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HAL Authorization

1 **Studies on plant cell toxicity of luminescent silica nanoparticles**
2 **(Cs₂[Mo₆Br₁₄]@SiO₂) and its constitutive components**

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21
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25 nanoparticle TEM micrographs (Plateforme THEMIS, UR1).

27 **Abstract**

28

29 As part of the risk evaluation before potential applications of nanomaterials, phytotoxicity of newly
30 designed multifunctional silica nanoparticles (CMB@SiO₂, average diameter of 47 nm) and their
31 components, i.e. molybdenum octahedral cluster bromide units (CMB, 1 nm) and SiO₂
32 nanoparticles (nSiO₂, 29 nm), has been studied using photosynthetic *Arabidopsis thaliana* cell
33 suspension cultures. CMB clusters presented toxic effects on plant cells, inhibiting cell growth and
34 negatively affecting cell viability and photosynthetic efficiency. Nevertheless, we showed that
35 neither nSiO₂ nor CMB@SiO₂ have any significant effect on cell growth and viability or
36 photosynthetic efficiency. At least part of the harmful impact of CMB clusters could be ascribed to
37 their capacity to generate an oxidative stress since lipid peroxidation greatly increased after CMB
38 exposure, which was not the case for nSiO₂ or CMB@SiO₂ treatments. Exposure of cells to CMB
39 clusters also lead to the induction of several enzymatic antioxidant activities (i.e. superoxide
40 dismutase, guaiacol peroxidase, glutathione peroxidase, glutathione reductase, and glutathione S-
41 transferase activities) compared to control and the other treatments. Finally, using electron
42 microscopy, we showed that *Arabidopsis* cells internalize CMB clusters and both silica
43 nanoparticles, the latter through, most likely, endocytosis-like pathway as nanoparticles were
44 mainly found incorporated into vesicles.

45

46

47 **Keywords**

48

49 Nanotoxicity; Silica nanoparticles; Molybdenum clusters; *Arabidopsis* cells; Oxidative stress.

50

51 **Introduction**

52

53 Manufactured nanoparticles (NPs) (one dimension < 100 nm) are being increasingly produced for a
54 wide range of applications and are present in hundreds of nanotechnology products (Buzea et al.
55 2007). However, they are also bringing new toxic effects on human and environmental health
56 (Buzea et al. 2007; Colvin 2003). Among nanomaterials, the use of functional synthetic amorphous
57 silicon dioxide or silica nanoparticles (nSiO₂) in information technology, biotechnology and
58 medicine is becoming increasingly accepted for a variety of therapeutic, diagnostic and imaging
59 applications (Selvan et al. 2010). The challenge for nanotechnologies at this point is to elaborate
60 non-toxic and aging resistant phosphorescent silica nanoparticles emitting in the near infrared
61 region (NIR). For this purpose, new functional silica nanoparticles incorporating luminescent
62 molybdenum hexanuclear cluster bromide units (Cs₂Mo₆Br₁₄, noted CMB, as the cluster precursor)
63 inside monodispersed and size-controlled silica nanoparticles (noted CMB@SiO₂) have been
64 recently developed in our group (Aubert et al. 2013). Besides, Mo₆-based clusters are already
65 involved in several patents for applications in biotechnology as contrast agents (Long et al. 1998),
66 oxygen sensors (Baker et al. 2010) and in display technologies (Cordier et al. 2015).

67 Silica nanoparticles are used as matrices because of their versatility and their relative
68 biocompatibility (Fruijtier-Pölloth 2012). However, this point of nSiO₂ safety is controversial, and
69 different studies reported toxic effects in some cells or organisms like humans (Brown et al. 2015;
70 Guarnieri et al. 2014; Napierska et al. 2010), other animals (Debnath et al. 2011; Lee et al. 2009;
71 Parveen et al. 2014), algae (van Hoecke et al. 2011) or bacteria (Adams et al. 2006). Concerning
72 higher plants, most of the studies reported null or positive effects (Le et al. 2014; Lee et al. 2010;
73 Lin et al. 2004; Nair et al. 2011; Siddiqui and Al-Whaibi 2014; Slomberg and Schoenfisch 2012),
74 nSiO₂ toxicity being only observed at very high concentrations (Le et al. 2014; Lee et al. 2010). The
75 toxic mechanisms of nSiO₂ exposure remain far from clear, but in some cases nSiO₂ toxic effects
76 were related to interactions with cellular surfaces (membrane or cuticle), oxidative stress and/or

77 genotoxicity (Adams et al. 2006; Brown et al. 2015; Debnath et al. 2011; Fruijtier-Pöllöth 2012;
78 Napierska et al. 2010; Parveen et al. 2014).

79 SiO₂ nanoparticles are a common nanomaterial which is used (either or not in admixture with
80 other elements) for a variety of applications in the medical (biomedicine, biosensor, disease
81 labeling) and technological (food processing, ceramics synthesis, industrial and household
82 applications) fields, but also in the environmental (wastewater treatment, water purification,
83 environmental remediation) and agriculture fields. In agriculture, silica nanoparticles are used in
84 different formulations, mainly as carriers in chemical delivery, or in uptake and translocation of
85 nutrient elements, and as active ingredients against insect pests (Gogos et al. 2012), thereby
86 fostering their dispersion in the environment. In this context, luminescent properties of CMB@SiO₂
87 nanoparticles are not without interest for biotechnological uses that could also be applied to plants,
88 or to be combined with silica nanoparticles intended for agronomical uses. From these current and
89 potential uses of silica nanoparticles, it is obvious that their potential to harm the environment is a
90 relevant issue. Plants, as important environmental components and sinks in terrestrial and aquatic
91 ecosystems, are essential living organisms for testing ecological effects of nanoparticles. Hence, it
92 is of great importance to study the impact of new functional silica nanoparticles on plant cells, and
93 to anticipate new potential risks derived from their accumulation into plants and their subsequent
94 fate within food chains. In earlier studies on the impact of CMB@SiO₂ nanoparticles and of CMB
95 clusters in plant growth (Aubert et al. 2012; 2013), we showed that silica nanoparticles containing
96 clusters have no effect on plant growth, whereas CMB clusters penetrated into roots and negatively
97 impacted growth. In these studies, roots were always much more affected than aerial parts, certainly
98 due to the root direct contact with clusters and the very low translocation of clusters into aerial part.
99 The latter makes it difficult to analyze the direct impact of these nanomaterials on photosynthetic
100 cells in these root-treated systems. Indeed, the absence of adverse effects reported in most works
101 evaluating silica nanoparticle phytotoxicity could be partially linked to low or no occurrence of
102 silica nanoparticles in photosynthetic cells, rather than the lack of inherent hazards. At this respect

