profile. Heavy metals are absorbed through plant roots from the soil solution and only a small part through the above ground atmosphere. Generally, there are two aspects of the interaction of plants and heavy metals: heavy metals can have negative influence on plants, or plants can express some of the protection mechanisms, including detoxification (Baker 1981; Kumar et al. 1995; Salt et al. 1995; Brooks 1998; Mejáre & Bülow 2001; Alkorta et al. 2004; Babula et al. 2008; Shah et al. 2010; DalCorso 2012; Ali et al. 2013). Ac-

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*Author for correspondence: dimeskag@yahoo.com

cording to Van Assche and Clijesters (1990), only some of the metals are phytotoxic and those are fractions that enter into the cell metabolism.

It was determined that plant species genotypes show significant differences in pollution tolerance (Antonovics et al. 1971; Gartside & McNeilly 1974; Shaw 1989; Hall 2002). Absorption of lead in most plants is more intensive through their roots, rather than through the above ground parts (Ouzounidou et al. 1995). Accumulation of lead in the roots could be one type of protection of the above ground parts from high concentration of this element from the surrounding environment. Lead is considered to be a general protoplasmic toxin, which is cumulative and affects slowly and with subtlety. Lead contaminated soil causes significant lowering productivity of different plants, and that represents serious problem in agriculture (Mulev 1997). Numerous data point to the damaging effects of the excess concentrations of lead and other heavy metals on the growth and development of a plant organism (Degrassi & Rizzoni 1981; Druskovic 1984; Lefebre & Vernet 1990; Neuman et al. 1995; Cuéllar et al. 1999; Cvetanovska et al. 2005; Vasilevska 2005; Dimeska et al. 2006, 2007). Taking into consideration the frequent toxic influence, this research was aiming to investigate the influence of the excess concentrations of lead on the growth and the development of the sunflower (*Helianthus annuus* L.), an annual plant and one of the most impotant spieces in Asteraceae family from economical point of view. Primarily, we examined the effects detected on cytogenetic level, i.e. in mitotic division of the root meristem of the sunflower, as well as the fertility of pollen seeds as an indicator for regularity of the meiotic karyokinesis. Aiming to precisely determine the pollution effects, known concentrations of $Pb(NO_3)_2$ are applied on soil.

Material and Methods

Mature commercial sunflower seeds were planted in containers of 10 kg each, and various concentrations of $Pb(NO_3)_2$ were applied on the soil (40, 80, 160, 320 mg/kg). Simultaneously, three groups were formed: a control group, the group consisting of treated sunflowers in elongation phase and the group consisting of sunflowers treated in flowering phase.

The material for cytological analyses was processed according to Tjio & Levan (1950), as well as the standard "squash" method. Isolated roots were treated with 8-hydroxyquinoline (0,002 M), for 24 hours, on 4^0C temperature, then fixed in a Clarke's solution (absolute ethanol – glacial acetic acid in 3:1 ratio), for 48 hours, on 4^0C temperature. After fixation, they were transferred in 75% ethanol, for 24 hours. Roots were put for hydrolysis in 0,1N HCL, for 15 minutes on 60^0C. Staining of the chromosomes is performed with leuco-basic fuchsin (Darlington & La Cour 1962) and Gomori's hematoxylin according to Konstantinov et al. (1985).

Mitotic index (MI) is calculated as a ratio of cells in mitosis to the total number of meristem cells, given in percentage.

For pollen analyses flower heads were treated with Clarke's fixative, and then transferred in 70% ethanol. Pollen grains were stained according to the Alexander method (1969), where fertile pollen grains are stained red and sterile green (Petrovic & Vuchenovic 1992). Pollen fertility was expressed in percentage, as a ratio of the number of viable pollen grains to the total number of pollen grains.

