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# Inorganic Hg toxicity in plants: a comparison of different genotoxic parameters

Raquel Azevedo[1], Eleazar Rodriguez[1], Rafael José Mendes[2], Nuno Mariz-Ponte[2], Sara Sario[2], José Carlos Lopes,[3] José Miguel P. Ferreira de Oliveira[4], Conceição Santos*[2]

[1] Laboratory of Biotechnology and Cytomics, University of Aveiro, 3810-123 Aveiro, Portugal

[2] $iB_2$Lab, Department of Biology, Faculty of Sciences, LAQV/REQUIMTE, University of Porto, Rua do Campo Alegre 4169-007, Porto, Portugal

[3] Department of Physics, University of Aveiro, 3810-123 Aveiro, Portugal

[4] LAQV, REQUIMTE, Laboratory of Applied Chemistry, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Porto, 4050-313 Porto, Portugal

* Corresponding author

Email: csantos@fc.up.pt

**Abstract**

Inorganic Mercury (Hg) contamination persists an environmental problem, but its cyto- and genotoxicity in plants remains yet unquantified. To determine the extent of Hg-induced cyto- and genotoxicity, and assess most sensitive endpoints in plants, *Pisum sativum* L. seedlings were exposed for 14 days to different $HgCl_2$ concentrations up to 100 µM. Shoots and roots from hydroponic exposure presented growth impairment and/or morphological disorders for doses >1 µM, being the roots more sensitive. Plant growth, ploidy changes, clastogenicity (HPCV), cell cycle dynamics ($G_1$-S-$G_2$), Comet-tail moment (TM), Comet-TD, Mitotic-index (MI) and cell proliferation index (CPI) were used to evaluate Hg-induced cyto/genotoxicity. Both leaf and root DNA-ploidy levels, assessed by flow cytometry (FCM), remained unaltered after exposure. Root cell cycle impairment occurred at lower doses ($\geq$1 µM) than structural DNA damages ($\geq$10 µM). Cytostatic effects depended on the Hg concentration, with delays during S-phase at lower doses, and arrests at $G_1$ at higher ones. This arrest was paralleled with decreases of both mitotic index (MI) and cell proliferation index (CPI). DNA fragmentation, assessed by the Comet assay parameters of TD and TM, could be visualized for conditions $\geq$10 µM, while FCM-clastogenic parameter (FPCV) and micronuclei (MNC) were only altered in roots exposed to 100 µM. We demonstrate that inorganic-Hg induced cytostaticity is detectable even at 1 µM (a value found in contaminated sites), while structural DNA breaks/damage are only visualized in plants at concentrations $\geq$10 µM. We also demonstrate that among the different techniques tested for cyto- and genotoxicity, TD and TM Comet endpoints were more sensitive than FPCV or MNC. Regarding cytostatic effects, cell cycle analysis by FCM, including the difference in % cell cycle phases and CPI were more sensitive than MI or MNC frequency. Our data contribute to better understand Hg cyto- and genotoxicity in plants and to understand the information and sensitivity provided by each of the genotoxic techniques used.

*Keywords:* Mercury; Cell cycle; Comet assay; Flow cytometry; Micronuclei; *Pisum sativum*

## 1. Introduction

The increasing environmental pollution with mercury (Hg) has raised a serious concern worldwide, with the European Union (EU) publishing in 2008 the Mercury repealing and replacing Regulation (EC) 1102/2008, and signing in 2013 the Minamata Convention on Mercury (http://ec.europa.eu/environment/chemicals/mercury/ratification_en.htm). Despite these efforts, many industries continue to release worldwide high amounts of metals/pollutants. A major use of mercury is in the chlor-alkali industry (Järup, 2003) and major forms of Hg released to the environment include mercuric ($Hg^{2+}$), mercurous ($Hg_2^{2+}$) or elemental (Hgo) (Wuana and Okeimen, 2011). Sewage sludge is a potential adsorbent of Hg (Natarajan and Manivasagan, 2015), and its wide use in agriculture as fertilizer potentiates the risk of exposing crops to Hg, and

eventually present phytotoxicity and/or transfer Hg through the food chain (Morgan, 2013; Roy and McDonald, 2013).

Maximum limits of Hg levels in soil, water, and sewage sludge have been established by several countries. These represent the limit beyond which Hg-induced toxicity can occur. For example, the American Environmental Protection Agency set the Hg maximum admissible contaminant level goals at 0.002 mg/L (https://www.epa.gov/), while the maximum admissible Hg concentration in sewage sludge (e.g., from industrial leakages, mining, pesticide industries) in several countries range ~16 mg/kg (EU, 2004; Kuusik *et al*., 2017), above which toxic effects are assumed as potentially occurring. Worldwide, the average content of Hg in soil is within the range of 0.01-1.5 mg/kg, but rarely surpasses the 1.0 mg/kg (Kabata-Pendias and Szteke, 2015). Moreover, the levels of Hg in the soils vary according to the type of soil, its location (e.g., the proximity to mining sites or pesticides industries). For example, in Spain, agriculture soils contain Hg within the range of 0.001-0.22 mg/kg (Rodríguez *et al*., 2009), whilst in some Brazil regions, the soil contents of Hg vary from 1.6 to 29.1 mg/kg (Kabata-Pendias *et al*., 2015). In India, Hg contamination in water was found to reach alarming values due to the discharge of Hg-containing industrial effluents ranging up to 0.268 mg/L (Srivastava, 2009), while in Japan the limit is 0.4 mg/kg (Akiyama *et al*., 2017). In some regions of China, Guo *et al*. (2011) also found Hg levels much above the permitted limits, including some rice fields' soil with levels varying between 2 and 186 mg/kg in sites near ores (Meng *et al*., 2014).

Urban/industrial sewage sludge wastes are emerging as potential sources of nutrients in sustainable agriculture (Kirchmann *et al*., 2017; Sánchez-Báscones, *et al*., 2016). This reuse brings, however, the concern that inorganic Hg commonly found in those sludge wastes will pose implicit toxicological risks. Moreover, as stressed by Boatti *et al*., (2017), little is known regarding molecular mechanisms regulating the interactions of Hg, which is even more dramatic in crops yield and food safety.