103 in particular, the use of plant cell cultures provides a way for *in vitro* exposing photosynthetic cells
104 directly to the action of nanoparticles. In fact this model system can mimic, as regards with plant
105 cell interaction with nanoparticles, the situation that could be found in photosynthetic cells of aerial
106 parts of plants exposed to CMB@SiO₂, nSiO₂ or CMB in a chronic way, where a higher
107 accumulation of nanoparticles in leaves can be achieved after a long-time exposure.

108 In order to go further in CMB@SiO₂, nSiO₂ and CMB toxicological research, and with the aim
109 of exploring the impact of weak doses on photosynthetic cells, we have chosen light-grown
110 *Arabidopsis thaliana* cell suspension cultures (ACSC) as a valuable cellular system in which to
111 investigate oxidative damage and cell response. ACSC join uniformity, homogeneity, repeatability,
112 decoupling of cellular processes from development and slow systemic effects between cells
113 (Menges et al. 2003), to the convenience of application of nanomaterial treatments. Here, we
114 present studies on biochemical and oxidative stress factors on *A. thaliana* cells under exposure to
115 functional CMB@SiO₂ nanoparticles and their components, nSiO₂ nanoparticles and CMB clusters.
116 The *in vitro* cytotoxicity of these nanomaterials was examined by investigating their influence on
117 cell growth and viability, photosynthesis, lipid peroxidation, and antioxidant enzyme activities (i.e.
118 superoxide dismutase (SOD), guaiacol peroxidase (POD), glutathione peroxidase (GPX),
119 glutathione reductase (GR), and glutathione S-transferase (GST) activities). Finally, the fate of
120 nanoparticles in the medium and their penetration into plant cells was detected by transmission
121 electron microscopy (TEM).

122

123

124 **Materials and Methods**

125

126 Chemicals, cluster units, and silica nanoparticles

127

128 Polyoxyethylene (4) lauryl ether (Brij30) and tetraethoxysilane (TEOS, 99.00%) were purchased
129 from Sigma-Aldrich. Ammonia (28 wt % in water) and n-heptane (99.00%) were purchased from
130 VWR. Ethanol (99.80%) was purchased from Fluka. $\text{Cs}_2\text{Mo}_6\text{Br}_{14}$ was used as the precursor of
131 $[\text{Mo}_6\text{Br}_{14}]^{2-}$ cluster units.

132 Hexamolybdenum cluster units are nanometric building blocks (1 nm) constituted of a Mo_6
133 octahedral cluster bonded to 8 inner Br^i (i = inner) ligands capping the faces of the octahedron and 6
134 apical Br^a (a = apical) ligands in terminal positions. The negative charge of the $[\text{Mo}_6\text{Br}_{14}]^{2-}$ cluster
135 unit is counter balanced by two Cs^+ cations. In solid state, the cluster units co-crystallize with the
136 cations to form a cluster compound denoted $\text{Cs}_2\text{Mo}_6\text{Br}_{14}$. The $\text{Cs}_2\text{Mo}_6\text{Br}_{14}$ cluster compound can be
137 dispersed as nanosized entities in ethanolic solution (Grasset et al. 2008).

138 All the silica nanoparticles have been prepared using a water-in-oil (W/O) microemulsion
139 process developed by our group since the earlier 2000 (Aubert et al. 2010; Grasset et al. 2002). In
140 this work, the complex water phase was prepared by dissolving the $\text{Cs}_2[\text{Mo}_6\text{Br}_{14}]$ cluster compound
141 in a mixture of ethanol and distilled water (1:1 volume ratio). The concentration of the cluster sol
142 was 0.02 M. For pure nSiO_2 , the complex water phase was free of cluster. Finally, the nanoparticles
143 were collected and washed by several centrifugation cycles to remove surfactant molecules before
144 to be dispersed in purified water at concentration around 15 g L^{-1} . The average hydrodynamic size
145 of the silica nanoparticles in water solution was estimated by dynamic light scattering
146 (Supplementary Fig. 1) using a Malvern Zetasizer Nano ZS apparatus. All the samples were studied
147 by TEM (Supplementary Fig. 2) using a microscope JEOL 2100 LaB₆ at 200 kV or JEOL JEM-
148 1400 microscope operating at 120 kV. Samples for TEM analysis were prepared by placing a drop
149 of the diluted solution in mesh copper grids, allowing the solvent in the grid to evaporate at room
150 temperature.

151

152 ACSC growth conditions and nanomaterial treatments

153

154 The *Arabidopsis thaliana* (ecotype Col-0) cell suspension cultures were kindly provided by the
155 Institute of Natural Resources and Agronomy from Salamanca (IRNASA-CSIC, Spain). ACSC
156 were maintained at 24°C under sterile conditions in 200 mL of liquid growth medium (Axelos et al.
157 1992; Jouanneau et al. 1967) by agitation at 120 rpm and under continuous illumination (50 $\mu\text{E m}^{-2}$
158 s^{-1}) in an incubator shaker (Innova 42R, NBS). For nanoparticle toxicity tests, we applied
159 luminescent silica nanoparticles (CMB@SiO₂) and their constituents (i.e. CMB clusters and nSiO₂)
160 into 9 days-old ACSC (cell density of 150-200 mg mL⁻¹). For these studies, three different
161 concentrations of CMB@SiO₂ and nSiO₂ (1, 10 and 100 mg L⁻¹), and of CMB clusters (1, 7.5 and
162 60 mg L⁻¹) were tested. Treated ACSC were incubated for up to three days under normal growth
163 conditions, and sample aliquots collected at 3, 24 and 72 h. Collected samples were centrifuged at
164 4000 rpm for 5 min, the supernatant was removed, and the cell pellet was weighed, frozen in liquid
165 nitrogen and finally stored at -80°C until further analysis.

