Genotoxicity of the food additive E171, titanium dioxide, in the plants *Lens culinaris* L. and *Allium cepa* L.

Lorenza Bellani[a,d], Simonetta Muccifora[a], Francesco Barbieri[a], Eliana Tassi[b], Monica Ruffini Castiglione[c], Lucia Giorgetti[d*].

[a]Department of Life Sciences, University of Siena, via A. Moro, 2 - 53100 Siena, Italy

[b]Research Institute on Terrestrial Ecosystems, National Research Council (IRET-CNR), via G. Moruzzi, 1 - 56124 Pisa, Italy

[c]Department of Biology, University of Pisa, via Luca Ghini, 13 - 56126 Pisa, Italy

[d]Institute of Agricultural Biology and Biotechnology, National Research Council (IBBA-CNR), via G. Moruzzi, 1 - 56124 Pisa, Italy

*Corresponding author: Lucia Giorgetti, Institute of Agricultural Biology and Biotechnology, National Research Council (IBBA-CNR), via G. Moruzzi, 1 - 56124 Pisa, Italy. E-mail: lucia.giorgetti@ibba.cnr.it

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Abstract

E171 (titanium dioxide, TiO_2), an authorized foods and beverage additive, is also used in food packaging and in pharmaceutical and cosmetic preparations. E171 is considered to be an inert and non-digestible material, not storable in animal tissues, but the possible presence of TiO_2 nanoparticles (NP) may present a risk to human health and the environment. We determined the presence of 15% TiO_2 NP in a commercial E171 food additive product, by electron microscopy. The biological effects of E171 were assessed in *Lens culinaris* and *Allium cepa* for the following endpoints: percentage of germination, root elongation, mitotic index, presence of chromosomal abnormalities, and micronuclei. The results indicated low phytotoxicity but dose-dependent genotoxicity. We also observed internalization of TiO_2 NP and ultrastructural alterations in the root systems.

Keywords: TiO_2 particles **-** Phytotoxicity - Chromosomal aberrations - Root ultrastructure – Particles internalization

1. Introduction

Titanium ore mineral (mainly ilmenite) is a natural source of TiO_2. Crystalline forms of TiO_2 include rutile, anatase, and brookite. After processing, TiO_2 is a white powder. Due to its brightness, high refractive index, and UV resistance, TiO_2 has many industrial uses, in construction, electronics, papermaking, manufacturing, pharmaceuticals, cosmetics, paints, etc. [1-3]. In the food industry, TiO_2 is used as a white pigment and as a preservative. For example, it may be found in cheeses, low-fat products such as skim milk and ice cream, candy coatings and chewing gum, glazing of confectionery products, and in dehydrated mixtures for drinks, as a clarifying agent [4-8].

TiO_2 is considered to be an inert, non-absorbable material, and therefore not to be accumulated in animal or human tissues [4]. Its use in food is not regulated [9]. In Europe, TiO_2 as a food additive is designated as "E171", and its ADI (Acceptable Daily Intake) is "not specified"; in the USA, it is designated as INS171 and allowed in foods up to a concentration of 1% [10].

In recent years, nanoparticle (NP) TiO_2, *i.e.* particles with at least one dimension <100 nm [1,7-8], has been widely used in preference to the bulk material. TiO_2 NP are transparent, suitable for UV protection applications in food packaging films, cosmetics, and sun lotions [11]. TiO_2 NP are not allowed in food because of their different behaviour and reactivity versus the bulk form, the NP being potentially carcinogenic to humans (Group 2B, IARC) if ingested [9] or inhaled [12]. Studies on rats have shown that NP can pass through the intestinal barrier and accumulate in the intestine, causing preneoplastic lesions [9,13], and can pass the blood-brain barrier if inhaled, inducing brain injury [14]. Accumulation and toxic effects of TiO_2 NP have also been demonstrated in plants [15]. Surveys have shown the presence of NP in commercial products [5-7], with possible

hazards to animals and humans [9,12,13,14]. NP have been evaluated as environmental contaminants [7,16] and as entrants into the food chain [15].

Environmental monitoring studies are performed with microorganisms (*e.g.*, *Vibrio fischeri*), eukaryotic organisms (*e.g.*, *Daphnia magna*), mammalian cells (*e.g.*, leukocytes), and plants [17]. Plants are very sensitive to environmental pollutants, due to their sessile lifestyles and direct contact with environmental matrices (air, water and soil) in which pollutants can accumulate. In some situations, plant tests are more sensitive than animal tests [18-19]. Plants such as *Lactuca sativa* L. and *Lepidium sativum* L. are usually used for phytotoxicity studies, *Allium cepa* L. and *Vicia faba* L. for genotoxicity studies [20-21]. Phytotoxicity tests consider endpoints such as seed germination and primary root length (acute effects) or morpho-physiological alterations such as biomass, length, chlorophyll content, etc. during the plant life cycle (chronic effects). Genotoxicity test endpoints include cytogenetic analysis in root meristems (c-metaphases, lagging chromosomes, bridges at anaphases, chromosome and chromatid breakages resulting in micronuclei). Cytotoxic effects can also be evaluated (effects on mitotic index and changes in percentages of mitotic phases) [21-25].

Since there is no regulation in European legislation of the maximum concentration of E171 additive in food products [26], and since TiO_2 NP are present at variable percentages in E171 composition, we have studied its potential hazard. The phytotoxic and genotoxic effects of E171 were investigated in two plant systems, *Allium cepa* L., a model plant in ecotoxicological studies, and *Lens culinaris* L., an edible legume, recently used in genotoxicity tests [27]. Ultrastructural analysis of root systems was also carried out.

