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Flow cytometric assessment of Cd genotoxicity in three plants with different metal accumulation and detoxification capacities

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ABSTRACT

Cadmium (Cd) is a widespread environmental contaminant, strongly mutagenic and known to cause DNA damage in plants. In this work, flow cytometry (FCM) was applied to determine if *in vivo* exposure to Cd would induce genotoxic effects at the genome level. The hyper-accumulator *Thlaspi caerulescens* (J. & C. Presl), the related non-accumulator *Thlaspi arvense* L. and the accumulator crop species *Lactuca sativa* L. were germinated in distilled water and grown in modified Hoagland's medium with increasing concentrations of $Cd(NO_3)_2$ (0, 1, 10 and 100 μM). After 28 days of exposure, shoot and root growth was recorded and the tissues were harvested for Cd and FCM analysis. In general, roots from treated plants contained higher content of Cd than leaves and growth inhibition was observed in the treated plants. Nuclear DNA content was estimated and the G_0/G_1 full peak coefficient of variation (FPCV), as an indicator of clastogenic damage, was recorded. In *T. arvense* and *T. caerulescens* no significant differences were detected between control and exposed plants. Leaves of *L. sativa* exposed to 10 μM Cd presented a statistically significant increase in FPCV values in comparison with the control group. Furthermore, roots exposed to 100 μM Cd presented a reduction in nuclear DNA content and an increase in FPCV when compared to the control. FCM data indicates that no major DNA damage was induced on both Cd-exposed *Thlaspi* species and *L. sativa* leaves. On the contrary, results obtained with *L. sativa* roots suggests clastogenic damage in these organs exposed to 100 μM of Cd.

1. Introduction

Cadmium (Cd) is a cytotoxic and mutagenic metal that can affect plant growth and development (Fodor, 2002). Atmospheric deposition, urban-industrial activities, coal ash and agricultural practices (e.g. use of agrochemical products and addition of sewage sludge) are the main anthropogenic sources of Cd in soils. The molecular mechanisms of Cd genotoxicity in organisms are not well understood, but it has been suggested that it may involve direct binding of Cd to the nucleotides guanine, adenine and thymine (Hossain and Huq, 2002; Valverde et al., 2001), direct inhibition of DNA mismatch repair (Hartwig, 1994; Jin et al., 2003), or may be processed indirectly by promoting the production of reactive oxygen species (ROS) that may then damage nucleic acids (Fodor, 2002). Cadmium is known to induce genotoxicity in plants (Panda and Panda, 2002); Borboa and de La Torre (1996) have shown clastogenicity and aneugenicity in *Allium cepa* as a consequence of Cd exposure.

Flow cytometry (FCM) is a technique that theoretically has the potential to detect minute differences in nuclear DNA (nDNA) content, as well as chromosomal damage, in exposed organisms. Otto and Oldiges (1980) were able to assess chromosomal damage induced by clastogenic agents and irradiation on Chinese hamster cell lines and mice through the analysis of the coefficient of variation (CV) of the G_0/G_1 peak. The increase in CV was positively correlated with the clastogenic effects observed by microscopic examination. Flow cytometry measurement of the dispersion in the nDNA content as induced by the interactions of DNA with environmental agents, emerged then as a powerful tool in cytogenetic investigations and in genotoxicity testing (Otto et al., 1981).

Although FCM is routinely used in animal toxicological studies (Bickham et al., 1998; Biradar and Rayburn, 1995; Easton et al., 1997; Whittier and McBee, 1999), the use of similar approaches in plant genotoxicity assessment remains much less common. Significant changes in nDNA content have been detected in maize plants (*Zea mays*) exposed to coal fly ash (McMurphy and

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Rayburn, 1993) and to the fungicides captan (Rayburn et al., 1993) and triticonazole (Biradar et al., 1994). Furthermore, the mean CV of the G_0/G_1 peaks also increased in *Z. mays* individuals subjected to coal fly ash treatments (McMurphy and Rayburn, 1993). More recently, Rayburn and Wetzel (2002) reported that an increase in the CV values of the G_0/G_1 peak in both a maize mutant and in wheat grown in soil with high levels of aluminium was correlated with the number of abnormal anaphase figures.

In combination with amplified fragment length polymorphism (AFLP) analysis, FCM was also successfully introduced as a new biomonitoring tool to assess soil genotoxicity (Citterio et al., 2002). These authors demonstrated that exposure of *Trifolium repens* to Cd and Cr resulted in a decrease in the DNA index with increasing concentrations of Cr, and to an increase of debris background at the highest concentrations of Cd and Cr. More recently, Aina et al. (2006) using the same method and the same species did not find any differences in nDNA content between plants exposed to different polycyclic aromatic hydrocarbons and the control.

To test the hypothesis that 28 days *in vivo* exposure to Cd will induce DNA damage in plants the dose–response relationship on nDNA content and CV of the G_0/G_1 peak was evaluated by FCM. For this, three plant species were chosen, on the basis that they might respond differently to Cd stress due to their different patterns of Cd accumulation and strategies to store and detoxify Cd: (i) the hyper-accumulator alpine pennycress (*Thlaspi caerulescens* J. & C. Presl) that accumulates high levels of Cd in shoot tissues (up to 10.000 mg kg^{-1} in the Ganges ecotype, Lombi et al., 2000) and mainly stores Cd in electron-dense granules inside vacuoles by means of complexation with malate (Ma et al., 2005; Ueno et al., 2005); (ii) the related field pennycress (*Thlaspi arvense* L.), which is a non-accumulator plant; and (iii) lettuce (*Lactuca sativa* L.), a Cd-accumulating plant and important human food crop recommended in several standard tests (e.g. ISO/CD 17126) (ISO, 1995); both *T. arvense* and *L. sativa* possess detoxification mechanisms in which phytochelatins play an important role (Ebbs et al., 2002; Maier et al., 2003).