Data on Hg phytotoxicity is scarce (revised by Mahbub *et al*., 2017b), and is even scarcer regarding Hg phyto-, cyto-, and genotoxicity. Plants can be contaminated by Hg because it interferes with some micronutrients (Merchant, 2010), and/or bind to sulfur- or nitrogen-rich ligands. Hg represses plant growth (Mondal *et al*., 2015; Mahbub *et al.*, 2017a) and induces morphological and physiological changes (Ortega-Villasante *et al* 2005; Cargnelutti *et al*., 2006; Turino *et al*., 2006; Clemens and Ma, 2016), including oxidative stress (Sahu *et al*., 2012; Tamás *et al*., 2015; Chen *et al*., 2017; Tamás and Zelinová, 2017) and impairments of net photosynthesis (Marrugo-Negrete *et al*., 2016). Regarding Hg-induced cyto- and genotoxicity, Subhadra and Panda (1994) reported that 100 µM methyl mercuric chloride induced abnormal anaphases and micronuclei (MNC) in *Hordeum vulgare*. High levels of Hg also induced MNC in *Cicia faba* and chromosomal aberrations in *Allium cepa* roots (e.g., Babu and Maheswari, 2006). It was proposed that Hg can interact with DNA, producing point mutations (Manikandan *et al.*, 2015), in addition

to alterations in chromosome structure and number (Patra *et al*., 2004), but information on its mechanisms remains insufficient. The maintenance of genomic material integrity is of vital importance, not only because DNA damages can seriously affect survival but also because in plants the successive accumulation of DNA damage could lead to disastrous consequences to the progeny (Singh *et al*., 2008). Thus, the evaluation of Hg cyto- and genotoxicity is a subject of extreme importance due to the high risk of exposure and severe toxicity of this metal.

To assess the genotoxicity induced by inorganic Hg, robust and accurate techniques must be applied, such as flow cytometry (FCM), a technique that allows rapid and highly accurate multiparametric assays. FCM was used to assess Cd and Cr(VI) genotoxicity in lettuce and pea (Monteiro *et al*., 2010; Rodriguez *et al*., 2011). Also, the Comet Assay requires a low number of cells and provides sensitive information, detecting double and single-strand DNA breaks (Koppen *et al*., 2017, Collins *et al*., 2008, Glei *et al*., 2016). Lastly, plant root meristems are actively proliferating and sensitive to the effects caused by pollutants or stress, being a good source for cytological studies, like mitotic index or MNC assay, which despite being highly time-consuming techniques, are frequently used as biomarkers of metals-induced genotoxicity (Feng *et al*., 2007).

The aim of this work was to characterize the cyto- and genotoxicity of inorganic Hg in *Pisum sativum* L., an important crop species for animal and human nutrition (Garousi *et al*., 2017), which is also widely used as a model in other toxicological approaches (e.g., Souguir *et al*., 2008). In order to accomplish this, increasing concentrations of mercury chloride ($HgCl_2$) (a form of Hg supply widely used in this kind of studies, (e.g., Li *et al*., 2006; Hussein *et al*., 2008) were administered using a hydroponic system (e.g., Monteiro *et al*., 2010). The Hg concentrations were selected based on levels that may be encountered in contaminated soils and tailings near e.g., chlor alkali industries or ore mining sites (mainly gold mines) (Rodríguez *et al*., 2009). Hg-induced cyto/genotoxicity was assessed by comparing several parameters including plant growth, ploidy changes, clastogenicity (HPCV), cell cycle dynamics (G1-S-G2), Comet-tail moment (TM), Comet-Tail DNA (TD), Mitotic-index (MI) and cell proliferation index (CPI). This information provides a better perception of inorganic Hg-induced phytogenotoxic mechanisms and provides a discussion on the most suitable endpoints in similar studies.

## 2. **Material and Methods**

### *2.1. Plant material, growth conditions, and treatments*

*Pisum sativum* (cv. Telephone) seeds were surface-sterilized with 70% ethanol (2 min) and ammonia hypochlorite (8 min), rinsed with sterile distilled water and germinated in Petri dishes covered with filter paper in the dark. Three-day-old seedlings were hydroponically grown with Hoagland's nutrient solution. A stock solution of $HgCl_2$ (Sigma, USA) was prepared in deionized water, and the required volume added to the nutrient solution to obtain the final concentrations of Hg: 0, 0.1, 1.0, 10 and 100 µM. Plantlets were cultivated for 14 days (14 days exposed to Hg)

with a day/night cycle of 16:8h at 21ºC, under a light intensity of 200 µmol $m^{-2}$ $s^{-1}$. Cultures were closed with disposable plastic to minimize Hg lost by volatilization (e.g., Moreno *et al*., 2008). Nutrient solution was constantly aerated and replaced twice a week during the experience (Monteiro *et al*., 2010). At the end of the experiment, shoot and root length were measured, and morphological characterization registered.

### *2.2. Hg content analysis*

Leaves (at the same stage of development) and roots were collected and lyophilized for further analysis. Roots were thoroughly rinsed in water, washed for 5 min in 0.5 mM $CaSO_4$ to remove (by cation exchange) Hg adsorbed and rinsed again with distilled water. Hg concentration in solutions and its content in both roots and leaves were measured in AMA 254 Mercury Analyzer (UK), with the limit detection of 0.001 µg/g. TORT-2 (0.27±0.06) and Peach Leaf (0.031±0.007) were used as internal references and three replicates for each individual were measured (Száková *et al*., 2004). Hg-exposed and control/reference plant organs were digested in 4 M $HNO_3$ for 5-6 h at 40 ºC prior to analysis. Analytical Hg concentrations found for nutrient solutions (with nominal Hg concentrations of 0.1, 1.0, 10 and 100 µM) were, respectively, of 0.10; 0.99, 9.93 and 99.7 µM.