Results and discussion

The results obtained in this research indicate that the applied concentrations of lead in the sunflower's soil cause changes of the mitotic index of the plant which is the main indicator of the proliferation status of the cell population. The values of the mitotic index of treated plants show relative decrease compared to control (Tab. 1 and Tab. 2). In elongation phase, mitotic index is higher than the one in the flowering phase. In the analysis of the mitotic system it was determined that certain percentage of aberrations is present. The results displayed in Tables 1 and 2, refer to the prepared concentrations by random choice, where cytogenetic analysis of 30 000 cells is performed. From these measurements, it is evident that the mitotic index of the control group of plants and the group exposed to the first concentration are very similar, considering the values for each separate phase (Fig. 1).

Table 1. Mitotic index (MI) of *H. annuus* root meristem, treated with various concentrations of $Pb(NO_3)_2$, in elongation phase

%		Control	I (40 mg/kg)	II (80 mg/kg)	III (160 mg/kg)	IV (320 mg/kg)
MI		5.7	5.1	4.33	3.26	3.22
INTERPHASE		95.1	94.9	96.1	96.7	96.7
PROPHASE		2.30	2.26	1.93	1.78	1.56
PROMETAPHASE		0.10	0.10	0.20	0.32	0.38
METAPHASE	Normal	0.11	0.07	0.53	0.42	0.68
	Abnormal	0.03	/	0.12	0.13	0.24
	Total	0.14	0.07	0.65	0.55	0.92
ANAPHASE	Normal	0.70	0.50	0.31	0.12	/
	Abnormal	/	/	0.01	/	/
	Total	0.70	0.50	0.32	0.12	/
TELOPHASE	Normal	0.60	1	0.53	0.02	/
	Abnormal	0.02	0.03	0.03	0.02	/
	Total	0.62	1.03	0.56	0.04	/
Other abnormalities		0.20	1.13	0.79	0.47	0.38

Table 2. Mitotic index (MI) of *H. annuus* root meristem, treated with various concentrations of $Pb(NO_3)_2$, in flowering phase

%		Control	I_2 (40 mg/kg)	II_2 (80 mg/kg)	III_2 (160 mg/kg)	IV_2 (320 mg/kg)
MI		4.6	3.85	3.65	3.13	3.03
INTERPHASE		95.8	96.15	96.28	95.78	95.31
PROPHASE		2.18	2.18	1.78	1.79	1.72
PROMETAPHASE		0.10	0.38	0.36	0.29	0.27
	Normal	0.10	0.46	0.92	0.76	0.93
METAPHASE	Abnormal	0.10	0.05	0.05	0.07	0.09
	Total	0.11	0.51	0.97	0.83	0.92
	Normal	0.01	0.05	0.07	0.08	1.00
ANAPHASE	Abnormal	0.04	0.05	/	0.06	0.05
	Total	0.50	0.10	0.07	0.14	1.05
	Normal	0.30	0.08	0.06	0.06	0.06
TELOPHASE	Abnormal	/	/	/	0.04	0.09
	Total	0.30	0.08	0.06	0.10	0.15
Other abnormalities		0.02	1.91	1.83	1.07	0.88

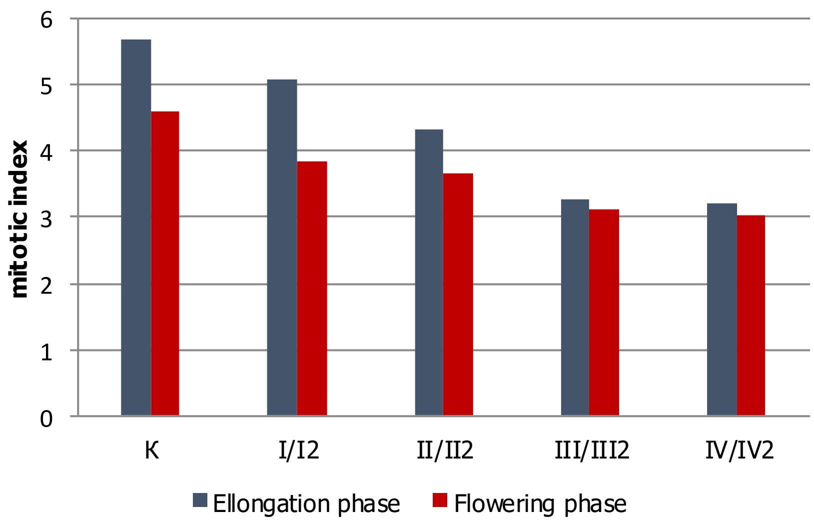

Figure 1. Mitotic index of *H. annuus* samples from plants treated with $Pb(NO_3)_2$ in elongation phase and flowering phase