2. Material and methods

2.1. Plant culture and growth conditions

Seeds of *L. sativa* (cv. Reine de Mai, Oxadis, France), *T. arvense* (Amsterdam) and *T. caerulescens* (Saint-Félix-de-Pallières, Ganges, France) were germinated under dark conditions in distilled water, using perlite as support media in polystyrene seedling trays. After germination, lettuce plants were grown on modified Hoagland's nutrient solution (Monteiro et al., 2007), and *Thlaspi* sp. were grown on modified Rorison's nutrient solution. Both distilled water and nutrient solutions were supplemented with 0, 1, 10 and 100 μM $Cd(NO_3)_2$. Plants were grown at 24 ± 2 °C, under light intensity of 200 μmol m^{-2} s^{-1} and photoperiod of 16 h/8 h (light/dark). Nutrient solution was continuously aerated and changed twice a week to avoid nutrient depletion and changes in Cd concentration (Mann et al., 2005). After 28 days of exposure, plants were harvested. Morphological symptoms of Cd toxicity were noted, and shoot and root length was recorded (n=15–17). Also, plant material was collected for Cd accumulation assessment and FCM analysis.

2.2. Cadmium accumulation assessment

Cadmium concentration in the hydroponic culture medium of control and Cd-treated plants was verified by inductively coupled plasma spectroscopy (ICPS, Jobin Ivon, JY70 Plus, Longjumeau Cedex, France). Accumulation of Cd was determined in leaves and roots (n=3) dried to constant weight at 60 °C. Roots were previously washed for 10 min in 0.5 mM $CaSO_4$ to remove, by cation exchange, the Cd adsorbed by the root surface. Dried tissues were treated as described by Santos et al. (2001) and subsequently analysed by ICPS.

2.3. Flow cytometric analysis

Nuclear suspensions from plant leaves and roots were prepared according to Galbraith et al. (1983). In brief, to release nuclei from the cells, leaf sections (1–2 cm^2) and root apices (1–2 cm from the tip of 3 to 4 roots), were chopped with a sharp razor blade together with a young leaf (2 cm^2) of the internal reference standard *Pisum sativum* cv. Ctirad (for *L. sativa*; 2C=9.09 pg DNA (Doležel et al., 1998)) or *Solanum lycopersicum* cv. Stupicke (for the two *Thlaspi* spp.; 2C=1.96 pg DNA (Doležel et al., 1992)), in LB01 buffer (Doležel et al., 1989) (15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine · 4HCl, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, pH 8.0). The suspension of nuclei was then filtered through an 80 μm nylon filter to remove large tissue fragments. Afterwards, 50 μg ml^{-1} of propidium iodide (PI; Fluka, Buchs, Switzerland) and 50 μg ml^{-1} of RNase (Sigma, St. Louis, MO, USA) were added to the samples to stain nDNA and prevent the binding of PI to double stranded RNA, respectively. At least 5000 nuclei per sample were analysed in a Coulter EPICS-XL (Coulter Electronics, Hialeah, Florida, USA) flow cytometer. The instrument was equipped with an air-cooled argon-ion laser (15 mW operating at 488 nm). Before starting the analysis, the instrument was