### *2.3. Cell cycle and DNA damage evaluation by FCM*

Nuclei suspensions were prepared using root apices (1 mm from root tip) and leaves from five individuals per condition, as described by Rodriguez *et al*. (2011). Briefly, 1 mL of nuclei suspension was filtered through a 50 µm nylon filter. Nuclei were stained with 50 mg/mL propidium iodide (PI) (Fluka, Buchs, Switzerland), and 50 mg/mL RNase (Sigma, St Louis, MO, USA) was added. After incubation (10 min), nuclei were analyzed in a Coulter EPICS XL flow cytometer (Hialeah, FL, USA). Results were acquired using the SYSTEM II software version 3.0 (Coulter Electronics). Forward light scatters (FS, relative size/volume of nuclei), and side light scatters (SS, relative optical complexity/granularity), relative fluorescence (FL, variation in DNA staining) were monitored. In the $G_1$ peak, the half peak coefficient of variation (%HPCV), and the full peak coefficient of variation (%FPCV) were evaluated as indicators of putative clastogenicity, and were measured according to Rayburn and Wetzel (2002). CPI was calculated as CPI=(%S+%$G_2$)/(%$G_1$+%S+%$G_2$) (Almeida *et al*., 2011).

### *2.4. Comet assay*

Unexposed and Hg-exposed roots and leaves were placed in a Petri dish kept on ice and spread with 300 µL of cold 0.4 M Tris buffer, pH 7.5 (Gichner *et al*., 2008 a,b) and modified for pea according to Rodriguez *et al* (2011). Fresh apical roots and leaves were gently sliced. For positive control, similar samples were immersed in 100 µM $H_2O_2$ for 20 min. Fifty microliters of the

nuclear suspension were gently dispersed in 50 μL of 1% LMP (Low Melting Point) agarose in PBS (Phosphate Buffer Solution) at 40 ºC and embedded into gels on glass microscope slides pre-coated with 1% NMP (Normal Melting Point) agarose, with a coverslip on top. The slides were cooled at 4 ºC for a minimum of 5 min, the coverslip was removed. A final layer of 0.5% LMP agarose (100 μL) was placed on the slides and they were cooled again for at least 5 min at 4 ºC, removing the coverslip posteriorly. The cells were incubated at 4 °C with electrophoresis buffer (1 mM $Na_2EDTA$ and 300 mM NaOH, pH >13). Subsequently, gels underwent electrophoresis (0.75 V/cm at 4 ºC, with dim light) for 30 min. After electrophoresis, the slides were rinsed three times with 400 mM Tris buffer, pH 7.5, stained with 80 μL ethidium bromide for 5 min, dipped in ice-cold water and covered with a coverslip. For each slide (3 slides per condition, each slide from a different individual), 25 nucleoids from randomly chosen fields were analyzed using a fluorescence microscope with a G-2A (long-pass emission) filter cube. A computerized image-analysis system Eclipse 80i fluorescence microscope (Nikon Corporation, Nikon Instech Co., Kanagawa, Japan) was employed. From the repeated experiments, the average median tail moment value (TM) and the percentage of tail DNA (TD) were calculated using CASP v1.2.2 software.

### *2.5. Mitotic Index (MI) and Micronucleus (MNC) assay*

Root tips (meristem zones) from three individuals per condition were cut and stored in the Carnoy fixation solution containing ethanol and glacial acetic acid (1:1) at 4 °C. Root tips were rinsed with distilled water and hydrolyzed with 1 N HCl for 8 min at 70 ºC. The root cap was removed before crushing the tissues and samples were stained with orcein. The slides were examined with a microscope and the MI was estimated (MI = number of cells in division per 1000 cells analyzed). MNC detection was performed according to Rodriguez *et al*. (2011). A computerized image-analysis system Eclipse 80i fluorescence microscope (Nikon Corporation, Nikon Instech Co., Kanagawa, Japan) was used to visualize the slides.

### *2.6. Statistical analysis*

Statistical significance of treatments was assessed by One-Way ANOVA with a post-hoc Holm-Sidak multiple comparison test, using SigmaStat 3.5 for WINDOWS (SPSS Inc., Chicago, IL, USA). Pearson's correlation was performed using SigmaPlot for Windows ver. 11.0 (Systat Software Inc). Unless otherwise referred, two independent experiments (each with at least five replicate individuals per condition) were performed to ensure the reliability and statistical robustness. Multivariate analyses for data correlation used Principal Component Analysis (PCA) and were performed with CANOCO for Windows v4.5 program.

## 3. Results

### *3.1. Plant growth, morphology and Hg accumulation*

No visible differences were found in size and morphology of both control and 0.1 µM exposed plants. Contrarily, plants exposed to doses ≥ 1 µM showed increasing chlorosis and necrotic spots, paralleled with a decrease in plant size in a dose-dependent manner (Table 1). Between 1-100 µM, exposed roots became increasingly brownish and with a reduced number of lateral roots. EC50 was calculated based on the reduction of roots and shoots length with a standard curve. For roots, EC50 was 53.28 µM of Hg and 61.53 µM for leaves. As we used environmentally real doses, they were not high enough to calculate LD50 with certainty, as the highest dose only induced ~20% mortality at the end of the experiment.

Table 1 also presents the mean Hg accumulation in Hg-treated organs. Results show that exposed plant roots and leaves accumulated Hg in a dose-dependent manner, with linear relation in the leaves ($y = 1.3006x + 13.249$, $R^2 = 0.964$) and polynomial relation for roots ($y = 2.5784x^2 + 3.4337x + 16.097$, $R^2 = 1$). Roots always showed higher levels and increments of accumulation ($p<0.05$) than leaves. Stems showed only trace amounts of Hg (data not shown).