2. Materials and Methods

2.1 Chemicals

Titanium dioxide (E171), food color in powder (Decora, Karma Srl – SA, Italy), was purchased from an Italian market. Five different concentrations of E171 (0.05, 0.1, 0.5, 1 and 2 g/L) were prepared in ultra-pure Milli-Q water (Merck Millipore). Suspensions were prepared immediately before use by sonication for 30 min at 80 W output power (Sonifier 250, Branson).

2.2 Laser light scattering (LD)

The size distribution of the aggregates was estimated by laser light scattering (Mastersizer 2000, Malvern Instruments). The suspension was previously prepared, as described above, at a nominal concentration of 0.05 g/L. Sizes of stable aggregates were recorded after 30 min in sonication mode (55%) and laser obscuration 11%. The appropriate amount of sample was previously tested using sub-samples of E171 suspended in water, to define the best obscuration limit conditions, reduce sources of error, provide a good detection signal-to-noise ratio, and avoid multiple scattering.

2.3 Environmental Scanning Electron Microscopy (ESEM)

Elemental composition analysis was performed by FEI Quanta 400 ESEM equipped with an electron probe X-ray microanalysis unit (EDAX), on a single drop of E171 suspension, 0.5 g/L, placed on a graphite stab and then dried. EDAX analyses were based on detection of X-rays generated from the interaction between the electron beam and the sample [28].

2.4 Plant material

Seeds of *Lens culinaris* L. var. Onano were kindly provided by the Company "Agricola Marco Camilli", Onano (VT, Italy) and stored at -20 ° C until use. Seeds of *Allium cepa* L. var. Tropea rossa tonda were purchased from a local market.

2.5 Phytotoxicity test

The phytotoxicity test was performed according to US EPA Guidelines (1996), which indicate seed germination and root elongation as essential parameters [29]. Seeds of *A. cepa* and *L. culinaris* were germinated at 24 ± 1 °C for 72 h in the dark in 5 cm diameter Petri dishes containing four layers of Whatman papers imbibed with water (control) and with E171 at the different concentrations. For all treatments, five Petri dishes with ten seeds each were prepared. Germination percentage (% G) and seedling root length were assessed after 72 h.

2.6 Genotoxicity test

After 72 h germination, roots of *A. cepa* and *L. culinaris* were fixed in ethanol:glacial acetic acid (3:1 v/v) for 12 h; then the meristems were stained and squashed following the Feulgen technique [30]. Analysis was performed by light microscopy on slides coded to prevent bias in the scoring and the slides were scanned methodically in order to cover the entire area. At least 25 fields were chosen randomly to count 1000 nuclei for each slide, with five replicates for each treatment. Disturbance in mitotic activity (mitotic index, MI = number of mitosis /100 nuclei) indicated cytotoxicity, while both micronuclei presence (number of micronuclei /1000 nuclei) and mitotic aberrations (aberration index, AI = number of aberrations /100 nuclei) indicated genotoxicity. The aberrations scored included chromosomal bridges, fragments, lagging chromosomes, stickiness,

aberrant metaphases and disturbed anaphases in dividing cells, and micronuclei in interphase cells [31].

2.7 Transmission electron microscopy (TEM)

To evaluate the sizes of the TiO_2 particles and the possible presence of TiO_2 NP, E171 suspension, 0.5 g/L, one drop (10 µl) was placed on TEM grids covered with formvar and allowed to settle and dry. TEM images were examined with the ImageJ programme to analyse particles dimensions, by measuring the major and minor axes. For TEM root observations, 0.1, 0.5, and 1 g/L concentrations were chosen, based on preliminary tests. Small cubes of roots were pre-fixed in Karnovsky solution [32], post-fixed in osmium tetroxide, dehydrated, and embedded in Epon 812-Araldite A/M mixture. Thin sections were stained with uranyl acetate and lead citrate. Isolated NP and root sections were observed under a FEI Tecnai G2 Spirit electron microscope at 100 kV.

2.8 Statistical Analysis.

Data are reported as means ± standard deviation (SD). One-way analysis of variance (ANOVA, $p \leq 0.05$) was applied to identify statistically significant differences among treatments (control, E 171 0.05, 0.1, 0.5, 1, and 2 g/L) for each plant species separately (*L. culinaris* and *A. cepa*). When the F value showed significance, a *post hoc* Tukey HSD (Honestly Significant Difference) multiple range test was performed at $p \leq 0.05$. The Statistica package (StatSoft) 6.0 version was used.

3. Results and Discussion

3.1 Particle size

E171 in water showed a bimodal size distribution (Fig. 1a) with no aggregates <100 nm; aggregates 250-900 nm comprised about 65% of the particles and those 950 nm-10 μm, about 32.5%. E171 particles, including those near or <100 nm (see below), tend to form aggregates and agglomerates in ultra-pure water, probably due to the tendency to minimize the high surface energy of particles caused by the high surface/volume ratio, a common condition for NP [33].

TEM analysis (Fig. 1b) showed pristine particles of E171 to have irregular shapes; therefore, both major and minor axes were measured. Sizes ranged from 40-350 nm. No particles <40 nm were observed. The average dimensions were 179 and 156 nm for the major and minor axes, respectively. Of the analyzed particles (Fig. 1c, d), 10% had both axes <100 nm, while 15% had at least one axis <100 nm. The most-represented frequency class (35%) had minor axis 101-150 nm, while particles >300 nm were only 2%. Since NP are particles with at least one dimension <100 nm, the sample contained about 15% NP. These results are in line with previous reports in which TiO_2 NP in commercial samples were 15-40% [5-8]

3.2 Chemical composition

ESEM showed generally aggregated electron-dense particles (Fig. 2a); the X-ray spectrum showed peaks corresponding to the emission energies of the α and β electrons of the L and K orbitals of Ti and of the K orbital corresponding to the O (Fig. 2b), while other elements were not detected. In contrast, previous work on commercial samples of E171 reported the presence of elements such as Al, Si, and P, even if within the limits allowed by law [8].