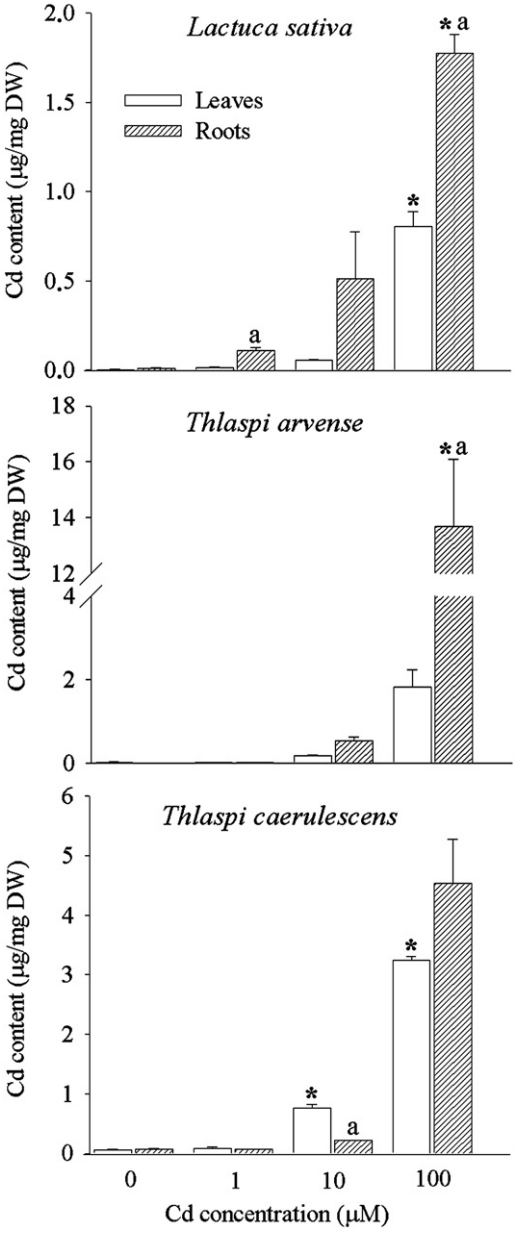

Fig. 1. Cadmium content in leaves and roots of *Lactuca sativa*, *Thlaspi arvense* and *Thlaspi caerulescens* plants after 28 days of exposure to 0, 1, 10 and 100 μM of Cd. Results are expressed as mean ± standard error of the mean. Significantly different values (*) when compared with the control group at $p<0.05$ and (a) when compared with leaf group at the same concentration of Cd.

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checked for linearity with fluorescent check beads (Coulter Electronics, Hialeah, FL) and the amplification was adjusted to position the G_0/G_1 peak of sample nuclei at channel 200. This setting was kept constant throughout the analysis. The results were acquired using the SYSTEM II software (v. 3.0, Beckman Coulter®) in the form of three histograms: linear-fluorescence light scatter (FL); FL pulse integral *versus* FL pulse height and forward angle (FS) *versus* side angle (SS)-light scatter in logarithmic scale. In the last two cytograms, "interest zones" were defined to separate intact nuclei from doublets and debris.

Three replicates per condition and tissue were analysed. In order to assess putative genotoxic effects on exposed plants, two different parameters were determined in each histogram: nuclear DNA content and full peak coefficient of variation (FPCV) of the G_0/G_1 nuclei. The nuclear DNA content was given by the ratio between the mean channel position of the sample and the internal standard multiplied by the nuclear DNA content of the reference standard. The FPCV was chosen instead of the more usual half peak coefficient of variation as it was our intention to analyse the whole dispersion of nDNA content, as diagnostic for clastogenic damage (as recommended for toxicological studies by Misra and Easton, 1999). Since the control samples of *Thlaspi* spp. roots presented very high FPCV values ($>10\%$) and debris background, the samples corresponding to exposed plants of these species were not analysed and only the results concerning the leaves are presented. The high FPCV values registered in roots might be related with the presence of secondary metabolites that might interfere with propidium iodide binding to DNA (Loureiro et al., 2006).

2.4. Statistical analysis

Statistical differences between control and exposed leaves and roots were analysed using one-way ANOVA, followed by the appropriate post-hoc tests (Dunnett's method). Where necessary, data were transformed to achieve normality and equality of variance. If these criteria were not satisfied even after data transformation, non-parametric tests were performed (Kruskal–Wallis one-way ANOVA). SigmaStat (version 3.01, SPSS, Chicago, IL, USA) was used to perform all statistical tests.

3. Results

3.1. Cd accumulation assessment

The concentration of Cd in the nutrient solution of control plants was below the ICPS detection limit (<0.01 μM). In the nutrient solutions with the nominal concentrations of 1, 10 and 100 μM $Cd(NO_3)_2$ the concentrations of Cd were 1.88, 10.50 and 96.97 μM for lettuce and 1.25, 11.56 and 100.52 μM for *Thlaspi* spp., respectively.

The accumulation of Cd in roots and leaves of lettuce and *Thlaspi* spp. plants at the 28th day of exposure is presented in Fig. 1. In general, roots from treated plants contained higher content of Cd than leaves. Specifically, plants exposed to 100 μM Cd presented about 2.0, 8.0 and 1.4-fold higher content of Cd in roots than in leaves of *L. sativa*, *T. arvense* and *T. caerulescens*, respectively. In contrast, among *T. caerulescens* plants exposed to 10 μM Cd, the content of Cd in the leaves was more than 3-fold higher than in roots.

3.2. Toxicity symptoms and plant growth

Physical manifestations of Cd toxicity were observed in plants within the 28 days of culture. Plants developed toxicity symptoms in the form of chlorotic lesions, especially in expanded leaves of *L. sativa* and *T. arvense* (Fig. 2). Expanded leaf-fall was observed among Cd-treated plants during exposure, and the roots of Cd-treated plants appeared darker than those of control plants.

In general, exposure to Cd in the nutrient solution led to growth inhibition in the studied plants (Fig. 3). The growth of lettuce shoots and roots was significantly reduced at Cd concentrations of 10 and 100 μM ($p<0.05$). In the case of *T. arvense* growth inhibition was evident, with statistically significant differences ($p<0.05$) being observed in leaves after an exposure to 10 and 100 μM of Cd and on roots at a Cd concentration of 100 μM ($p<0.05$). Finally, growth in *T. caerulescens* was significantly reduced ($p<0.05$) only after exposure to 100 μM Cd.

3.3. Flow cytometric analysis

Cadmium treated plants of *T. caerulescens* and *T. arvense* presented similar nDNA content and FPCV values to those obtained for the control group (see Table 1). The small differences that were observed were not statistically significant ($p>0.05$). The nDNA contents of *T. caerulescens* and *T. arvense* were estimated to be 0.63 ± 0.006 and 1.19 ± 0.005 pg/2C, respectively. To our knowledge, this is the first estimation of nDNA content for the species *T. caerulescens*. FPCV values were much higher in *T. caerulescens* than in *T. arvense*, which is likely associated with the higher impact that autofluorescent particles (such as chloroplasts) may have on the nuclei with lower fluorescence (those of *T. caerulescens*).