166 For stock solutions to be used for nanoparticle toxicity tests, CMB@SiO₂ and nSiO₂ were
167 suspended in water at 5 g L⁻¹, and CMB in 50% ethanol at 60 g L⁻¹. CMB@SiO₂ used here are
168 composed of 7.5% clusters and 92.5% SiO₂ (Aubert et al. 2013). Thus, the intermediate CMB
169 concentration (7.5 mg L⁻¹) used for treatments corresponds to the cluster content associated to the
170 intermediate concentration of CMB@SiO₂ nanoparticles (100 mg L⁻¹). From this intermediate
171 CMB concentration, we have set the lowest CMB concentration at 1 mg/L, which correspond
172 (rounded to the nearest unit) to the cluster content present in 10 mg/L of CMB@SiO₂, and the
173 highest CMB concentration at 60 mg/L (in order not to exceed 0.05% of ethanol in cell culture
174 medium). As control, we used ACSC without added nanomaterial but containing the equivalent
175 volume of the corresponding solvent or medium as used for the nanomaterial tested.

176

177 Cell viability assay

178

179 Cell mitochondria and metabolic activities were measured by the MTT (3-(4,5-dimethylthiazol-2-
180 yl)-2,5-diphenyl tetrazolium bromide) test following manufacturer's indications (Cell growth
181 determination kit CGD-1, Sigma-Aldrich). Cell density was adjusted to 25 mg mL⁻¹ at the
182 beginning of the treatment, and cell dilution for MTT assay was the same for all the samples.
183 Relative cell viability was expressed as the percentage of control untreated cells and calculated by
184 $[\text{Absorbance}_{570\text{ nm}} - \text{Absorbance}_{690\text{ nm}}]_{\text{test}} / [A_{570} - A_{690}]_{\text{control}} \times 100$.

185

186 Pigment analysis

187

188 The chlorophyll and carotenoid contents were determined spectrophotometrically following
189 Lichtenthaler and Wellburn (1983) equations. Collected cells were lyophilised (Christ ALPHA 1-
190 2LDplus) and pigments extracted from 20 mg dry weight (DW) by overnight pure acetone
191 extraction at 4°C. The absorbance was quantified at 470, 645 and 663 nm using a micro plate
192 spectrophotometer (SAFAS, Xenius).

193

194 Chlorophyll fluorescence measurements

195

196 Modulated chlorophyll fluorescence measurements were made in ACSC (previously dark adapted
197 for 30 min) with a PAM-210 chlorophyll fluorometer (Heinz Walz). Maximum quantum yield of
198 photosynthesis was estimated by the F_v/F_m ratio from dark-adapted ACSC, where F_v is calculated
199 subtracting the minimal fluorescence (F_o) to the maximal fluorescence (F_m).

200

201 Lipid peroxidation

202

203 The level of lipid peroxidation was determined by measuring the amount of TBARS (thiobarbituric
204 acid reactant species) produced by the thiobarbituric acid (TBA) reaction, according to the corrected
205 TBA method as described by Hodges et al. (1999) adapted to 96-well plates.

206

207 Antioxidant enzyme extraction and activity assays

208

209 Enzyme extracts correspond to supernatants obtained after homogenizing *A. thaliana* cells in
210 sodium phosphate buffer (50 mM, pH 7.5) with Na-EDTA (1 mM), polyvinyl-pyrrolidone (5 %
211 w/v), sodium ascorbate (5 mM) and Protease Inhibitor Cocktail (0.5 % v/v, Sigma-P9599) at a ratio
212 of 1 mL per 20 mg DW. Protein contents were determined according to Bradford (Bradford 1976),
213 using bovine serum albumin as the standard protein. Enzyme extracts were frozen in liquid nitrogen
214 and kept at -80°C until their use for enzymatic assays. All enzyme assays were adapted to 96-well
215 plates (final reaction volume of 300 µL).

216 SOD activity was determined based on the inhibition of the reduction of nitro-blue tetrazolium
217 (NBT) into formazan in the presence of riboflavin as described by Giannopolitis and Ries (1977).
218 Formazan formation was determined measuring the absorbance at 560 nm after 10 min of
219 incubation under white light at 25 °C. The reaction mixture consisted of 10 µL of enzyme extract,
220 potassium phosphate buffer (50 mM, pH 7.8), EDTA (0.1 mM), NBT (75 µM), methionine (13
221 mM) and riboflavin (2 µM).

222 POD activity was measured by the method of Srivastava and van Huystee (1977) with a reaction
223 mixture consisting of 5 µL of enzyme extract, potassium phosphate buffer (100 mM, pH 6.5), H₂O₂
224 (0.05% v/v) and guaiacol (15 mM). The enzymatic activity was determined from the maximum rate
225 of tetragaiacol formation by monitoring the increase in absorbance at 470 nm ($\epsilon_{\text{Tetragaiacol}} = 26.6$
226 $\text{mM}^{-1} \text{cm}^{-1}$).