**Table 1.** Organ content of Hg (mg $kg^{-1}$) (with the increase regarding the control in brackets), and length (cm) of pea shoots and roots after 14 days exposure to different Hg concentrations (µM). Values given are the mean value ± standard deviation (SD). (*) significantly different from control ($p \leq 0.05$).

| Organ | Exposure (µM) | Hg Quantification (mg $kg^{-1}$) ± SD | Length (cm) |
|---|---|---|---|
| **Leaves** | 0 | 2.1±0.44 | 37.0 ± 2.6 |
| | 0.1 | 3.1±0.21 (1.5x) | 37.4± 3.1 |
| | 1 | 26.7 ± 6.85* (12.5x) | 34.5 ± 3.7 |
| | 10 | 36.5± 8.68* (17.1x) | 33.5 ± 6.5 |
| | 100 | 142.1 ± 63.18* (66.43x) | 17.3 ± 4.0* |
| **Roots** | 0 | 4.7 ± 1.25 | 17.5 ± 4.5 |
| | 0.1 | 11.7 ± 5.98* (2.5x) | 17.2 ± 3.9 |
| | 1 | 40.2 ± 6.14* (8.5x) | 15.5 ± 1.5 |
| | 10 | 306.3 ± 22.66* (64.8x) | 15.5 ± 2.0 |
| | 100 | 2614.5 ± 2731.72* (552.7x) | 4.3 ± 1.5* |

### *3.2. FCM analysis*

Analysis performed by FCM with extracted nuclei showed that exposure to Hg-induced an increase ($p < 0.05$) in the nuclei FS and SS parameters in roots nuclei (not shown). The FCM histograms showed the typical diploid level expected for pea (with a major $G_1$ peak and a second $G_2$ peak), and no changes in these peaks were observed in Hg-treated organs ($p > 0.05$), which shows the absence of aneuploidy or polyploidy mutations. Also, histograms of control leaves and roots presented HPCV values for the $G_1$ peaks of 1.96%± 0.26 and 1.62% ± 0.14, respectively, supporting that the technique was highly reliable and sensitive (Table 2). Whilst the FPCV and

HPCV values of the $G_1$ peak from Hg-treated leaves did not change ($p > 0.05$), these values increased in roots exposed to 100 µM Hg. Moreover, it should be noted that in roots exposed to 100 µM, to run at least 3000 nuclei, 3-fold more root apices were needed, in comparison with the other conditions.

**Table 2.** Half peak coefficient of variation (%HPCV), and Full Peak Coefficient of Variation (%FPCV) of roots and leaves of plants exposed to different Hg concentrations (µM). Values are given as mean ± SD. (***) significantly different from control ($p \leq 0.001$).

| | Hg [µM] | %HPCV | %FPCV ± SD |
|---|---|---|---|
| **Leaves** | 0 | 1.96 ± 0.26 | 3.16 ± 0.57 |
| | 1 | 1.80 ± 0.31 | 3.23 ± 0.37 |
| | 10 | 1.64 ± 0.27 | 2.82 ± 0.16 |
| | 100 | 1.68 ± 0.13 | 3.37 ± 0.44 |
| **Roots** | 0 | 1.62 ± 0.15 | 3.50 ± 0.41 |
| | 1 | 1.96 ± 0.21 | 4.14 ± 0.70 |
| | 10 | 2.25 ± 0.27 | 3.77 ± 0.58 |
| | 100 | 2.10 ± 0.38 | 6.16 ± 0.93 *** |

Cell cycle progression was also evaluated to assess Hg putative cytostatic effects. The FCM histograms for control leaves displayed a main peak, corresponding to nuclei at $G_1$ with 75.9% of the events, a smaller peak corresponding to $G_2$ with 13.5% of the events and an S phase with 10.6% of the total events, and no changes were detected as a result of the exposure (data not shown). The FCM histogram of control root apices presented a small peak for $G_1$ with 30.2% of the events, a main peak corresponding to nuclei in $G_2$ (53.3% of the events) and 16.5% of the nuclei analyzed were on S phase (Figure 1). Contrarily to leaves, cytostatic effects were visualized at doses ≥ 1 µM Hg. A decrease in G2 population was observed with the increase of the Hg concentration. Roots treated with 1 µM had a 2-fold increase of the S phase when compared to control ($p \leq 0.001$), which was accompanied by a decrease of the $G_2$ (33% lower). Root apices exposed to 10 µM Hg showed a significant blockage of the pre-mitotic phase $G_1$ (41 % vs 30.2% in the control group), 11% higher than control ($p \leq 0.05$). The CPI for this concentration presented a significant decrease of 15% ($p \leq 0.001$) when compared to all other conditions. In leaves, the profile of cell cycle progression showed little variation among the tested conditions ($p > 0.05$) (data not shown).

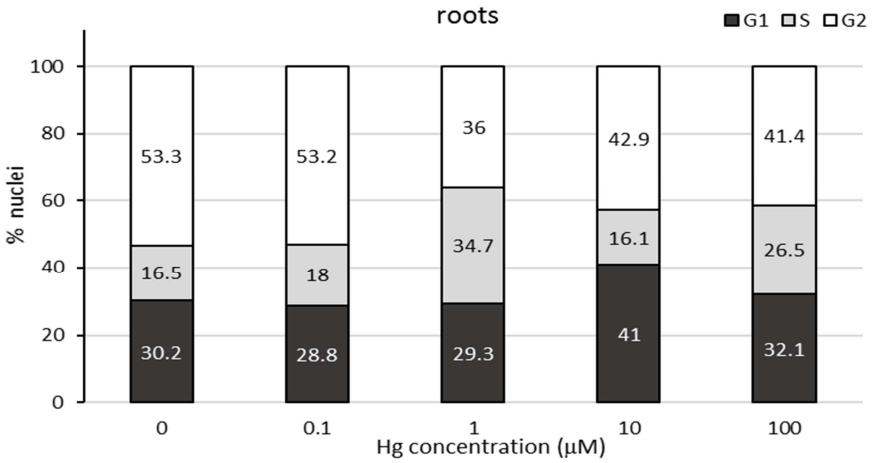


**Figure 1.** Nuclei (%) in $G_1$, S and $G_2$ of roots exposed to Hg. Values are given as mean ± standard deviation. (***) significantly different from control ($p \leq 0.001$).

### *3.3. Comet assay*

The TM and TD were used as biomarkers of the Comet assay to detect DNA damage induced by Hg in both roots and leaves. Analysis of nuclei extracted from control leaves and roots were round with only occasional comets visualized (Figure 2a), while the nucleoids of the positive control were on average ~300-400 U.A., meaning comets' scoring of class 3 and 4. Contrarily to the values of positive controls (TD=78.60 and TM=124.27 for leaves and TD=50.51 and TM=56.93 for roots), TD and TM of exposed leaves did not show significant differences in regards the negative control (p>0.05). Exposed roots showed a dose-related increase of both TM and TD, but only at ≥10 µM the TD differences regarding the control were significant ($p \leq 0.05$) (Figure 2a-e), while only at 100 µM the TM increases were significant. At this dose, there was an increase of 22-fold in TM and 80% more TD.