Various concentrations of $Pb(NO_3)_2$ applied on the sunflower in elongation phase, caused decrease in pollen viability proportional to the concentration increase (Tab. 3). In plants treated with $Pb(NO_3)_2$ in their soil in elongation phase, decrease in pollen viability was evident. This change points to the fact that the sunflower, in its growth phase, absorbed part of the applied lead through the root, which caused irregularities in meiosis, resulting in increase of the sterile pollen grains. It is important to indicate that within the frame of fertile seeds' morphological structure, i.e. their appearance, size and form remain unchanged, regardless of the applied lead concentration (Fig. 2). These characteristics are especially important for the fertilization ability and cell selection, emphasized in these processes. This is a result of the great resistance of this crop towards the heavy metals, especially lead, depending on the age of the plant (Cvetanovska et al. 2007). There is not significant decrease in pollen viability of plants treated with lead in the flowering phase (Fig. 3). Although soil and plants in the elongation phase were treated with the same concentrations of $Pb(NO_3)_2$ no significant differences were detected. Therefore, there are indications that *H. annuus* can be considered as a potential phytoremediator of lead, one that manages to protect itself from the excess concentrations and fast translocation of the lead in the upper parts of the plant does not exist (Lefebre & Vernet 1990; Couland et al. 1999). When the application of lead is during the elongation phase, before flowering, which is a period of microsporogenesis, that process remains unimpaired for the mutagen and results in normal cells leading to normal pollen seeds formation (Vasilevska 2005).

Mitotic index, acting as main indicator in confirming the effects of the lead, perfectly correlates to the plant condition and its cytogenetics, in various cytotoxic concentrations. In fact, the index constantly gradually decreases as the lead concentrations increase. Differences occur at the mitotic index depending on the phase of development of the sunflower, which has been confirmed in the literature data (Dimeska et al. 2011). With applying of different concentrations in the elongation phase, mitotic index indicated higher values which were different in the flowering phase, which correlates more to the development stage and the nature of this phytoremediator that possesses individual adaptive value mechanisms of protection.

References

Aitken, R. J. & Krausz, C. (2001). Oxidative stress, DNA damage and the Y chromosome. *Reproduction* **122** (4): 497-506.

Alexander, M.P. (1969). Differential staining of aborted and non-aborted pollen. *Stain Technology* **11**(3): 117-123.

Ali, H., Khan, E. & Sajad, M. A. (2013). Phytoremediation of heavy metals—concepts and applications. *Chemosphere* **91**(7): 869-881.

Alkorta, I., Hernández-Allica, J., Becerril, J. M., Amezaga, I., Albizu, I., Garbisu, C. (2004). Recent findings on the phytoremediation of soils contaminated with environmentally toxic heavy metals and metalloids such as zinc, cadmium, lead, and arsenic. *Reviews in Environmental Science and Biotechnology* **3**(1): 71-90.

Antonovics, J., Bradshaw, A. D., Turner, R. G. (1971). Heavy metal tolerance in plants. *Advances in ecological research* **7**: 1-85.

Atlagić, J., Panković, D. Pekanović, A. (2003). Backcrosses in interspecific hybridization in sunflower. *Genetika* **35**(3): 187-197.