227 GPX activity was measured by a coupled assay system in which oxidation of GSH was coupled
228 to NADPH oxidation catalyzed by glutathione reductase according to the method of Floh and

229 Günzler (1984). The reaction mixture consisted of 5 μL of enzyme extract, potassium phosphate
230 buffer (100 mM, pH 7.0), cumene hydroperoxide (0.5 mM), GSH (4 mM), NADPH (0.2 mM) and
231 0.5 units of yeast glutathione reductase. The enzymatic activity was determined at 25°C from the
232 maximum rate of NADPH oxidation by monitoring the decrease in absorbance at 340 nm ($\epsilon_{\text{NADPH}} =$
233 $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

234 GR activity was measured according to the method of Carlberg and Mannervik (1985), following
235 the oxidized glutathione (GSSG)-dependent oxidation of NADPH. The assay mixture consisted of
236 10 μL of enzyme extract, HEPES buffer (50 mM, pH 8.0), EDTA (0.5 mM), GSSG (0.5 mM) and
237 NADPH (0.25 mM). The enzymatic activity was determined at 25°C from the maximum rate of
238 NADPH oxidation by monitoring the decrease in absorbance at 340 nm ($\epsilon_{\text{NADPH}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

239 GST activity was measured by the method of Habig and Jacoby (1981) using CDNB (1-chloro-2,
240 4-dinitrobenzene) as the substrate. The assay mixture consisted of 5 μL of enzyme extract,
241 potassium phosphate buffer (100 mM, pH 7.4), GSH (1 mM) and CNDB (1 mM). The enzymatic
242 activity was determined at 25°C from the maximum rate of GSH/CNDB conjugate formation by
243 monitoring its absorbance at 340 nm ($\epsilon_{\text{CNDB}} = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

244 All enzymatic activities but SOD were expressed as nkat mg^{-1} protein. SOD activity was
245 expressed as U mg^{-1} protein, U (a unit) being the amount of enzyme causing 50% inhibition of the
246 NBT reduction observed in the absence of enzyme.

247

248 ACSC TEM analysis

249

250 TEM samples were prepared following standard procedures. Roughly, collected cell samples were
251 centrifuged at 1700 g for 5 min, the supernatants were removed, and the cell pellets were washed
252 once with cacodylate buffer, chemically prefixed in 2.5 % (v/v) glutaraldehyde for 1.5 h, washed 3
253 times in sodium cacodylate buffer (0.2 M, pH 7.1), then post fixed in 0.5 % (v/v) osmium tetroxide
254 for 1 h, and washed 3 times in sodium cacodylate buffer (0.2 M, pH 7.1). The samples were then

255 included in low melting agar (4%) and dehydrated in several ethanol baths with increasing
256 concentrations. The specimens were embedded in an Araldite/Epon epoxy resin from which
257 ultrathin sections (thickness: 90 nm) were cut using an ultramicrotome (LEICA UC7) and directly
258 deposited on copper grids. The grids were visualized in a JEOL 1400 microscope operated at 120
259 kv and using a Gatan 2kX2k Orius camera. Image analysis on the silica nanoparticles and clusters
260 was carried out on 35 TEM images. The processing of the image files was performed on more than
261 500 particles using standard ImageJ analysis software (<http://rsbweb.nih.gov/ij/>). Particle size is
262 presented as mean \pm standard deviation (SD).

263

264 Statistical analysis

265

266 Statistical analyses were performed with R software version 3.2.1 (<http://www.r-project.org/>).
267 Normality and homoscedasticity were confirmed with Shapiro and Bartlett tests for each assay. The
268 results are presented as mean \pm standard error of the mean (SEM) of three independent experiments.
269 Differences between means were evaluated for significance by Student's t-test for pairwise
270 comparisons, and by one-way analysis of variance (ANOVA) followed of Tukey's test for multiple
271 comparisons. Statistical significance was accepted when $p < 0.05$.

272

273

274 **Results**

275

276 Particle characterization

277

278 The hydrodynamic diameter of the two types of silica nanoparticles was found to be comprised
279 between 40-60 nm from the dynamic light scattering data in aqueous dispersion at pH = 7.4, which
280 indicates that the nanoparticles are not or slightly aggregated in the solution. The result obtained for

281 CMB@SiO₂ is represented in Supplementary Fig. 1 as example. These results are in the same range
282 as the size observed by scanning electron microscopy (not shown) and TEM. The TEM images of
283 the nSiO₂ and CMB@SiO₂ are as shown in Supplementary Fig. 2. Diameter sizes of ‘as produced’
284 CMB@SiO₂ and nSiO₂ obtained from the TEM image are of 47 ± 3 and 29 ± 2 nm, respectively.

285

286 Impacts of nanomaterials on cell growth and cell viability

287

288 In our conditions, ACSC grew with a doubling time of about 2.1 days and had a cell density around
289 200 mg mL⁻¹ at the beginning of the stationary phase (between 9 to 11 days after subculture). No
290 changes in cell growth or viability were observed 3 h after exposure, regardless of nanomaterial
291 (Fig. 1a-c). However, depending on the type of nanomaterial, significant changes in cell growth
292 were detected during longer treatment periods. Thus, while ACSC exposed to CMB@SiO₂ or nSiO₂
293 at concentrations up to 100 mg L⁻¹ are capable to continue normal growth up to 72 h after treatment
294 (Fig. 1a, b), 60 mg L⁻¹ CMB significantly impacted ACSC growth (18.5 and 21.3 % of growth
295 inhibition after 24 and 72 h of treatment, respectively) (Fig. 1c).

296 On the other hand, *Arabidopsis* cell viability was assessed by the MTT assay (Fig. 1d-f). In
297 agreement with the impact of nanomaterials on cell growth, only CMB clusters at their highest used
298 concentration had significant cytotoxic effects, provoking a 45.6 and 27.7 % decrease of cell
299 viability after 24 and 72 h of exposure respectively (Fig. 1f).

300

301 Changes in chloroplast pigment content and photosynthetic efficiency

302

303 The impact of nanomaterials on chloroplasts was evaluated through the analyses of chlorophyll and
304 carotenoid contents, and PSII photochemical efficiency. Light-grown ACSC used for nanomaterial
305 treatments were pale green and contained about 240 µg chlorophyll and 85 µg carotenoid per gram
306 DW. Chlorophyll content remained unchanged during treatment in light-grown control, and in

307 CMB@SiO₂ and nSiO₂ treated ACSC, whereas ACSC treated with 60 ppm CMB experienced a 13
308 and 21% decline in chlorophyll content after 24 and 72 h of treatment respectively (Fig. 2a–c).
309 Concerning carotenoid content, we roughly observed a similar behavior as for chlorophyll, CMB
310 clusters being the only nanomaterial to have a significant impact on it (Fig. 2d–f). Finally, it should
311 also be noted that the chlorophyll a/b and chlorophyll/carotenoid mass ratios in control ACSC at the
312 beginning of the treatment were approximately 3.7 and 2.7 respectively, and that they were not
313 affected by any of the nanomaterials (data not shown).