In *L. sativa* (Table 1, Fig. 4), roots exposed to 100 μM Cd presented a statistically significant reduction ($p<0.05$) in nDNA content (5.89 ± 0.056 pg/2C) when compared to the estimation obtained for the control plants (6.13 ± 0.055 pg/2C). On the other hand, in leaves, no statistically significant differences in nDNA content were obtained between control and exposed plants. There was a statistically significant increase in FPCV after the exposition of leaves to 10 μM Cd and of roots to 100 μM Cd.

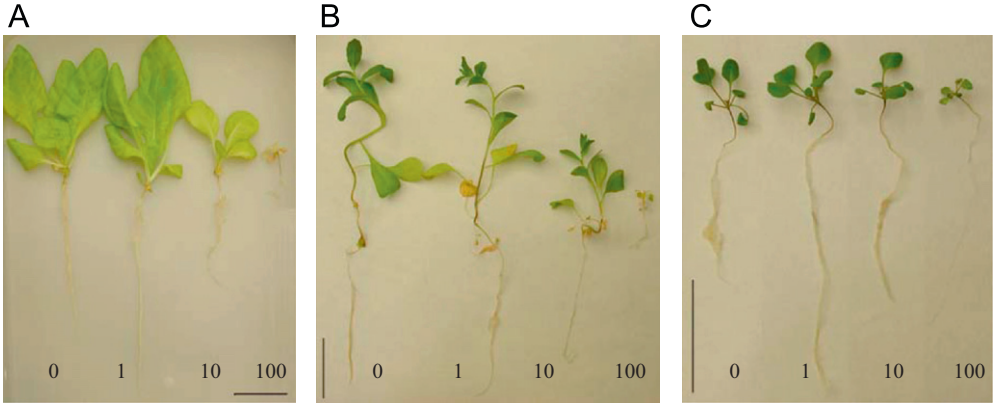

Fig. 2. Morphological aspect of the plants of *Lactuca sativa* (A), *Thlaspi arvense* (B) and *Thlaspi caerulescens* (C) after 28 days of exposure to 0, 1, 10 and 100 μM of Cd. Bars represent 5 cm.

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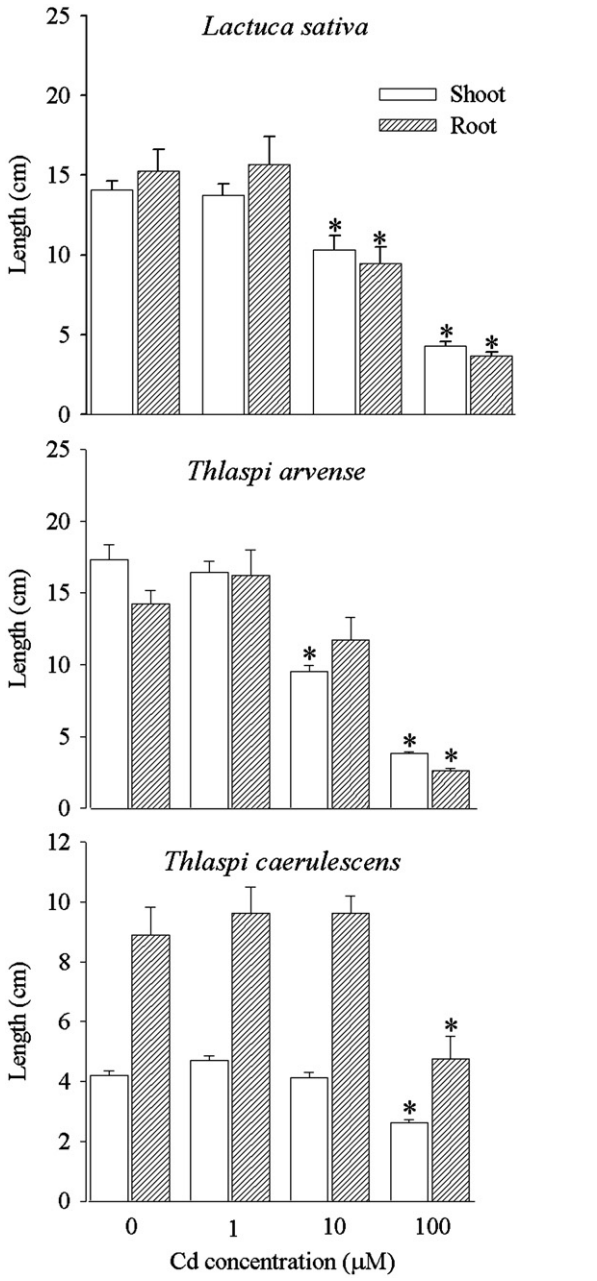

Fig. 3. Shoot and root length of *Lactuca sativa*, *Thlaspi arvense* and *Thlaspi caerulescens* plants after 28 days of exposure to 0, 1, 10 and 100 μM of Cd. Results are expressed as mean ± standard error of the mean. (*) Significantly different values when compared with the control group at $p < 0.05$.

Table 1
Nuclear DNA content and FPCV of plants exposed to 0, 1, 10 and 100 μM Cd.