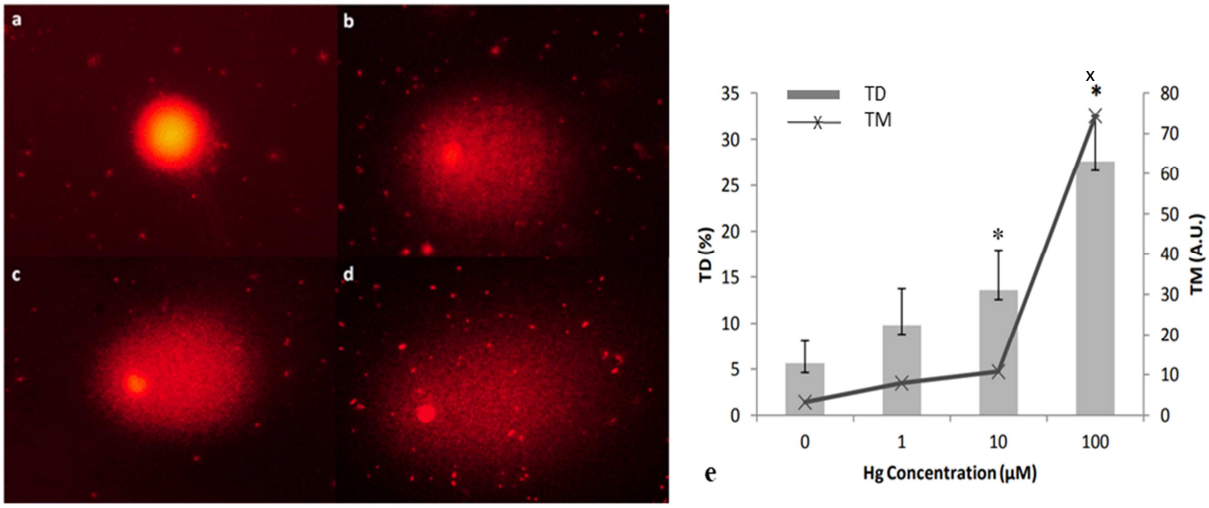


**Figure 2. a-d** Comet assay representative images of nuclei extracted from roots: **a)** control; **b)** 1 µM; **c)** 10 µM; **d)** 100 µM; **e)** % of Tail DNA and TM of roots exposed to different $HgCl_2$ concentrations. Values are given as mean ± standard deviation of at least 3 replicates with at least 75 nuclei per replicate. TM values are given in arbitrary units. * TD significantly different from control ($p \leq 0.05$); x TM significantly different from control ($p \leq 0.05$)

### 3.4. Mitotic Index and MNC formation

The cell division frequency of exposed root apices was determined in the form of MI and is displayed in Figure 3a. It can be observed that the decrease of mitotic events correlated with the increase of Hg. Plants exposed to 10 and 100 µM showed a significant difference from control ($p \leq 0.05$), with decreases of 3- and 5-fold, respectively. As for MNC, 100 µM was the only condition inducing the formation of MNC with an average rate of 4 MNC per 1000 cells (Figure 3b).

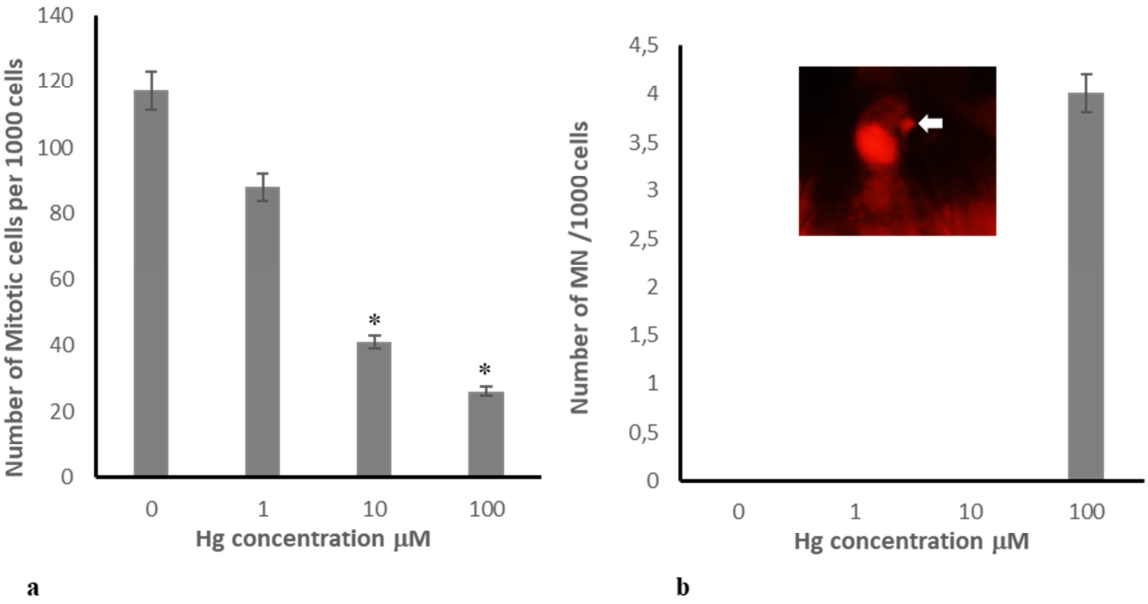


**Figure 3.** Rate of mitotic cells and micronuclei detection in exposed to different $HgCl_2$ concentrations. a) Number of mitotic cells (MC) per 1000 cells counted with Orcein Acetic method. * significantly different from control ($p \leq 0.05$); b) Number of micronuclei (MNC) per 1000 cells counted with PI method. (*) significantly different from control ($p \leq 0.05$); arrow: example of a micronucleus.