314 The maximum quantum yield of photosynthesis is generally influenced by stress situations, and
315 is usually estimated by the ratio F_v/F_m. The maximum quantum yield of photosynthesis in light-
316 grown ACSC reached a level of around 0.45-0.55 in 9-days-old ACSC used for nanomaterial
317 treatments, and changed little during treatment time course. CMB@SiO₂ treatments did not affected
318 F_v/F_m values (Fig. 2g, h) but CMB significantly did (Fig. 2i) through 24-72 hours of treatment at all
319 the tested concentrations.

320

321 Oxidative impact and enzymatic antioxidant response

322

323 We studied the mechanisms of cytotoxicity caused by nanomaterials with respect to oxidative stress
324 through the oxidative impact on lipids (lipid peroxidation) and the antioxidant response (antioxidant
325 enzymatic activities). The oxidative degradation of lipids by reactive oxygen species (ROS), called
326 lipid peroxidation, results in the formation of highly reactive and unstable lipid peroxides which
327 decomposed into TBARS, including malondialdehyde (MDA). Thus, TBARS level give a
328 convenient estimation of the relative lipid peroxide content. The TBARS content of control 9-day-
329 old ACSC was $\approx 14-16$ nmol MDA_{equivalents} g⁻¹. After nanomaterial exposure, a significant increase
330 of lipid peroxidation was only observed for 60 mg L⁻¹ CMB-treated cells (Fig. 3). In this case, lipid
331 peroxidation increased with time treatment, being of 119% after 24 h and 143% after 72 h of
332 treatment.

333 In order to understand the adaptability and to determine the nature of the antioxidant responses
334 of *A. thaliana* cells to the different nanomaterials, we analyzed the activities of five antioxidant
335 enzymes, i.e. superoxide dismutase (SOD; EC 1.15.1.1), guaiacol peroxidase (POD; EC 1.11.1.7),
336 glutathione peroxidase (GPX; EC 1.11.1.9), glutathione reductase (GR; EC 1.8.1.7) and glutathione
337 S-transferase (GST; EC 2.5.1.18), in ACSC treated for 3, 24 and 72 hours with investigated
338 nanoparticles at different concentrations. The only nanomaterial affecting SOD (Fig. 4a–c), POD
339 (Fig. 4d–f) and GR (Fig. 4g–i) activities were CMB clusters. Thus, *A. thaliana* cells undergoing 60
340 ppm CMB treatment showed, relative to control, a 50% transitory increase in SOD activity after 24
341 h, and a marked increase in POD (2 and 1.9 times) and GR (1.6 and 1.5 times) activities after 24
342 and 72 h of treatment. On the other hand, GPX activity (Fig. 4j–l) was increased by all the
343 nanomaterials tested, but with different induction patterns. Thus, while 60 ppm CMB clusters
344 induced GPX after 24 and 72 h of treatment, CMB@SiO₂ and nSiO₂ slightly induced GPX after 3
345 h, and nSiO₂ was able to provoke a second wave of inductions in a concentration-dependent way
346 after 72 h. Finally, concerning GST activity (Fig. 4m–o), all the nanomaterials at the different
347 concentrations were able to early induce GST (3 h after treatment). The highest increase of GST
348 activity was obtained after nSiO₂ treatment, but only 60 ppm CMB maintained GST induction over
349 time.

350

351 Nanoparticle interaction with plant cells as examined by TEM

352

353 In culture medium ('as exposed' state), CMB clusters showed tendency to aggregate forming
354 particles of a diameter around 83 ± 14 nm, which agglomerate to form different shape branched
355 structures under the micrometer range (Fig. 5a). On the other hand, 'as exposed' CMB@SiO₂ and
356 nSiO₂ stayed non aggregated and spherical in shape with diameters, as measured from TEM
357 micrographs, of 44 ± 4 and 27 ± 2 nm, respectively (Fig. 5b, c). These nanoparticle sizes were not

358 significantly different from ‘as produced’ sizes measured from TEM micrographs (Supplementary
359 Fig. 2).

360 In addition, *Arabidopsis* cells were also observed by TEM to determine if the different
361 nanomaterials entered the plant cells. In the case of CMB treated cells (Fig. 5a, d), they display
362 altered cell wall ultrastructure, presenting a loosely structured cell wall (reduced electron density)
363 but more than twice as thick compared with that of control cells (Supplementary Fig. 3). Cluster
364 aggregates seem to be present inside this loose cell wall and central vacuole, but are not observed
365 inside vesicles. Furthermore, we cannot rule out the presence of individual nanometric clusters
366 which, as already mentioned, are not detectable when they are at their synthesis size (1 nm
367 diameter) due to resolution limit of the TEM available for this work. In contrast, CMB@SiO₂ (Fig.
368 5b, e) and nSiO₂ (Fig. 5c, f) were observed inside plant cells and seemed to conserve their ‘as
369 produced’ and ‘as exposed’ sizes. Furthermore, CMB@SiO₂ and nSiO₂ were observed inside
370 vesicles (Fig. 5d, e), pointing endocytosis as a mechanism of cell uptake for these nanoparticles.