Species and organ	Cd (μM)	DNA content (pg/2C)	FPCV (%)
Thlaspi caerulescens **Leaf**	0	0.63 ± 0.006	8.60 ± 0.441
	1	0.64 ± 0.012	8.71 ± 0.232
	10	0.66 ± 0.002	8.35 ± 0.930
	100	0.65 ± 0.017	9.83 ± 0.990
Thlaspi arvense **Leaf**	0	1.19 ± 0.005	4.53 ± 0.456
	1	1.17 ± 0.008	4.66 ± 0.269
	10	1.17 ± 0.022	4.91 ± 0.335
	100	1.18 ± 0.027	4.90 ± 0.689
Lactuca sativa **Leaf**	0	6.41 ± 0.044	3.65 ± 0.287
	1	6.36 ± 0.073	4.26 ± 0.590
	10	6.32 ± 0.077	**4.90 ± 0.512***
	100	6.25 ± 0.194	4.64 ± 0.43
Lactuca sativa **Root**	0	6.13 ± 0.055	4.77 ± 0.643
	1	6.27 ± 0.045	4.63 ± 0.358
	10	6.23 ± 0.081	4.94 ± 0.283
	100	**5.89 ± 0.056***	**6.63 ± 0.619***

Results are expressed as mean ± standard deviation of the mean ($n=3$).

* significantlly different to controls ($p < 0.5$).

4. Discussion

Exposure of the three plants to Cd resulted in an array of dose dependant morphological effects (e.g. inhibition of leaf and root growth, chlorosis in the leaves and browning of the roots) that are symptoms of Cd toxicity found in general in plants (Prasad, 1995), and in particular in *Thlaspi* species (Ozturk et al., 2003; Wójcik et al., 2005) and lettuce (Michalska and Asp, 2001).

Flow cytometric analyses of the leaves of *T. arvense* and *T. caerulescens* revealed homogeneity in nDNA content and FPCV values, suggesting that no clastogenic damage occurred due to Cd exposure. The two *Thlaspi* species were expected to have different responses to Cd stress, based on their different patterns of Cd accumulation and detoxying mechanisms. These results seem to indicate that besides the severe inhibition of growth and the accumulation of Cd in roots and subsequent translocation to the leaves, even at higher levels than the hyper-accumulator species *T. caerulescens*, *Thlaspi arvense* possess mechanisms to cope with Cd translocation to leaves. These mechanisms involve the chelation of Cd by phytochelatins (Ebbs et al., 2002) and may prevent the accumulation of ionic Cd^{2+} in the cells where it could cause DNA damage. On the other hand, the absence of DNA damage in *T. caerulescens* leaves is in agreement with what was expected; this species should be less sensitive to Cd toxicity effects since it has highly efficient mechanisms to chelate and store Cd in leaf vacuoles without showing toxicity symptoms (Ma et al., 2005; Ueno et al., 2005).

Lactuca sativa has been for some time a "model" species for Cd genotoxicity tests in our laboratory. In preliminary trials using the same methodology, no changes in nDNA content and in CV (HPCV) values were detected in 5-week-old lettuce plants exposed to 100 μM Cd for 14 days (Monteiro et al., 2004). The effects of metals are strongly dependent on the age of the plant at the time of exposure: the older the plant the larger the amount of metal that can be tolerated, because metals accumulate at metabolically inactive sites such as cell walls and vacuoles (Fodor, 2002). This might explain the absence of observable effects in a previous study (Monteiro et al., 2004). Therefore, in the present work it was decided to assess the genotoxic effects in younger plants. In lettuce, statistically signifcant changes in nDNA content and FPCV values have been detected. These results suggest that Cd stress may be leading to clastogenic DNA damage as a consequence of loss of chromosome portions, because nDNA content is depressed. Still, other factors may be governing the observed differences: (i) higher condensation of nDNA in exposed tissues, which could lead to a lower binding of PI (a chromatin state sensitive fluorochrome) to DNA structure and thus to a lower estimation of nDNA content (Doležel and Bartos, 2005); (ii) higher amounts of secondary metabolites (e.g. phenolic compounds) in the cytosol of cells exposed to Cd. It was previously demonstrated that phenolic compounds affect the fluorescence and light scatter properties of plant nuclei by interfering with the stoichiometric binding of PI to DNA and by aggregating other particles to plant nuclei, which leads to higher CV values (Loureiro et al., 2006). Several authors have already demonstrated that part of the mechanism for metal tolerance in plants involves the production of organic acids and the release of phenolic compounds to the