Babula, P., Adam, V., Opatrilova, R., Zehnalek, J., Havel, L., Kizek, R. (2008). Uncommon heavy metals, metal-

Table 3. Viability of *H. annuus* pollen grains (%) obtained from plants treated in elongation phase and plants treated in flowering phase

	Control	I (40 mg/kg)	II (80 mg/kg)	III (160 mg/kg)	IV (320 mg/kg)
Elongation phase	95.7	95.4	92.5	87.5	83.78
Flowering phase	95.7	87.6	90.0	96.3	94.5

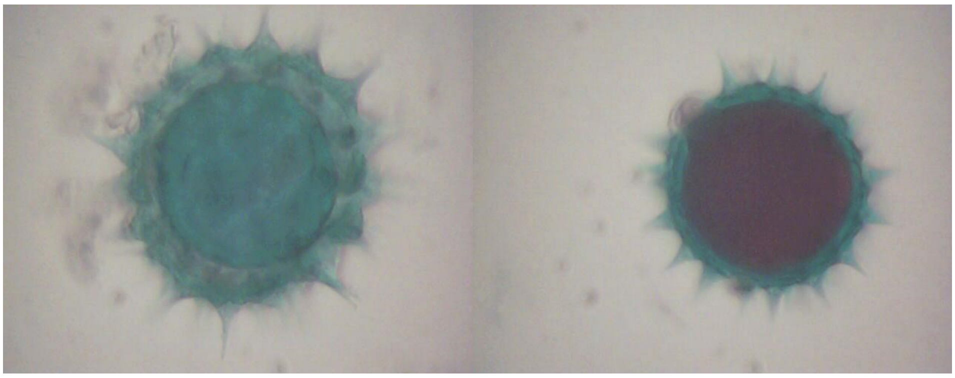

Figure 2. Sterile pollen seeds with visible thickening of the cell wall (l), fertile pollen seeds with visible local thickening of the cell and both cell coverings (right) in treatment with $Pb(NO_3)_2$, concentration I

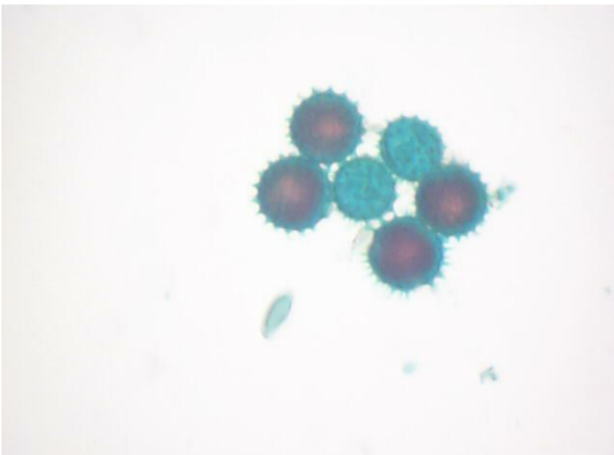

Figure 3. Fertile and sterile pollen seeds of *H. annuus*, treated with 40 mg/kg $Pb(NO_3)_2$, concentration I_2

loids and their plant toxicity: a review. *Environmental Chemistry Letters* **6**(4): 189-213.

Baker, A. J. (1981). Accumulators and excluders-strategies in the response of plants to heavy metals. *Journal of plant nutrition* **3**(1-4): 643-654.

Bal, W., & Kasprzak, K. S. (2002). Induction of oxidative DNA damage by carcinogenic metals. *Toxicology letters* **127**(1): 55-62.

Barceló, J., Poschenrieder, C. (1990). Plant water relations as affected by heavy metal stress: a review. *Journal of Plant Nutrition* **13**(1): 1-37.

Brooks, R. R. (1998). Plants that hyperaccumulate heavy metals, their role in phytoremediation, microbiology, archaeology, mineral exploration and phytomining. CAB International, Wallingford, UK.

Bowen, C. C. (1956). Freezing by liquid dioxide in making slides permanent. *Stain Technology* **31**(2):87-90.

Cuéllar, T., Orellana, J., Belhassen, E., Bella, J. L. (1999). Chromosomal characterization and physical mapping of the 5S and the 18S-5.8 S-25S ribosomal DNA in *Helianthus argophyllus*, with new data from Helianthus annuus. Genome **42**(1): 110-115.