3.3 Phytotoxicity tests on *L. culinaris* and *A. cepa*

The % G and primary root length (phytotoxicity parameters) of *L. culinaris* and *A. cepa* analysed following 72 h exposure are reported in Table 1. Differences for the two parameters among the treatments (0, 0.05, 0.1, 0.5, 1, and 2 g/L E171) were not statistically significant in the two plant systems, with no phytotoxic effects in terms of % G ($F_{(5, 12)} = 0.67$, $p = 0.65$ for *L. culinaris* and $F_{(5, 16)} = 0.58$, $p = 0.72$ for *A. cepa*) or root length ($F_{(5, 12)} = 0.37$, $p = 0.858$ for *L. culinaris* and $F_{(5, 15)} = 2.178$, $p = 0.111$ for *A. cepa*). These results agree with previous observations in *Vicia narbonensis*, *Zea mays* [31], and *Vicia faba* [21] after nano and bulk TiO_2 exposure during seed germination. Positive effects on germination, seedling growth, plant development, and yield were reported; for example, in spinach these effects were correlated to improved photosynthetic activity (enhancement of Rubisco Activase activity) induced by TiO_2 NP but not by bulk material [34]. In *Brassica napus*, no phytotoxic effects were detected after TiO_2 NP exposure (0.5, 2.5 and 4 g/L) and all analyzed morphological and physiological parameters were improved; no ultrastructural changes in chloroplasts were observed [35].

A greater reactivity than their bulk counterpart was observed for TiO_2 NP, based on their surface and photocatalytic properties. Small size and higher reactivity could favour their entry into plant cells and their participation in (or induction of) processes related to oxidative stress and reactive oxygen species (ROS) homeostasis [36-37]. Similar results on the effect of E171 and TiO_2 NP were reported in intestinal epithelial cells, explained on the basis of induction of oxidative stress and accumulation of ROS [38].

In general, these effects depended on the plant species and on the properties of the TiO_2 particles: dimension, crystalline form, dose, type of application, and duration of treatment [39-40]. However, in our studies, commercial E171 induced phytotoxicity in neither species tested.

3.4 Cytogenetic analysis of root meristems

To measure cytotoxic/ genotoxic effects, cytological analysis was performed on the two plants and the following cytogenetic endpoints were evaluated: mitotic index (MI), % of mitotic phases (for cytotoxicity), % of abnormal mitoses and presence of micronuclei (for genotoxicity). MI data for *L. culinaris* and *A. cepa* is shown in Fig. 3a, b. In both plants, no effects were observed on MI; mitotic activities in root meristems were similar (around 6% in *L. culinaris* and 8% in *A. cepa*) regardless of E171 treatment and differences were not statistically significant ($F_{(5, 24)} = 0.848$, $p = 0.53$ for *L. culinaris* and $F_{(5, 19)} = 1.77$, $p = 0.166$ for *A. cepa*) (Fig. 3a-b). This is in agreement with the phytotoxicity results reporting no effects on % G and root length.

In contrast, the analysis of mitotic phase frequencies (Table 2, *L. culinaris*; Table 3, *A. cepa*) indicated negative effects of E171 in both plants. The percentage of prophase, however, was not significantly influenced by the treatments. In *L. culinaris*, E171 treatments induced a significant decrease ($F_{(5, 24)} = 13.819$, $p = 2.079 \times 10^{-6}$) in percentage of normal metaphases, but no influence was attributable to the effect of dose (Table 2). In *A. cepa*, no significant differences between control (16.5%) and treatments were observed for normal metaphases, with the exception of E171 1 g/L (8.34%) ($F_{(5, 18)} = 9.711$, $p = 0.0001$) (Table 3). The frequency of abnormal metaphases increased significantly in *L. culinaris* from E171 0.1 g/L, reaching the highest value at 2 g/L (23.56%) ($F_{(5, 24)} = 7.939$, $p = 0.0002$), and in *A. cepa* from 1 g/L, reaching 22.6% at 2 g/L ($F_{(5, 20)} = 13.199$, $p = 9.195 \times 10^{-6}$). Percentages of normal ana/telophases were not significantly influenced by E171 treatments with the exception of 1 and 2 g/L in *L. culinaris* (Table 2) ($F_{(5, 24)} = 3.23$, $p = 0.0227$), and 2 g/L in *A. cepa* (Table 3) ($F_{(5, 20)} = 4.57$, $p = 0.006$). A similar trend to abnormal metaphases was observed for abnormal ana/telophases (Tables 2 and 3), with a gradual increase from control to 2 g/L (2.84% and 14.44% in *L. culinaris*, 7.5% and 20.1% in *A. cepa*, respectively)

($F_{(5, 24)}$ = 6.351, p = 0.0007 for *L. culinaris* and $F_{(5, 20)}$ = 6.627, p = 0.0009 for *A. cepa*). The percentages of total cytological anomalies for the two plant systems are shown in Fig. 4, a-c. The results confirmed the genotoxic effect of E171, which was maximum at the highest dose, 2 g/L (37.2% for *L. culinaris* and 42.63% for *A. cepa*), but not significantly different from other treatments (0.1 g/L in *L. culinaris* and 0.5 g/L in *A. cepa*) ($F_{(5, 24)}$ = 9.928, p = 3.05×10^{-5} for *L. culinaris* and $F_{(5, 20)}$ = 14.52, p = 4.523×10^{-6} for *A. cepa*). The two plant systems gave similar responses to E171 treatment. While in *A. cepa* there was an increase in the percentage of anomalies with increasing doses (Fig. 4b, E171 0.05 g/L = 16%; 0.1 g/L = 23.3%; 0.5 g/L = 32.06%; 1 g/L = 37.69%), for *L. culinaris,* E171 0.1, 0.5, and 1 g/L induced about the same percentage of anomalies (around 27%), all significantly different from control (Fig. 4a).