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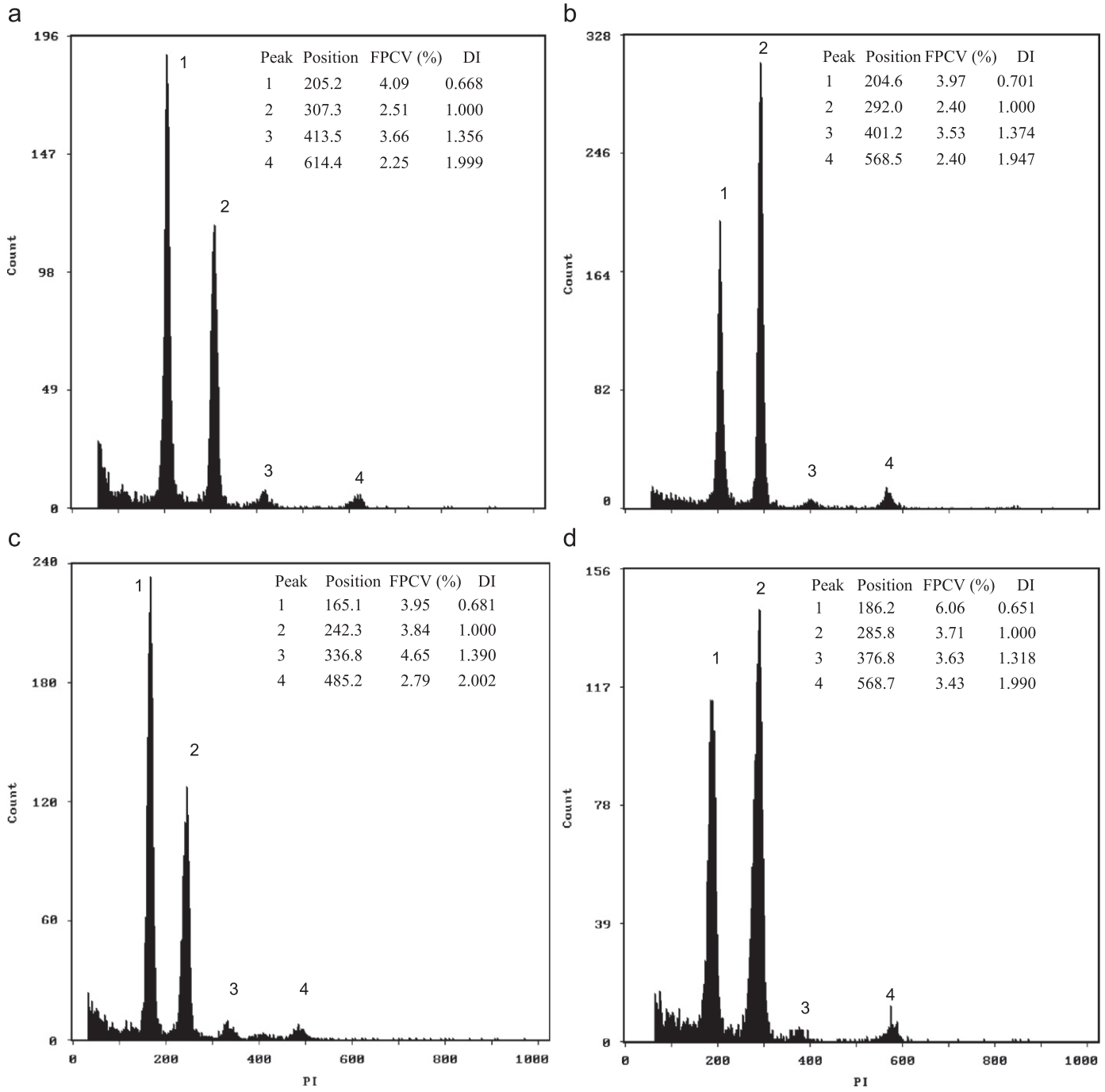

Fig. 4. Histograms of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from *Pisum sativum* cv. Ctirad (as reference standard) and *Lactuca sativa* leaves (a and c) and roots (b and d) exposed to 0 (a and b) and 100 μM Cd (c and d). In all FL histograms four peaks were observed: 1 – nuclei at G_0/G_1 phase of *L. sativa*; 2 – nuclei at G_0/G_1 phase of *P. sativum*; 3 – nuclei at G_2 phase of *L. sativa*; 4 – nuclei at G_2 phase of *P. sativum*. For each peak the following information is given: mean fluorescence in arbitrary units (Mean FL), full peak coefficient of variation (FPCV) and the DNA index (ratio between the mean FL of each peak and the mean FL of the G_0/G_1 peak of the internal standard, DI).

cytosol (Delhaize et al., 1993a, 1993b; Mullet et al., 2002). In the particular case of Cd, Irtelli and Navari-Izzo (2006) revealed an increase of phenolic compounds in leaves of *Brassica juncea* that were under Cd exposure.

Previous work by Monteiro et al. (2007) support the hypothesis that Cd stress has indeed resulted in DNA damage. Using microsatellite markers these authors demonstrated a mutation rate of 3.7% in roots of lettuce exposed to 10 μM Cd. Moreover, in the present study and in the work of Monteiro et al. (2007), the genotoxic effects occurred mainly in the roots and not in the leaves (except the increase in FPCV registered in leaves of 10 μM Cd-exposed plants). Therefore, the results obtained by FCM should be due to DNA damage induced by Cd stress and not to the other factors mentioned above. Actually, Cd was accumulated in far greater concentrations (2-fold) in the roots than in the leaves of *L. sativa* exposed to 100 μM Cd, which may well be related with the genotoxicity patterns that were detected. Gichner and co-workers obtained similar results, with Cd

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exposure inducing DNA damage only in the roots of *Nicotiana tabacum* L., but not on the leaves (Gichner et al., 2004), and with the absence of Cd genotoxicity in the leaves of *Solanum tuberosum* L. subjected to short-term treatments with Cd (Gichner et al., 2008). In both studies the authors related the absence of genotoxic effects of Cd in plant leaves, to the lower accumulation of this metal in this organ and to the presence of a better antioxidant defence system that might protect the nuclear DNA in leaf cells from Cd-induced oxidative stress (Gichner et al., 2004, 2008). Gichner et al. (2004) found that the activity of catalase (an antioxidant enzyme) was about 30 times higher in tobacco leaves than in roots, which underscores the differences in sensitivity to Cd exposure in leaves and roots.