371

372

373 **Discussion**

374

375 Luminescent functional silica nanoparticles based on Mo₆ clusters possess a huge potential for
376 application in the field of nanobiotechnology or nanophotonics (Cordier et al. 2015). For a
377 reasonable and responsible development of their use, potential toxic effects must be deeply studied.
378 Here, the cytotoxicity of low/medium doses of functional silica nanoparticles and their components
379 was investigated under *in vitro* conditions using photosynthetic *A. thaliana* cell cultures. CMB
380 clusters at 60 ppm concentration negatively impacted cells, significantly reducing cell growth and
381 viability (Fig. 1) in agreement with their reported negative impact on plant root growth (Aubert et
382 al. 2012). In the case of silica nanoparticles, none negative effect were observed on ACSC growth
383 or viability after neither CMB@SiO₂ nor ‘empty’ nSiO₂ treatments at any tested concentration.

384 Slomberg and Schoenfisch (2012) already showed that nSiO₂ did not caused toxic effects on *A.*
385 *thaliana* plants up to 1 g L⁻¹, but in this case nSiO₂ contact with photosynthetic cells was negligible
386 since they reported minimal upward translocation to foliage.

387 The impact of CMB@SiO₂, nSiO₂ and CMB on chloroplast functioning has never been
388 evaluated. We have first showed that although photosynthesis is not necessary for ACSC survival,
389 the photosynthetic electron transport chain of thylakoid membranes in light-grown ACSC is active
390 (Fig. 2). Thus, we measured quantum yield of photosynthesis (F_v/F_m) values around 0.6 for control
391 ACSC, which is in agreement with those from the literature for *A. thaliana* cell cultures (González-
392 Pérez et al. 2011) and indicates that *A. thaliana* cell cultures produce functional chloroplasts, even if
393 this ratio is lower than the 0.8 determined for green leaves (Zhang et al. 2008). In addition, the
394 levels of chlorophylls (240 µg g⁻¹ DW) and carotenoids (85 µg g⁻¹ DW) were in perfect agreement
395 with those described in the literature for *A. thaliana* cell cultures (González-Pérez et al. 2011; Doyle
396 et al. 2010) and represent, respectively, about 2.5% and 5.5 % of the levels described in leaf tissues
397 (Zhang et al. 2008; Doyle et al. 2010). Furthermore, while the chlorophyll *a/b* ratio (around 3.7)
398 was close to those for mature chloroplasts of *A. thaliana* leaves (around 3.3), the
399 chlorophyll/carotenoid mass ratio (around 2.7) was much lower than in *A. thaliana* leaves (around
400 6.4) (Zhang et al. 2008). Photosynthetic apparatus parameters such as pigment content (chlorophylls
401 and carotenoids), pigment ratios, and photosynthesis yield are good indicators for stress detection
402 and tolerance (Doyle et al. 2010; Zhang et al. 2008). In our work, only CMB clusters significantly
403 impacted these parameters, decreasing chlorophyll and carotenoid contents as well as F_v/F_m values,
404 but without affecting Chl *a/b* or chlorophyll/carotenoid ratios. It is worth to be noted that a
405 significant decreased in maximum quantum yield of photosynthesis was observed for CMB doses as
406 low as 1 ppm. This photosynthetic unbalance can generate excess energy, which is extremely
407 harmful and dangerous for plant cell metabolism, notably because it provokes the accumulation of
408 ROS which may lead to damages in the thylakoid membranes and protein modulation (Ruban
409 2015). In the light of the foregoing, photosynthetic apparatus seems to be more sensitive to CMB

410 under light conditions than cell growth or viability. This can be attributed to the fact that light
411 enhances ROS production by clusters, precisely $^1\text{O}_2$ (Aubert et al. 2013), and that this could
412 synergically interact with ROS production in different organelles, notably those associated to
413 photosynthetic light-driven process: $^1\text{O}_2$ in PSII, superoxide radical ($\text{O}_2^{\cdot-}$) in PSI, and hydrogen
414 peroxide (H_2O_2) in the chloroplast stroma (Gill and Tuteja 2010).

415 We have shown in previous work that $^1\text{O}_2$ production involving CMB clusters can be prevented,
416 to some extent, by the encapsulation of the cluster units in silica nanoparticles (Aubert et al. 2013).
417 However, the capacity of CMB to provoke an oxidative stress in cells, and the impact of silica
418 encapsulation on this, has never been studied. It is well known that the generation of ROS as natural
419 by-products during cell metabolism is enhanced in the different plant cell compartments after the
420 exposure of plants to environmental stresses, provoking subsequent damage in cell biomolecules
421 and metabolism. We chose to follow MDA production (through TBARS quantification) because
422 MDA is a product of the peroxidation of unsaturated fatty acids and it has been used as an indicator
423 of free radical damage to cell membranes under stress conditions (Gill and Tuteja 2010). Under
424 nanomaterial treatment, the TBARS content was found to be increased only after CMB cluster
425 exposure. This could be explained on the basis of the above mentioned $^1\text{O}_2$ production by CMB
426 under light conditions, as it has been shown that $^1\text{O}_2$ mediate lipid peroxidation (Triantaphylidès et
427 al. 2008), and matches with previous studies showing that the CMB@SiO_2 nanoparticles are
428 particularly stable and do not liberate clusters (Aubert et al. 2013). Furthermore, even if oxygen has
429 been shown to still have access to some cluster units from CMB@SiO_2 and produce $^1\text{O}_2$, we
430 showed here that silica encapsulation of CMB clusters prevents $^1\text{O}_2$ production at levels able to
431 provoke lipid peroxidation in *A. thaliana* cells.