Micronuclei (Figs. 4c and 6g-h) were observed principally in *A. cepa* root meristems at 72 h with a significant increase (more than double, from 5‰ in control to about 13‰ at highest concentrations of E171 1-2 g/L, $F_{(5, 14)}$ = 14.118, p = 4.79×10^{-5}), while in *L. culinaris*, micronuclei were observed occasionally with a frequency similar to the control (about 1‰) (data not shown).

Fig. 5 (*L. culinaris*) and Fig. 6 (*A. cepa*) show representative cytological anomalies.

Although *L. culinaris* (2n = 14), unlike *A. cepa* (2n = 16), is not a model plant for cytogenetic studies, its chromosomes also are readily distinguishable for the determination of chromosomal anomalies. Our analysis, in the two plants, showed anomalies in metaphase (c-metaphases, lagging chromosomes, sticky chromosomes) and in anaphase (chromosome bridges, lagging chromosomes) and the presence of micronuclei. These anomalies have already been observed after NP treatment in other plant and animal systems [31,41,42] and were associated with photocatalytic proprieties of TiO_2 NP and to the formation of intracellular ROS, which can directly or indirectly damage DNA and chromosomes. Recent reports on the effect of E171 and TiO_2 NP in

epithelial intestinal animal cells cultured *in vitro* confirmed oxidative stress and associated DNA damage [38,43].

Cytological anomalies could be the consequence of unrepaired double- and single-strand DNA breaks. Damaged chromosomes could become sticky and rearrange with other injured chromosomes giving rise to acentric or dicentric chromosomes. During mitosis, acentric chromosomes cannot congregate in the mitotic spindle, with the formation of lagging chromosomes and later micronuclei, while dicentric chromosomes could generate chromosome bridges. Moreover, disturbance of mitotic spindle could give rise to new anomalies, such as c-metaphases in which no spindle is formed and chromosomal distribution does not occur, giving rise to polyploidy. In other case, spindle irregularities could cause the incorrect distribution of sister chromatids in daughter cells, giving rise to aneuploidy [44,45].

From our experience testing potentially toxic substances on model plants, similar results were obtained, genotoxic effects not always being associated with phyto-cytotoxic ones [21,31]. A test article might induce DNA damage at non-cytotoxic/phytotoxic concentrations, with toxicity occurring first at the molecular level (e.g., changes in enzyme activities, ROS formation, DNA damage) and subsequently affecting growth and development. This seems to be the case for E171.

3.5 TEM analysis

Electron microscope observations of control root cells showed typical ultrastructure of root cells at 72 h imbibition. Cells had round-shaped nuclei with dispersed chromatin, numerous well-structured organelles such as mitochondria, endoplasmic reticulum, dictyosomes and plastids with starch granules (Fig. 7 a-b). In *A. cepa*, numerous electron-dense bodies were seen (Fig. 7 b). All

the cells of the treated roots of both *A. cepa* and *L. culinaris* showed ultrastructure alterations (Fig. 7 c-f). These consisted of more or less intense plasmolysis, swelling of the endoplasmic reticulum cisterns, destruction of the organelle ultrastructure such as dictyosomes and mitochondria, in which the cristae were often no longer recognizable (Fig. 9 c-f). The nuclei lost their round shape and often had numerous more or less deep invaginations of the nuclear membrane (Fig. 7 c). In addition, numerous large vesicles with electron-transparent content appeared in the cytoplasm (Fig. 7 c, e-f).

Ultrastructural observations showed the presence of TiO_2 particles in all the treated roots of both *A. cepa* and *L. culinaris* (Fig. 8 a-f). The particles were predominantly localized in the vacuoles in the form of aggregates of two to six particles (Fig. 8 c-f). They were also observed in the cytoplasm, generally as single particles (Fig. 8 b) and often in the space between the wall and plasmalemma, in the plasmolyzed areas, as aggregates of numerous particles (Fig. 8 a). The size range was about 40-250 nm (Fig. 8 a-f).

In all treatments of both species, the same ultrastructure alterations were seen, mainly plasmolysis, swelling of mitochondria cristae and reticulum cisternae, probably caused by the presence of TiO_2 particles as reported in *V. faba* and *Pisum sativum* [25,15]. In both species, the particles were mostly detectable in E171 0.5 g/L treated root cells. The lower concentration (0.1 g/L) may contain only a small amount of particles, limiting intake by the root; on the other hand, at 1 g/L, the high concentration may favour particle aggregations, also limiting intake.

4. Conclusions

E171 did not induce acute effects in *L. culinaris* or *A. cepa*, in terms of phyto-cytotoxicity (seed germination, root length, and mitotic index). The similar responses observed in *L. culinaris* and in the model plant *A. cepa*, allow us to conclude that *L. culinaris* may also be considered an excellent material for ecotoxicological tests. Cytogenetic results (cytogenetic anomalies and micronuclei) indicated general genotoxic effects. TEM analysis of root cells showed alterations in cell ultrastructure and E171 particle internalization, confirming this approach as a marker of further damages not yet revealed by phytotoxicity tests.