### 3.5. Principle Component Analysis

The PCA of the root data showed a clear separation between four groups regarding the Hg treatments (Figure 4). PC1 explained 63.8% of the variance, and PC2 explained 23.1% of the variance. Both control and 0.1 µM scores are quite similar, forming a single group located at the down-left quadrant, and being positively associated with G2, MI and root length, and negatively related with genotoxic parameters (TM, TD, FPCV, MNC) and increasing Hg content (Figure 4). Ranking in opposite direction scores 100 µM, directly related with the genotoxic parameters. The 1.0 µM scores are near the control and 0.1 mM and is positively related with the CPI, while 10 µM is positively related with the G1 blockage.

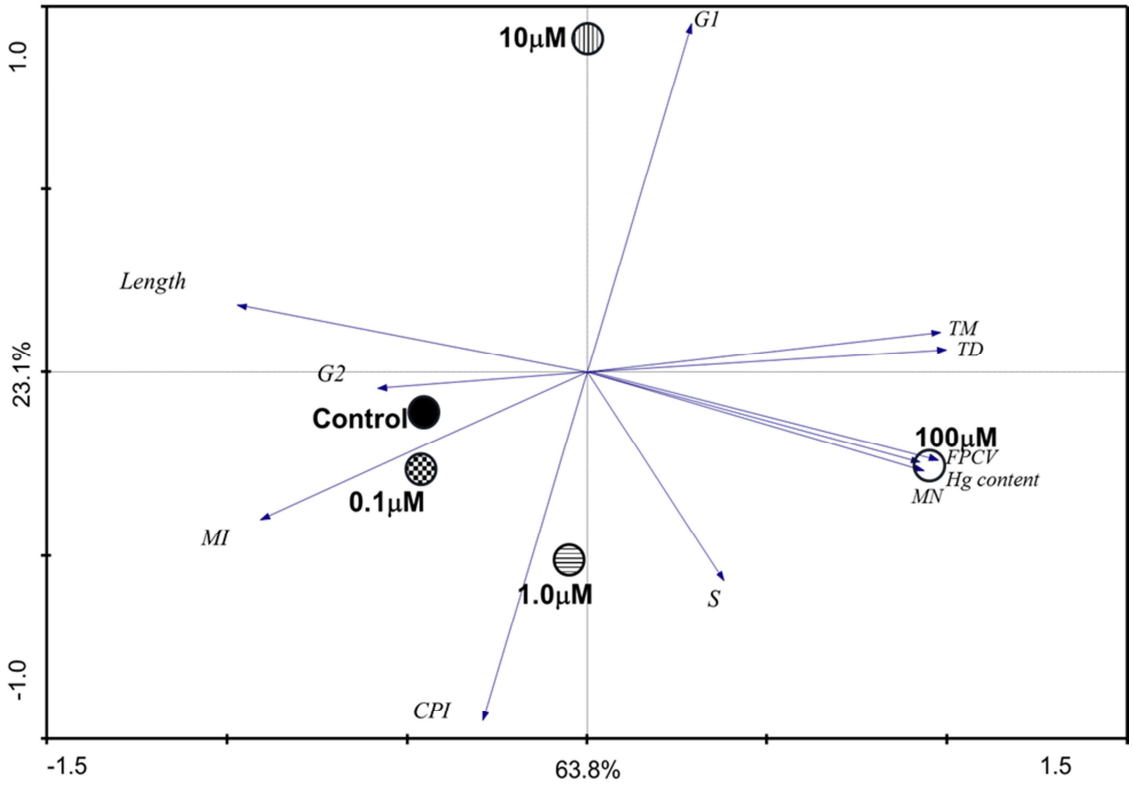


**Figure 4.** PCA analyses of genotoxic responses of pea roots exposed to increasing concentrations of Hg.

## 4. Discussion

Due to its bioavailability and bioaccumulation in organisms through the food chain, Hg contamination of agricultural soils is of great concern. However, and contrarily to animal cells, its cyto and genotoxic effects remain to unveil, allied to the urgent need to develop sensitive biomarkers as highlighted by Hou *et al.*, (2016).

In the model crop *Pisum sativum*, a dose-dependent accumulation of Hg was observed proving that this species is able to accumulate significant amounts of Hg in roots even at the lower concentrations, at which plants showed high tolerance with no morphological toxic symptoms. These data are, as expected, in line with the described Hg effects on plant growth and Hg accumulation and allocation (Mondal *et al.*, 2015; Sheetal *et al.*, 2016). This accumulation was paralleled by the decrease of pea organs' growth. These decreases negatively correlate with the external Hg concentrations as shown by the Pearson's correlation between these parameters ($r$ = -0.997; $p$ = 0.002 for shoots and $r$ = -0.984; $p$ = 0.015 for roots) and the PCA analyses.

Plant growth depends on both cell division and cell elongation. We demonstrate here that the effect of inorganic Hg on the cell cycle dynamics depends on the Hg dose. For example, in the lowest Hg doses, an increase of S phase is evident, maintaining the MI and CPI in roots exposed

up to 1 µM, and suggesting only a delay in the cell cycle as reported for this species exposed to other metals as Cd (Monteiro *et al*.**,** 2012). However, in roots exposed to 10 µM, an effective arrest of the cell cycle was observed at the $G_1$ to S checkpoint. These cytostatic data support the decreased biomass in the exposed plants, as also demonstrated for tomato seed germination and plant biomass reduction (Hou *et al.,* 2015). These data are also similar to the cell cycle arrest found in Hg-exposed sea urchins cells exposed to 10 µM Hg showed 100 % of the embryos remaining blocked at the first division (Marc *et al*.**,** 2002). The similar response obtained in both animal and plant cell models indicates a common cell strategy when facing toxic Hg: a blockage at the $G_1$ to S checkpoint preventing the cell from entering cell division by avoiding/retarding new DNA synthesis. Also, the slime mold model *Dictyostelium discoideum*, exposed to Hg, exhibited changes at the nuclear level, including changes in histones, increased nuclear protein carbonylation, evidencing genotoxicity and being also visible increases of micronuclei (Boatti *et al*.**,** 2017).