5. Conclusions

The results suggest that on the basis of FCM analysis, a 28-day exposure to Cd induced no DNA damage on both Cd-exposed *Thlaspi* species and *L. sativa* leaves. On the other hand, results obtained with *L. sativa* roots suggests clastogenic damage in these organs exposed to 100 μM of Cd.

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References

Aina, R., Palin, L., Citterio, S., 2006. Molecular evidence for benzo[a]pyrene and naphthalene genotoxicity in *Trifolium repens* L. Chemosphere. 65, 666–673.

Bickham, J.W., Mazet, J.A., Blake, J., Smolen, M.J., Ballachey, B.E., 1998. Flow cytometric determination of genotoxic effects of exposure to petroleum in mink and sea otters. Ecotoxicology 7, 191–199.

Biradar, D.P., Pedersen, W.L., Rayburn, A.L., 1994. Nuclear DNA analysis of maize seedlings treated with the triazole fungicide, triticonazole. Pest. Sci. 41, 291–295.

Biradar, D.P., Rayburn, A.L., 1995. Flow cytogenetic analysis of whole cell clastogenicity of herbicides found in groundwater. Arch. Environ. Contam. Toxicol. 28, 13–17.

Borboa, L., de La Torre, C., 1996. The genotoxicity of Zn(II) and Cd(II) in *Allium cepa* root meristematic cells. New Phytol. 134, 481–486.

Citterio, S., Aina, R., Labra, M., Ghiani, A., Fumagalli, P., Sgorbati, S., Santagostino, A., 2002. Soil genotoxicity assessment: a new strategy based on biomolecular tools and plant bioindicators. Environ. Sci. Technol. 36, 2748–2753.

Delhaize, E., Craig, S., Beaton, C.D., Bennet, R.J., Jagadish, V.C.R., Randall, P.J., 1993a. Aluminum tolerance in wheat (*Triticum-Aestivum* L.). I. Uptake and distribution of aluminum in root apices. Plant Physiol. 103, 685–693.

Delhaize, E., Ryan, P.R., Randall, P.J., 1993b. Aluminum tolerance in wheat (*Triticum-Aestivum* L.). II. Aluminum-stimulated excretion of malic-acid from root apices. Plant Physiol. 103, 695–702.

Doležel, J., Bartos, J., 2005. Plant DNA flow cytometry and estimation of nuclear genome size. Ann. Bot. 95, 99–110.

Doležel, J., Biranová, P., Lucretti, S., 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. Biol. Plant. 31, 113–120.

Doležel, J., Greilhuber, J., Lucretti, S., Meister, A., Lysák, M., Nardi, L., Obermayer, R., 1998. Plant genome size estimation by flow cytometry: interlaboratory comparison. Ann, Bot. 82, 17–26.

Doležel, J., Sgorbati, S., Lucretti, S., 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. Physiol. Plant. 85, 625–631.

Easton, M.D.L., Kruzynski, G.M., Solar, I.I., Dye, H.M., 1997. Genetic toxicity of pulp mill effluent on juvenile chinook salmon (*Oncorhynchus tshawytscha*) using flow cytometry. Water Sci. Technol. 35, 347–355.

Ebbs, S., Lau, I., Ahner, B., Kochian, L., 2002. Phytochelatin synthesis is not responsible for Cd tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescens* (J. & C. Presl). Planta 214, 635–640.

Fodor, F., 2002. Physiological responses of vascular plants to heavy metals. In: Prasad, M.N.V., Strzałka, K. (Eds.), Physiology and Biochemistry of Metal Toxicity and Tolerance in Plants. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 149–177.

Galbraith, D.W., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P., Firoozabady, E., 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220, 1049–1051.

Gichner, T., Patková, Z., Száková, J., Demnerová, K., 2004. Cadmium induces DNA damage in tobacco roots, but no DNA damage, somatic mutations or homologous recombination in tobacco leaves. Mut. Res. 559, 49–57.

Gichner, T., Patkova, Z., Szakova, J., Znidar, I., Mukherjee, A., 2008. DNA damage in potato plants induced by cadmium, ethyl methanesulphonate and γ-rays. Environ. Exp. Bot. 62, 113–119.

Hartwig, A., 1994. Role of DNA-repair inhibition in lead-induced and cadmium-induced genotoxicity – a review. Environ. Health Perspect. 102, 45–50.

Hossain, Z., Huq, F., 2002. Studies on the interaction between Cd^{2+} ions and nucleobases and nucleotides. J. Inorg. Biochem. 90, 97–105.

Irtelli, B., Navari-Izzo, F., 2006. Influence of sodium nitrilotriacetate (NTA) and citric acid on phenolic and organic acids in *Brassica juncea* grown in excess of cadmium. Chemosphere 65, 1348–1354.

ISO, 1995. Soil quality – determination of the effects of pollutants on soil flora – Part 2: Effects of chemicals on the emergence of higher plants. ISO – International organization for standardization, Genéve, pp. 2–7.

Jin, Y.H., Clark, A.B., Slebos, R.J.C., Al-Refai, H., Taylor, J.A., Kunkel, T.A., Resnick, M.A., Gordenin, D.A., 2003. Cadmium is a mutagen that acts by inhibiting mismatch repair. Nat. Genetics 34, 326–329.