The observed internalization of NP and particles and the genotoxic and ultrastructural effects of E171 agree with recent warnings from the scientific community supporting the need for regulation of E171 use in foods and limiting its release into the environment.

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Figure Legends

Fig. 1 Size characterization of E171 food additive**:** (a) Size distribution of E171 aggregates/agglomerates by Laser Diffraction; (b) TEM observation of E 171 particles; Size distribution of E 171 particles grouped in frequency classes for major (c) and minor (d) axis, after ImageJ program elaboration of TEM images.

Fig. 2 Composition analysis of the elements present in the E171 sample performed with an ESEM (Environmental Scanning Electron Microscope) equipped with an EDAX probe. (a) Area of analysis on the sample; (b) X-ray spectrum of the sample: the peaks showed the identified elements.

Fig. 3 Mean values of Mitotic index in (a) *L. culinaris* and (b) *A. cepa* recorded after 72h of imbibition in control (water) and in the presence of 0.05, 0.1, 0.5, 1, 2 g/L of E171. Bars represent standard deviation. Means followed by a common letter are not significantly different by the HSD – test. HSD = Tukey's honestly significant difference at $p \leq 0.05$

Fig. 4 Total cytological anomalies (abnormal metaphases + abnormal ana/telophases) in (a) *L. culinaris* and (b) *A. cepa*; (c) micronuclei frequency in *A. cepa*. Analysis were carried out in root meristems after 72h germination in control (water) and in the presence of 0.05, 0.1, 0.5, 1, 2 g/L of E171. Bars represent standard deviation. Means followed by a common letter are not significantly different by the HSD – test. HSD = Tukey's honestly significant difference at $p \leq 0.05$

Fig. 5 Cytogenetic analysis in *L. culinaris* treated with different concentrations of E171; cytological anomalies: (a-b) c-metaphase (0.5 g/L E171); (c-d) metaphases with not congregated chromosomes (0.5, 1 g/L E171, respectively); (e-f) sticky metaphases (1 g/L E171); (g-h) sticky metaphases with non-congregated chromosomes (2 g/L E171); (i-j) anaphase with chromosomal bridges (2 g/L

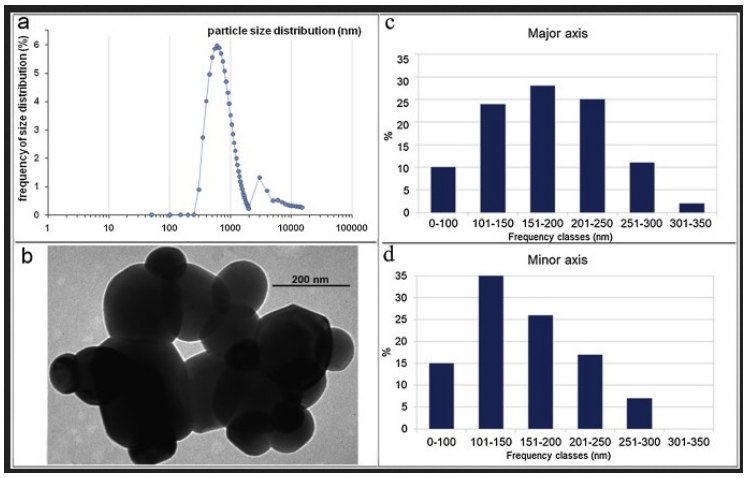
E171); (k) anaphases with non-congregated chromosomes (1 g/L E171); (l) cytoplasmic vesicles (1 g/L E171).

Fig. 6 Cytogenetic analysis in *A. cepa* treated with different concentrations of E171: cytological anomalies: (a) sticky metaphase (1 g/L E171); (b) metaphase with lagging chromosome (2 g/L E171); (c-d) anaphase with lagging chromosomes and bridges (1, 2 g/L E171, respectively); (e) sticky anaphases(0.5 g/L E171); (f) spindle anomalies at anaphase (1 g/L E171); (g-h) micronuclei (1, 2 g/L E171, respectively).

Fig. 7 TEM images of: cell portion of control root of (a) *L. culinaris* and (b) *A. cepa*; (c) E171 0.5 g/L treated *L. culinaris* root; (d) E171 0.1 g/L treated *A. cepa* root; (e) E171 1 g/L treated *L. culinaris* root; (f) E171 0.5 g/L treated *A. cepa* root. The arrows indicate endoplasmic reticulum cisternae, the asterisks plasmolysis area. B, electron dense bodies, D, dictyosome; M, mitochondria; N, nucleus; P, plastids; S, starch; V, vacuole; Ve, vesicles; W, cell wall.

Fig. 8 TEM images of cell portions of: (a-b) E171 0.5 g/L treated *L. culinaris* root; (c) E171 1 g/L treated *L. culinaris* root; (d) E171 0.1 g/L treated *A. cepa* root; (e-f) E171 0.5 g/L treated *A. cepa* root. The arrows indicate particle aggregates; the arrowhead indicates single particle; the asterisks indicate plasmolysis area. M, mitochondrion; N, nucleus; Pl, plasmalemma; V, vacuole; Ve, vesicles; W, cell wall.

Figure 1.