Interestingly, at 100 µM, $G_1$ and S phases remained larger than those of the control (at the expenses of a decrease of $G_2$), continuing the cell blockage. However, it should be noted that the total number of nuclei obtained in 100 µM was three times lower than the number of nuclei found in the root apices of the other conditions. This fact, together with the apparent delay in S and the CPI value suggest that only a subpopulation of root cells survived to this higher concentration, and was able to progress through the cell cycle, though with a delay. This hypothesis is supported by data found in animal cells also exposed to Hg. Marc *et al*. (2002) observed in sea urchins' embryos exposed to Hg that some cells showed apoptotic phenotypes and only 30% reached the swimming blastula stage, which is in line with our proposed theory for a Hg-resistant subpopulation of cells that is able to progress through the cell cycle and develop. Therefore, for doses higher than 10 µM, Hg-induced a blockage at the $G_1$ to S transition, while even higher doses (100 µM) lead to cell death but surviving/tolerant cells showed a delay in DNA synthesis. Therefore, our data support that a similar interference may occur in plant cells exposed to Hg. The effects of metals/metalloids in plants growth remain limited to a few studies, and as far as we know, this is the first study regarding cytostatic effects of Hg in plants using FCM.

The assessment of clastogenic damage using the FPCV demonstrated that Hg can induce breaks in the genetic material, already shown in animal cells (e.g., Falluel-Morel, 2007). Most of the DNA damage caused by metal stress is originated by indirect means, namely through reactive oxygen species (ROS) formation or by interacting with proteins associated with DNA replication/repair mechanisms (Beyersmann and Hartwig, 2008). Hg, however, has the ability (due to being positively charged) to bind directly with negatively charged centers of DNA, mainly to phosphorous, causing mutagenesis (Onyido *et al*., 2004). Besides, Hg is also capable of interacting with sulfhydryl (SH) groups of proteins (Patra *et al*., 2004) associated with DNA replication and alters genetic information and replication fidelity (Rao *et al*., 2001). In pea plants

exposed to 100 µM, the DNA damage measured by FPCV has the highest value of all concentrations. This parameter presented a strong correlation with Hg accumulation ($r$ = 0.977; $p$ = 0.02 for roots), TM ($r$ = 0.983; $p$ = 0.0166) and TD ($r$ = 0.952; $p$ = 0.0482), reinforcing the idea that DNA breaks were induced by Hg exposure. Also from the PCA analysis, it is evident that up to 10 µM cytogenetic parameters detect mostly functional impairments, whilst structural damages are evident only for doses above 10 µM (Figure 4).

The most common Comet assay DNA damage marker in plant applications is the TM (Santos *et al*., 2015). However, Collins *et al*. (2008) suggested that the TD covers the widest range of damage. Moreover, the TD is linearly related to break frequency, allowing better inter-laboratory comparison. Rodriguez *et al*. (2011) demonstrated in *P. sativum* plants exposed to Cr(VI) that, despite the high correlation between TM and TD, the latter correlated better with the FPCV and with the amount of Cr(VI) accumulated. In pea plants, both parameters allowed detecting DNA damage and showed a high Pearson's correlation coefficient for roots ($r$ = 0.968; $p \leq 0.05$), supporting that they can be used with confidence in Hg phytogenotoxic assessments. In our results, the TM presented better Pearson's correlation with both FPCV and Hg accumulation than the TD. These data demonstrate that a positive Pearson correlation is found between Hg accumulation and DNA damage ($r$ = 0.996, $p \leq 0.05$ for roots). Whilst Comet assays has not yet been applied to study Hg-induced DNA damage in plants, studies in animals indicate that this technique is sensitive enough to detect DNA damage in cells exposed to low concentrations of this metal, Ben-Ozer *et al*. (2000) observed a significant increase in the comet's tail length, dependent of the dosage administrated (between 0 and 5 µM). Our findings in root cells, like those of Ben-Ozer *et al*. (2000) for animal cells, indicate that Hg induces DNA damage in a dose-dependent manner.

In Hg-treated plants, the MI decreased with increased dosage indicating that MI is dose-dependent. Similarly, Asita and Matobole (2010) described a high decrease of the MI in onion and broad bean roots when exposed to Hg for 24 h. These results indicate that the MI is a reliable predictor of the cell proliferation in tissue, and support the cell cycle dynamics data of FCM indicating that, with increasing concentrations of Hg, there is a tendency to decrease cell division, either by a delay or an arrest of the cell cycle. On the other hand, the results regarding MNC formation, with 4 MNC detected per 1000 cells, are in agreement with the report of Souguir *et al*. (2008). In that article, exposure of *P. sativum* to maleic hydrazide and 50 mM of chromium resulted in MNC formation. The authors explained that due to *P. sativum*'s short chromosomes, MNC formation was more difficult to assess than in other, more common models for this assay like *Vicia faba* (Feng *et al*., 2007) or *Allium cepa*, which possess larger chromosomes. We demonstrate here that FCM-cytostatic detection is more sensitive than MI and genotoxic parameters and that among genotoxic parameters, those associated with the comet assay are more sensitive than FPCV and MNC.

In conclusion, this is the most comprehensive evidence in plants of the Hg-induced cyto- and genotoxic effects, including cytostaticity, using a large battery of biomarkers. From all the biomarkers used, the functional cytostatic data is more sensitive (detecting cell cycle delays even for doses > 0.1 µM Hg), which is a dose environmentally realistic. Next, the parameters provided by the Comet assay also show high sensitivity, detecting significant levels of DNA-fragmentation at low Hg doses and FCM-cytostatic endpoints. Other biomarkers as ploidy, MNC or MI were less sensitive. Despite this, the data presented here suggest that all the methodologies provide complementary data, allowing us to enlighten the role of Hg as a genotoxic element. Recently, Hou *et al*., (2015; 2016) proposed three genes related with antioxidant and secondary metabolism pathways (glutathione *S*-transferase parA, chlorophyll a–b binding protein 13, and geranylgeranyl pyrophosphate synthase 1) as candidates to detect Hg-contaminated soil. Our results complement the information, focused on cyto and genotoxic mechanisms. Figure 5 summarizes a proposed mechanism of cyto- and genotoxicity, considering the complex effects according to the Hg dose. As demonstrated elsewhere, Hg may interact directly with DNA or induce oxidative stress, and both may lead to visible DNA damage (for doses >0.1 µM Hg). This may lead to cell cycle delay (for lower doses) or blockage (for higher doses) for DNA repair, allowing restored cell cycle progression. Eventually, cells with no DNA repaired may proceed with cell cycle progression and eventually lead to mitotic disorders together with decreased cell proliferation ending in abnormal root development.