Couland, J., Barghi, N., Lefebrve, C., Siljak-Yakovlev, S. (1999). Cytogenetic variation in populations of *Armeria maritima* (Mill.) Willd. In relation to geographical distribution and soil stress tolerances. *Canadian journal of botany* **77**(5): 673-685.

Cvetanovska, L., Dimeska, G., Kratovalieva, S., Veselinova -Stavrevska, S. (2005). Morpho-anatomical and genetic anomalies due to heavy metal intoxication. In: *Proceedings of the I Congress for Plant Protection in Republic of Macedonia*: 237-240. (in Macedonian). [Цветановска, Л., Димеска, Г., Кратовалиева, С., Веселинова-Ставревска, С. Морфо-анатомски и генетски аномалии при интоксикација со тешки метали. I Конгрес за заштита на растенијата во Република Македонија. Зборник на трудови. 237-240.]

Cvetanovska, L., Dimeska, G., Kratovalieva, S., Suma, S. (2007). Plant defects due to heavy metal intoxication. In: *Proceedings of the III Stockbreeding Syphosium with International Participation*, 636: 767-772. (in Macedonian) [Растителни дефекти при интоксикација со тешки метали. III Симпозиум за сточарство со меѓународно учество. Зборник на трудови, 636: 767-772.]

DalCorso, G. (2012). Heavy metal toxicity in plants. In: *Plants and heavy metals*. pp. 1-25. Springer Netherlands.

Darlington, C. D., La Cour, L. F. (1962). The handling of chromosomes. Allen and Unwin, London, 264 p.

Degrassi, F., Rizzoni, M. (1981). Set up of a micronucleus test in root tips of *Vicia faba* to detect mutagenic damages in aquatic environmental pollution. *Mutation Research/Environmental Mutagenesis and Related Subjects* **85**(4): 246-247.

Dimeska, G. (2003). The effects of ionizing radiation (^{60}Co) on development of faba bean. In: *Proceedings of the 8th Croatian Biological Congress with International participation, Croatia*.

de Vries, W., Groenenberg, J. E., Lofts, S., Tipping, E., Posch, M. (2013). Critical loads of heavy metals for soils. In: *Heavy metals in soils.* Springer Netherlands. pp. 211-237.

Dimeska, G., Cvetanovska, L., Josifovska, S., Stamatovski, B., Koleva-Gudeva, Lj. (2006). Effects of different concentrations of lead in the cell cycle in the chloroplast pigments *Allium cepa* L. In: *Annual of the Institute of Agriculture Skopje* XXIV-XXV: 89-96. (in Macedonian). [Влијание на различни концентрации на олово врз клеточниот циклус кај хлоропласните пигменти кај *Allium cepa* L. Годишен зборник на Земјоделски Институт, Скопје XXIV-XXV: 89-96.]

Dimeska, G., Cvetanovska, L., Josifovska, S., Stamatovski, B. (2007). Morphological and genetical changes in *Allium cepa* L. due to increased concentrations of $ZnSO_4$. In: *Proceedings of the III Stockbreeding Syphosium with International Participation*, 636: 571-578. (in Macedonian) [Морфолошки и генетски промени кај *Allium cepa* L. во услови на зголемени концентрации на $ZnSO_4$. III Симпозиум за сточарство со меѓународно учество. Зборник на трудови, 636: 571-578.]

Dimeska, G., Bilbiloska, G., Sekovski, Ž., Cvetanovska, L. (2011). Aberration frequency during mitosis in *Hellianthus annus* L, after the treatments with lead applied in the form $Pb(NO_3)_2$. Ecosystems. International *conference SDED-SPP*, 85, Sarajevo BIH.

Druskovic, B. (1984). The impact of environmental pollution on the genetic changes in plant populations. Doctoral dissertation, University of Novi Sad. (In Serbian). [Druskovic, B. Uticaj zagadjenja sredine na genetske promene u biljnim populacijama. Doktorska disertacija, Univerzitet u Novom Sadu]

Ercal, N., Gurer-Orhan, H., Aykin-Burns, N. (2001). Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. *Current topics in medicinal chemistry* **1**(6): 529-539.