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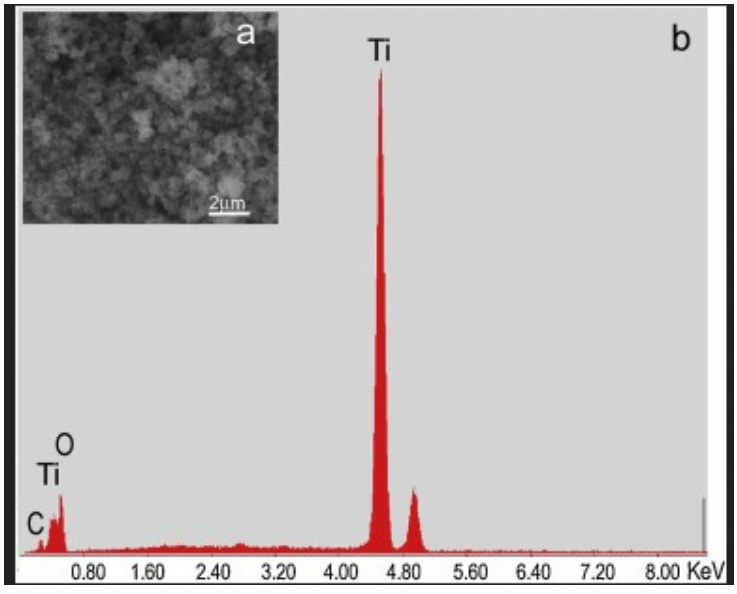

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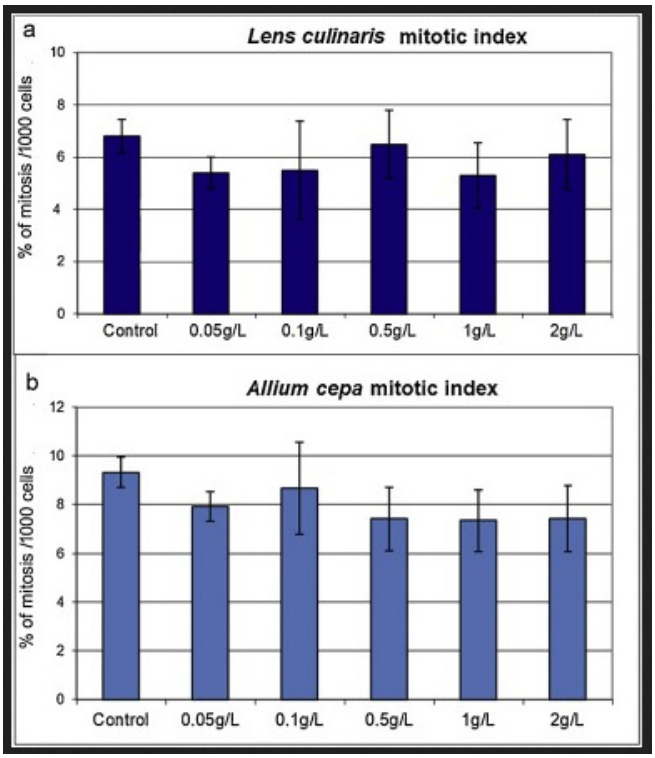

a
Lens culinaris mitotic index
% of mitosis /1000 cells
10
8
6
4
2
0
Control
0.05g/L
0.1g/L
0.5g/L
1g/L
2g/L
b
Allium cepa mitotic index
% of mitosis /1000 cells
12
10
8
6
4
2
0
Control
0.05g/L
0.1g/L
0.5g/L
1g/L
2g/L

Figure 4.

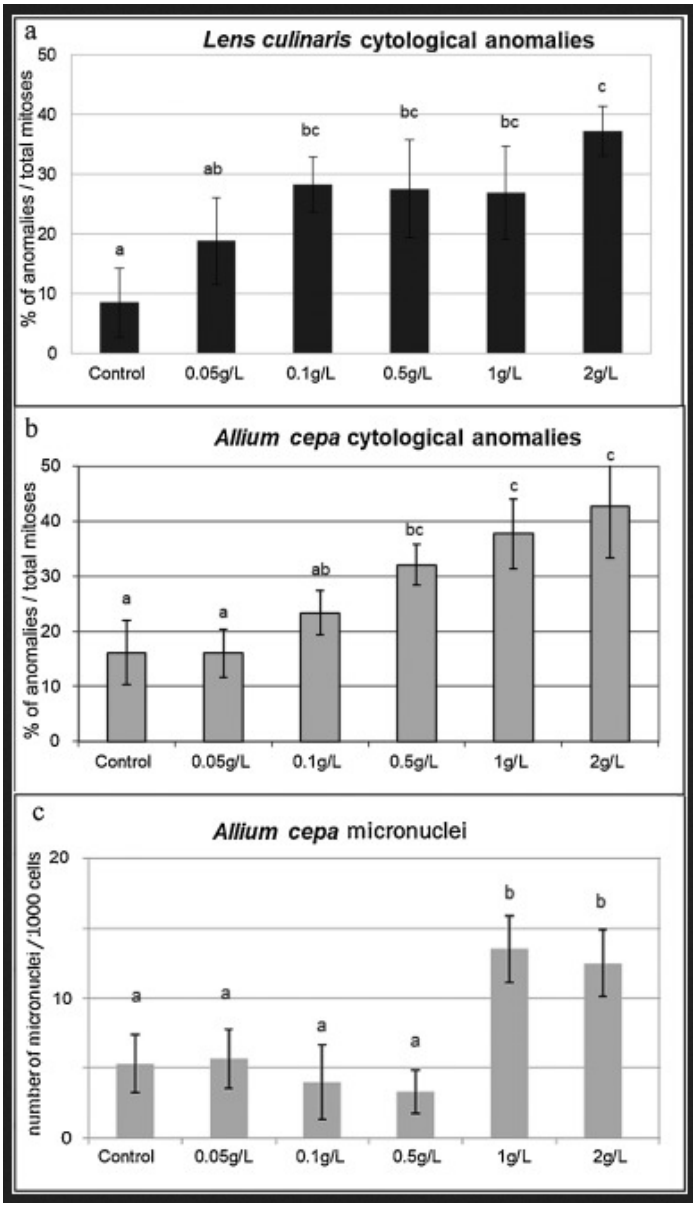
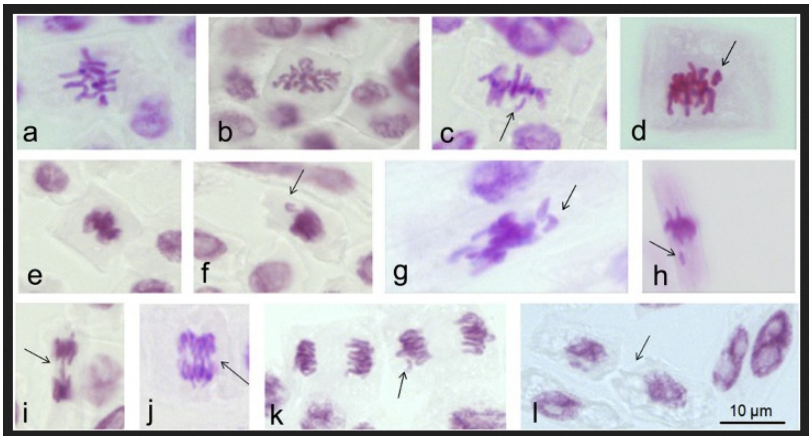