Lombi, E., Zhao, F.J., Dunham, S.J., McGrath, S.P., 2000. Cadmium accumulation in populations of *Thlaspi caerulescens* and *Thlaspi goesingense*. New Phytol. 145, 11–20.

Loureiro, J., Rodriguez, E., Doležel, J., Santos, C., 2006. Flow cytometry and microscopic analysis of the effect of tannic acid on plant nuclei and estimation of DNA content. Ann, Bot. 98, 515–527.

Ma, J.F., Ueno, D., Zhao, F.J., McGrath, S.P., 2005. Subcellular localisation of Cd and Zn in the leaves of a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. Planta 220, 731–736.

Maier, E.A., Matthews, R.D., McDowell, J.A., Walden, R.R., Ahner, B.A., 2003. Environmental cadmium levels increase phytochelatin and glutathione in lettuce grown in a chelator-buffered nutrient solution. J. Environ. Qual. 32, 1356–1364.

Mann, R.M., Matos, P., Loureiro, S., Soares, A.M.V.M., 2005. Foundation studies for cadmium accumulation studies in terrestrial isopods–diet selection and diet contamination. Eur. J. Soil Biol. 41, 153–161.

McMurphy, L.M., Rayburn, A.L., 1993. Nuclear alterations of maize plants grown in soil contaminated with coal fly ash. Arch. Environ. Contam. Toxicol. 25, 520–524.

Michalska, M., Asp, H., 2001. Influence of lead and cadmium on growth, heavy metal uptake, and nutrient concentration of three lettuce cultivars grown in hydroponic culture. Commun. Soil Sci. Plant Anal. 32, 571–583.

Misra, R.K., Easton, M.D.L., 1999. Comment on analyzing flow cytometric data for comparison of mean values of the coefficient of variation of the G1 peak. Cytometry 36, 112–116.

Monteiro, M., Santos, C., Mann, R.M., Soares, A.M.V.M., Lopes, T., 2007. Evaluation of cadmium genotoxicity in *Lactuca sativa* L. using nuclear microsatellites. Environ. Exp. Bot. 60, 421–427.

Monteiro, M.S., Lopes, T., Loureiro, J., Mann, R.M., Santos, C., Soares, A.M., V.M., 2004. An assessment of genetic stability in cadmium-treated *Lactuca sativa* L. by flow cytometry and microsatellite analysis. In: Fourth SETAC World Congress and 25th Annual Meeting in North America, Portland, Oregon, USA, 442 pp.

Mullet, J.E., Klein, R.R., Klein, P.E., 2002. *Sorghum bicolor* – an important species for comparative grass genomics and a source of beneficial genes for agriculture. Cur. Op. Plant Biol. 5, 118–121.

Otto, F.J., Oldiges, H., 1980. Flow cytogenetic studies in chromosomes and whole cells for the detection of clastogenic effects. Cytometry 1, 13–17.

Otto, F.J., Oldiges, H., Göhde, W., Jain, V.K., 1981. Flow cytometric measurement of nuclear DNA content variations as a potential *in vivo* mutagenicity test. Cytometry. 2, 189–191.

Ozturk, L., Karanlik, S., Ozkutlu, F., Cakmak, I., Kochian, L.V., 2003. Shoot biomass and zinc/cadmium uptake for hyperaccumulator and non-accumulator *Thlaspi* species in response to growth on a zinc-deficient calcareous soil. Plant Sci. 164, 1095–1101.

Panda, B.B., Panda, K.K., 2002. Genotoxicity and mutagenicity of metals in plants. In: Prasad, M.N.V., Strzałka, K. (Eds.), Physiology and Biochemistry of Metal Toxicity and Tolerance in Plants.. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 395–414.

Prasad, M.N.V., 1995. Cadmium toxicity and tolerance in vascular plants. Environ. Exp. Bot. 35, 525–545.

Rayburn, A.L., Pedersen, W.L., McMurphy, L.M., 1993. The fungicide captan reduces nuclear DNA content in maize seedlings. Pest. Sci. 37, 79–82.

Rayburn, A.L., Wetzel, J.B., 2002. Flow cytometric analyses of intraplant nuclear DNA content variation induced by sticky chromosomes. Cytometry 49, 36–41.

Santos, C.L.V., Campos, A., Azevedo, H., Caldeira, G., 2001. *In situ* and *in vitro* senescence induced by KCl stress: nutritional imbalance, lipid peroxidation and antioxidant metabolism. J. Exp. Bot. 52, 351–360.

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Ueno, D., Ma, J.F., Iwashita, T., Zhao, F.J., McGrath, S.P., 2005. Identification of the form of Cd in the leaves of a superior Cd-accumulating ecotype of *Thlaspi caerulescens* using Cd-113-NMR. Planta 221, 928–936.

Valverde, M., Trejo, C., Rojas, E., 2001. Is the capacity of lead acetate and cadmium chloride to induce genotoxic damage due to direct DNA–metal interaction? Mutagenesis 16 265–270.

Whittier, J.B., McBee, K., 1999. Use of flow cytometry to detect genetic damage in mallards dosed with mutagens. Environ. Toxicol. Chem. 18, 1557–1563.

Wójcik, M., Vangronsveld, J., Tukiendorf, A., 2005. Cadmium tolerance in *Thlaspi caerulescens*: I. Growth parameters, metal accumulation and phytochelatin synthesis in response to cadmium. Environ. Exp. Bot. 53, 151–161.

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