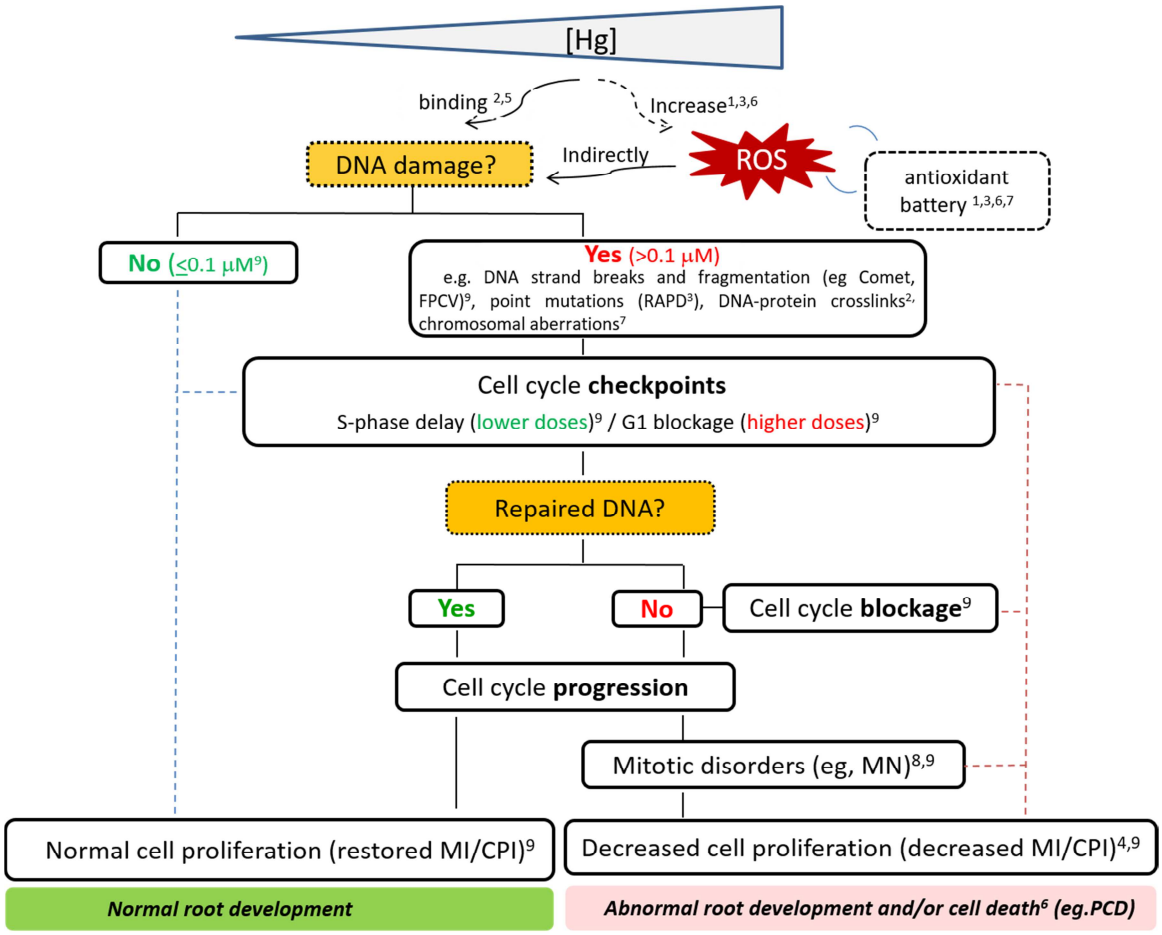


**Figure 5.** Proposed model for Hg-induced cytogenotoxicity in plant roots, according to Hg-dose, and considering the direct (eg Hg-DNA interaction ([2,5])) and indirect effects such as oxidative stress ([1,3,6,7]). In pea roots, measurable DNA damage and cytostaticity is not significant for doses ≤0.1 mM Hg. For higher doses, evident cell cycle delay (S delay for lower doses) or blockage (at $G_1$ checkpoint for higher doses) putatively allowing DNA repair, and later restoration of cell cycle progression. Eventually, for higher Hg doses, some cells may fail to repair DNA, proceeding with cell cycle progression and leading to increased mitotic disorders ([8,9]) together with decreased cell proliferation (MI and CPI) ([4,9]) ending in abnormal root development. ROS: reactive oxygen species; RAPD: random amplified polymorphism DNA; MI:mitotic index; CPI: cell proliferation index; MNC: micronuclei. [1)] Sahu *et al*., (2012); [2)]Onyido *et al*., (2004); [3)]Manikandan *et al*., (2015); [4)]Babu and Maheswari (2006); [5)]Patra *et al*., (2004); [6)]Tamás *et al*., (2015); [7)]Cargnelutti *et al*., (2006); [8)]Subhadra and Panda (1994); [9)]this paper.

## Contributions

C. Santos, J.M.P. Oliveira, and J.C. Lopes planned the experiments. R. Azevedo and E. Rodriguez performed all experiment assays and some statistical analysis. N. Mariz-Ponte, S. Sario, and R.J. Mendes supported R. Azevedo and E. Rodriguez in the experimental assays and statistical analyses and reviewed the manuscript.

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**Highlights**

- Cytostatic effects of Hg are detectable at 1 µM;
- DNA break/damage is visualized in plants at concentrations >10 µM;
- Comet endpoints are more sensitive than flow cytometry (FCM) for genotoxicity
- Cytostasis is best detected by FCM or Cell Proliferation vs Mitotic Index or MN

## Contribution

C. Santos planned the experiments. R. Azevedo and E. Rodriguez performed all experiment assays and statistical analysis. N. Mariz-Ponte, S. Sario and R.J. Mendes supported R. Azevedo and E. Rodriguez in the experiment assays and reviewed the manuscript.