432 To protect themselves against ROS production and uncontrolled lipid peroxidation, plant cells
433 possess and induce an array of antioxidant defense systems (Gill and Tuteja 2010). We analyzed the
434 activities of an array of antioxidant enzymes (SOD, POD, GPX, GR and GST) under nanomaterial
435 treatment conditions. Within these activities, SOD, which catalyze disproportionation of $\text{O}_2^{\cdot-}$ into

436 H₂O₂ and O₂, belong to the first line of defense. The H₂O₂ produced in the cell, by SOD or other
437 processes, may be scavenged by catalases and peroxidases, the latter including POD and GPX.
438 Additionally, GPX may also reduce lipid hydroperoxides. For their part, GST catalyze the
439 conjugation of electrophilic substrates to reduced glutathione, and can also function as glutathione
440 peroxidases. Finally, the glutathione oxidized in cells is regenerated by GR utilizing NADPH. The
441 increment in the activity of SOD after 24 h of exposure to CMB, and the higher increase of POD
442 and GR activities after CMB treatment at 24 and 72 h, suggested their role in the defense system
443 against CMB induced oxidative stress, either by the removal of ROS and of toxic products of
444 organic peroxidation. Moreover, the induction of POD, which are mainly considered extracellular
445 proteins, in interplay with apoplastic SOD could participate in initial oxidative burst and signal
446 transduction pathways (Francoz et al. 2015) as well as cell wall loosening (Minibayeva et al. 2015).
447 Interestingly, the latter have been observed in CMB treated cells (Fig. 5 and Supplementary Fig. 3).
448 It should also be pointed out that, in agreement with the absence of physiological (cell growth and
449 viability, pigments, and quantum yield of photosynthesis) and oxidative (lipid peroxidation)
450 impacts, CMB@SiO₂ and nSiO₂ treatments did not have a marked impact on antioxidant activities,
451 exception done of the induction of GR activity by nSiO₂ after a long exposure period (72 h), and the
452 early induction of GST activity by both silica nanoparticles.

453 It is well-known that the properties of nanomaterials can change from the form in which they are
454 synthesized to the form to which biological test systems are exposed, and that these potential
455 changes in size, shape or aggregation, among other, could influence toxicity. In previous studies we
456 showed that the CMB clusters and cluster aggregates can be found in a wide range of sizes
457 depending on the dispersing medium, and that their concentration-dependent toxicity depends on
458 their aggregation state (Aubert et al. 2012). Consequently, here we analyzed using TEM the
459 different nanomaterials in the exposure medium and in intimate contact with living cells. Even if
460 silica nanoparticles can have tendency to agglomerate and aggregate in high ionic strength medium
461 like growth medium (Guarnieri et al. 2014), we observed that hydrophilic CMB@SiO₂ and nSiO₂

462 do not aggregate in plant cell growth medium and presented similar size and shape that ‘as
463 produced’ (Fig. 5b, c). It should be mentioned that due to the resolution limit of the TEM available
464 for this work, it is not possible to see the nanosized metal cluster inside the CMB@SiO₂ silica
465 nanoparticles. For this particular point, the reader should see Grasset et al. (2008). In contrast, as
466 expected from our previous work, 1 nm CMB clusters aggregate forming structures with “spheric-
467 like” shapes that further agglomerates into ramified structures of different shapes and sizes (Fig.
468 5a). Actually, even if cluster units are nanosized entities, they are hydrolyzed in presence of water
469 and co-precipitate with water molecules to form the crystalline compound
470 [(Mo₆Brⁱ₈)(OH)^a₄(H₂O)^a₂]•12H₂O. However, it is worth to be noted that the sizes of aggregates (up
471 to one hundred nanometers) in present work conditions were smaller than in previous ones (from
472 several hundred nanometers to few micrometers), and that shapes are also different to the disc-like
473 aggregates previously observed. The TEM grid preparation could be one reason to explain this
474 difference.

475 The uptake and bioaccumulation of nanoparticles by plants is crucial in many respects, such as
476 environmental issues, food-chain transfer, biotechnological applications and interaction with cell
477 organelles or toxicity. There have been only a few studies examining silica nanoparticle uptake by
478 plants. These studies reported different degrees of nanoparticle root uptake and internalization onto
479 plant cells, and more rarely their upward translocation into shoots (Le et al. 2014; Nair et al. 2011;
480 Slomberg and Schoenfisch 2012; Torney et al. 2007; Vivero-Escoto et al. 2012). In our system, we
481 observed that both silica nanoparticles intimately interact with the cell wall, and seem to be
482 internalized into *Arabidopsis* cells by endocytosis since they were mainly found encapsulated in
483 vesicles (Fig. 5e, f). Indeed, recent studies have shown that plant cells are able to accomplish
484 endocytosis for the internalization of molecules from the extracellular environment in a process
485 resembling mammalian cell endocytosis (Fan et al. 2015). In contrast, CMB clusters were observed
486 forming aggregates inside cell walls and vacuoles, but not inside vesicles, suggesting that clusters

487 mainly penetrate by passive diffusion as nanosized entities. We already described this situation in
488 root cells of *A. thaliana* seedlings treated with CMB (Aubert et al. 2012).

489 A final consideration concerns the potential participation of ion release in the toxicity of the
490 evaluated nanomaterials. The observed perturbation of cell growth and metabolism in response to
491 Mo-based clusters cannot be ascribed to the eventual release of metal ions as we already showed
492 that only the apical Br ligands (6 atoms) and the Cs counter cations (2 atoms) were liberated from
493 clusters in culture medium, but no Mo was released in the solution as ionic species. At the highest
494 concentration of CMB clusters ($60 \text{ mg L}^{-1} = 0.0306 \text{ mM}$) used in the present study, levels of Cs^+
495 and Br^- ions liberated would be 0.0612 and 0.1836 mM respectively, which is far down toxic
496 concentrations for these ions (Aubert et al. 2012).

497

498

499 **Conclusion**

500

501 ACSC showed to be an appropriate screening system to assess plant biological responses to
502 nanomaterials, allowing proper interactions of the biological system with the evaluated
503 nanomaterials. We showed in this study that Mo_6 -based clusters, even at low doses, present a
504 significant toxicity for plant cells, negatively affecting growth, viability and photosynthesis, and
505 increasing oxidative impact, which provoked stimulation of antioxidant enzymatic activities. Based
506 on the results presented here, it is also concluded that the encapsulation of the clusters into silica,
507 which showed to be biologically compatible in our conditions, protected the plant cells by avoiding
508 direct contact of harmful clusters with cellular structures and the generation of oxidative stress.
509 Thus, deleterious impacts were not observed after CMB@SiO_2 nanoparticle exposure, and nSiO_2
510 nanoparticles neither showed cytotoxic effects, despite intimate contact with cells and their
511 internalization.