Gartside, D. W., McNeilly, T. (1974). The potential for evolution of heavy metal tolerance in plants. *Heredity* **32**: 335-348.

Gichner, T., Patková, Z., Száková, J., Demnerová, K. (2006). Toxicity and DNA damage in tobacco and potato plants growing on soil polluted with heavy metals. *Ecotoxicology and environmental safety* **65**(3): 420-426.

Hall, J. L. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of experimental botany* **53**(366): 1-11.

Hengstler, J. G., Bolm-Audorff, U., Faldum, A., Janssen, K., Reifenrath, M., Götte, W., Bienfait, H. G. (2003). Occupational exposure to heavy metals: DNA damage induction and DNA repair inhibition prove co-exposures to cadmium, cobalt and lead as more dangerous than hitherto expected. *Carcinogenesis* **24**(1): 63-73.

Konstantinov, G.H., Belcheva, R.G., Goranov, A.K. (1985). Rykovodstvo za prakticheski zanaytiy po genetika. Sofia.

Kumar, P. N., Dushenkov, V., Motto, H., Raskin, I. (1995). Phytoextraction: the use of plants to remove heavy metals from soils. *Environmental science & technology* **29**(5): 1232-1238.

Lefebvre, C. & C. Vernet. 1991. Microevolutionary process on contaminated depositis. In: Shaw, A. J. (eds*.). Heavy Metal Tolerance in Plants: Evolutionary Aspects*. CRC Press. pp. 286-297.

Mejáre, M. & Bülow, L. (2001). Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. *Trends in Biotechnology* **19**(2): 67-73.

Mulev, M. (1997). Environmental protection. Wordbook, Skopje. p. 300. (in Macedonian). [Заштита на животната средина. Ворлдбук, Skopje.]

Nagajyoti, P. C., Lee, K. D., Sreekanth, T. V. M. (2010). Heavy metals, occurrence and toxicity for plants: a review. *Environmental Chemistry Letters* **8**(3): 199-216.

Neumann, D., Zurnieden, U., Lichtenberger, O., Leopold, I. (1995). How does Armeria maritime tolerance high heavy metal concentrations. *Journal of Plant Physiology* **146** (5-6): 704-717.

Ouzounidou, G., Chiamporova, M., Moustakas, M., Karataglis, S. (1995). Responses of maize (*Zea mays* L.) plants to copper stress - I. Growth, mineral content and ultrastructure of roots. *Environmental and Experimental Botany* **35**: 167-176.

Påhlsson, A. M. B. (1989). Toxicity of heavy metals (Zn, Cu, Cd, Pb) to vascular plants. *Water, Air, & Soil Pollution* **47**(3): 287-319.

Petrović, S., Vučenović, M. (1992). Laboratory manual in cytogenetics. Faculty of Agriculture, University of Novi Sad. (In Serbian) [Petrović, S., Vučenović, M. (1992). Praktikum iz citogenetike. Poljoprivredni fakultet, Univerzitet u Novom Sadu].

Salt, D. E., Blaylock, M., Kumar, N. P., Dushenkov, V., Ensley, B. D., Chet, I., Raskin, I. (1995). Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. *Nature biotechnology* **13**(5): 468-474.

Shah, F. U. R., Ahmad, N., Masood, K. R., Peralta-Videa, J. R. (2010). Heavy metal toxicity in plants. In: *Plant Adaptation and Phytoremediation*. Springer Netherlands. pp. 71-97.

Shaw, J. (1989). Heavy metal tolerance in plants: evolutionary aspects. CRC Press.

Tjio, J. H., Levan, A., (1950). The use of oxyquinoline in chromosomes analysis. *Anal. Extac. Exp. Aula Dei* **2** (1): 21-64.

Van Assche, F. & Clijsters, H. (1990). Effects of metal on enzyme activity in plants. *Plant Cell Environment* **13**:195-206

Vasilevska, M. (2005). Cytogenetic status of some plant species from natural and cultivated populations treated in clean and polluted environment. Institute of Biology, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University, Skopje.