a
Lens culinaris cytological anomalies
% of anomalies / total mitoses
50
40
30
20
10
0
a
ab
bc
bc
bc
c
Control
0.05g/L
0.1g/L
0.5g/L
1g/L
2g/L
b
Allium cepa cytological anomalies
% of anomalies / total mitoses
50
40
30
20
10
0
a
a
ab
bc
c
c
Control
0.05g/L
0.1g/L
0.5g/L
1g/L
2g/L
c
Allium cepa micronuclei
number of micronuclei / 1000 cells
20
10
0
a
a
a
a
b
b
Control
0.05g/L
0.1g/L
0.5g/L
1g/L
2g/L

Figure 5.



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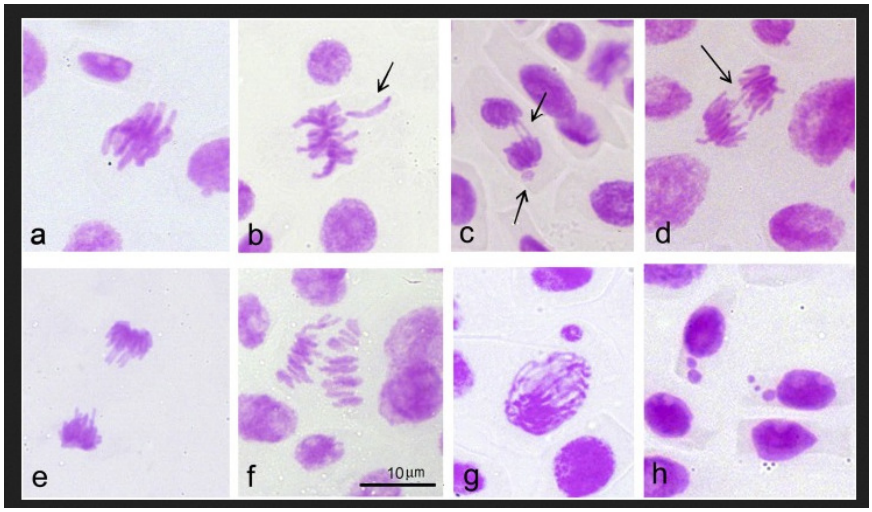

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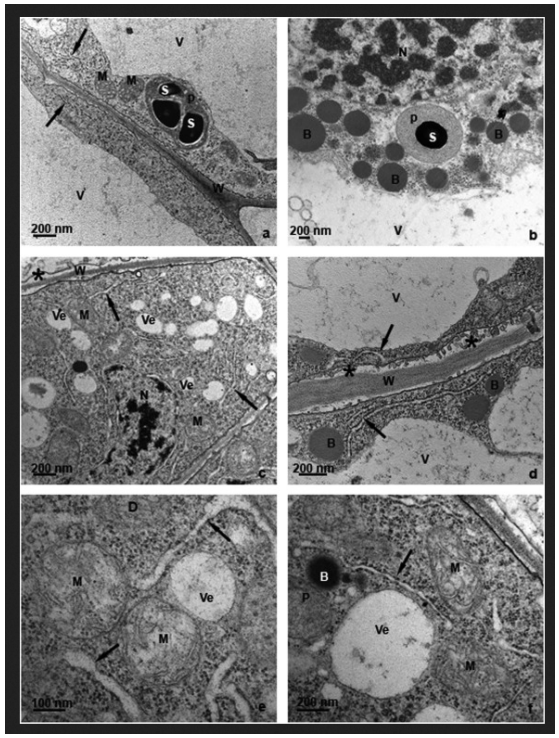

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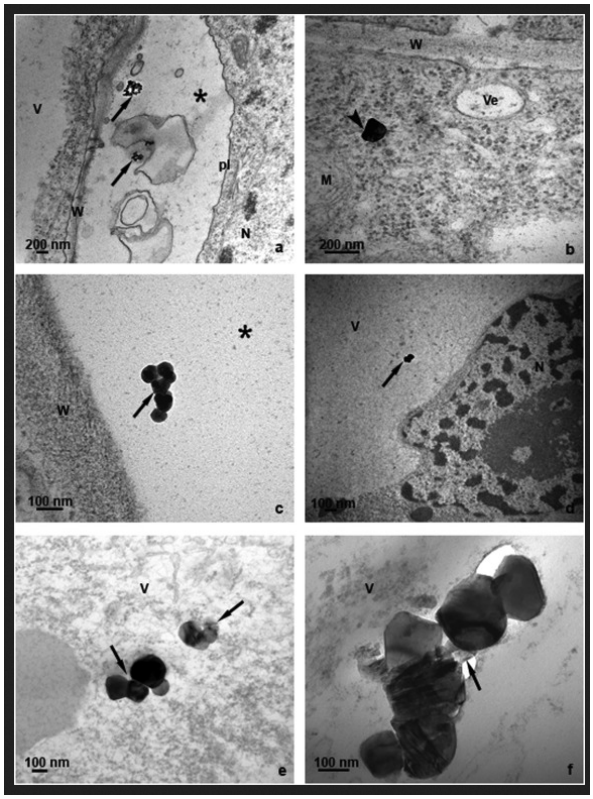

Table 1. Percentage of germination and primary root length in *L. culinaris* L. and *A. cepa* L. determined after 72 h of imbibition in control (water) and in the presence of 0.05, 0.1, 0.5, 1, 2 g/L of E171. Values were expressed as mean of 5 replicates ± standard deviation.

	Lens culinaris		*Allium cepa*	
Treatment	**Germination %**	**Root length cm**	**Germination %**	**Root length cm**
Control	80.0 ± 17.3	2.3 ± 0.4	96.7 ± 4.7	0.9 ± 0.1
0.05 g/L	70.0 ± 10.0	2.0 ± 0.4	99.2 ± 1.7	0.7 ± 0.1
0.1 g/L	66.7 ± 5.8	2.1 ± 0.6	98.3 ± 1.9	0.8 ± 0.1
0.5 g/L	80.0 ± 10.0	2.0 ± 0.5	96.7 ± 2.7	1 ± 0.2
1 g/L	73.3 ± 20.8	2.2 ± 0.4	96.7 ± 5.8	0.9 ± 0.1
2g/L	66.7 ± 15.3	2.1 ± 0.4	98.9 ± 1.9	0.9 ± 0.1

Table 2. Cytological analysis of *L. culinaris* root meristems in control and after 72 h treatments with 0.05, 0.1, 0.5, 1, 2 g/L of E171. Normal and abnormal mitotic phases (prophases, metaphases and ana/telophases) were expressed as mean values ± standard deviation on 100 mitoses analyzed. Within column, means followed by a common letter are not significantly different by the HSD – test. HSD = Tukey's honestly significant difference at $p \leq 0.05$

Cytological analysis of *Lens culinaris*					
Treatment	**% Prophases**	**% Normal Metaphases**	**% Abnormal Metaphases**	**% Normal Ana/telophases**	**% Abnormal Ana/telophases**
Control	32.1 ± 8.7	26.1 ± 4.3c	5.2 ± 3.1a	33.9 ± 9.2b	2.8 ± 2a
0.05 g/l	41.7 ± 5.1	14 ± 3.7ab	12.2 ± 3.3ab	24.5 ± 3ab	7.6 ± 4.2ab
0.1 g/l	31 ± 9.5	17.9 ± 2.8b	17.5 ± 4.5bc	23 ± 6.8ab	10.6 ± 3.9b
0.5 g/l	30.4 ± 10.6	15.8 ± 1.3ab	17.5 ± 7.1bc	26.5 ± 5.7ab	9.8 ± 3.5ab
1 g/l	40 ± 7.7	12.5 ± 3ab	14 ± 6.2abc	21 ± 7.3a	12.5 ± 3.9b
2g/l	32 ± 6.9	11 ± 3.4a	23.6 ± 3.9c	19.1 ± 5.2a	14.4 ± 3.9b

Table 3. Cytological analysis of *A. cepa* root meristems in control and after 72 h treatments with 0.05, 0.1, 0.5, 1, 2 g/L of E171. Normal and abnormal mitotic phases (prophases, metaphases and ana/telophases) were expressed as mean values ± standard deviation on 100 mitoses analyzed. Within column, means followed by a common letter are not significantly different by the HSD – test. HSD = Tukey's honestly significant difference at $p \leq 0.05$

Cytological analysis of *Allium cepa*					
Treatment	**% Prophases**	**% Normal Metaphases**	**% Abnormal Metaphases**	**% Normal Ana/telophases**	**% Abnormal Ana/telophases**
Control	39.6 ± 1.9	16.5 ± 4bc	8.6 ± 4.1ab	27.8 ± 2.4b	7.5 ± 2a
0.05 g/l	34.3 ± 8.5	22.3 ± 4c	7.3 ± 4.4a	27.3 ± 9.3b	8.7 ± 0.6ab
0.1 g/l	44 ± 9.5	17.0 ± 2.6bc	7.7 ± 2.1ab	15 ± 5.3ab	15.7 ± 5.7abc
0.5 g/l	36.4 ± 8.3	10.8 ± 1.8ab	15.9 ± 3.4bc	20.8 ± 7.6ab	16.2 ± 2.7bc
1 g/l	30.5 ± 6.5	8.3 ± 2.2a	18.4 ± 3.2c	23 ± 5.1ab	19.3 ± 6.3c
2g/l	37.1 ± 5.1	9.5 ± 3.8ab	22.6 ± 4.2c	13.5 ± 4.4a	20.1 ± 5.2c