512

513

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515

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647 FIGURE CAPTIONS

648

649 **Fig. 1** *A. thaliana* cell biomass concentration (g fresh weight mL⁻¹) (**a–c**) and cell viability (**d–f**). ACSC
650 were treated with CMB@SiO₂ (**a, d**), nSiO₂ (**b, e**) and CMB clusters (**c, f**) at different concentrations for 3,
651 24 and 72 hours. Relative cell viability is expressed as percentage related to control at each time point. *
652 Significant differences between nanomaterial treatment and control ($p < 0.05$)

653

654 **Fig. 2** ACSC chlorophyll (**a–c**) and carotenoid (**d–f**) contents ($\mu\text{g g}^{-1}$ dry weight), and PSII maximum
655 quantum yield (F_v/F_m) (**g–i**). ACSC were treated with CMB@SiO₂ (**a, d, g**), nSiO₂ (**b, e, h**) and CMB
656 clusters (**c, f, i**) at different concentrations for 3, 24 and 72 hours. * Significant differences between
657 nanomaterial treatment and control ($p < 0.05$)

658

659 **Fig. 3** Level of lipid peroxidation in *A. thaliana* cells. ACSC were treated with CMB@SiO₂ (**a**), nSiO₂ (**b**)
660 and CMB clusters (**c**) at different concentrations for 3, 24 and 72 hours. TBARS content is expressed as
661 percentage related to control at each time point. * Significant differences between nanomaterial treatment
662 and control ($p < 0.05$)

663

664 **Fig. 4** Antioxidant enzymatic activities in *A. thaliana* cells. ACSC were treated with CMB@SiO₂ (**a, d, g, j,**
665 **M**), nSiO₂ (**b, e, h, k, n**) and CMB clusters (**c, f, i, l, o**) at different concentrations for 3, 24 and 72 hours.
666 Different letters above bars indicate statistical significance ($p < 0.05$) at each time point

667

668 **Fig. 5** TEM images of *A. thaliana* cells in culture medium after 72 hours of treatment with 60 mg L⁻¹ CMB
669 clusters (**a, d**), 100 mg L⁻¹ CMB@SiO₂ (**b, e**), and 100 mg L⁻¹ nSiO₂ (**c, f**). The cell wall (CW), and the
670 nanomaterials outside (black arrows) and inside cells, cell wall or vesicles (white arrows) are shown

Figure 1

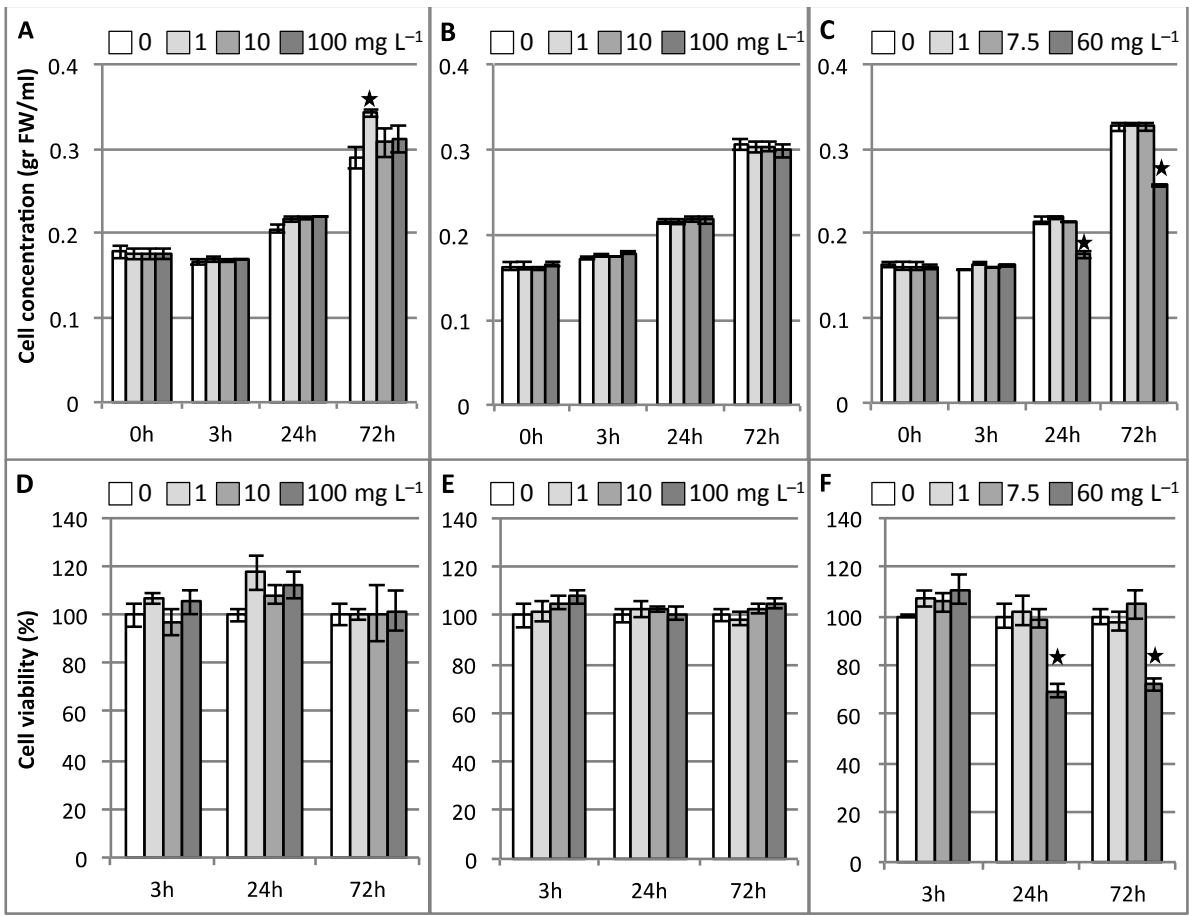


Fig. 1

Figure 2

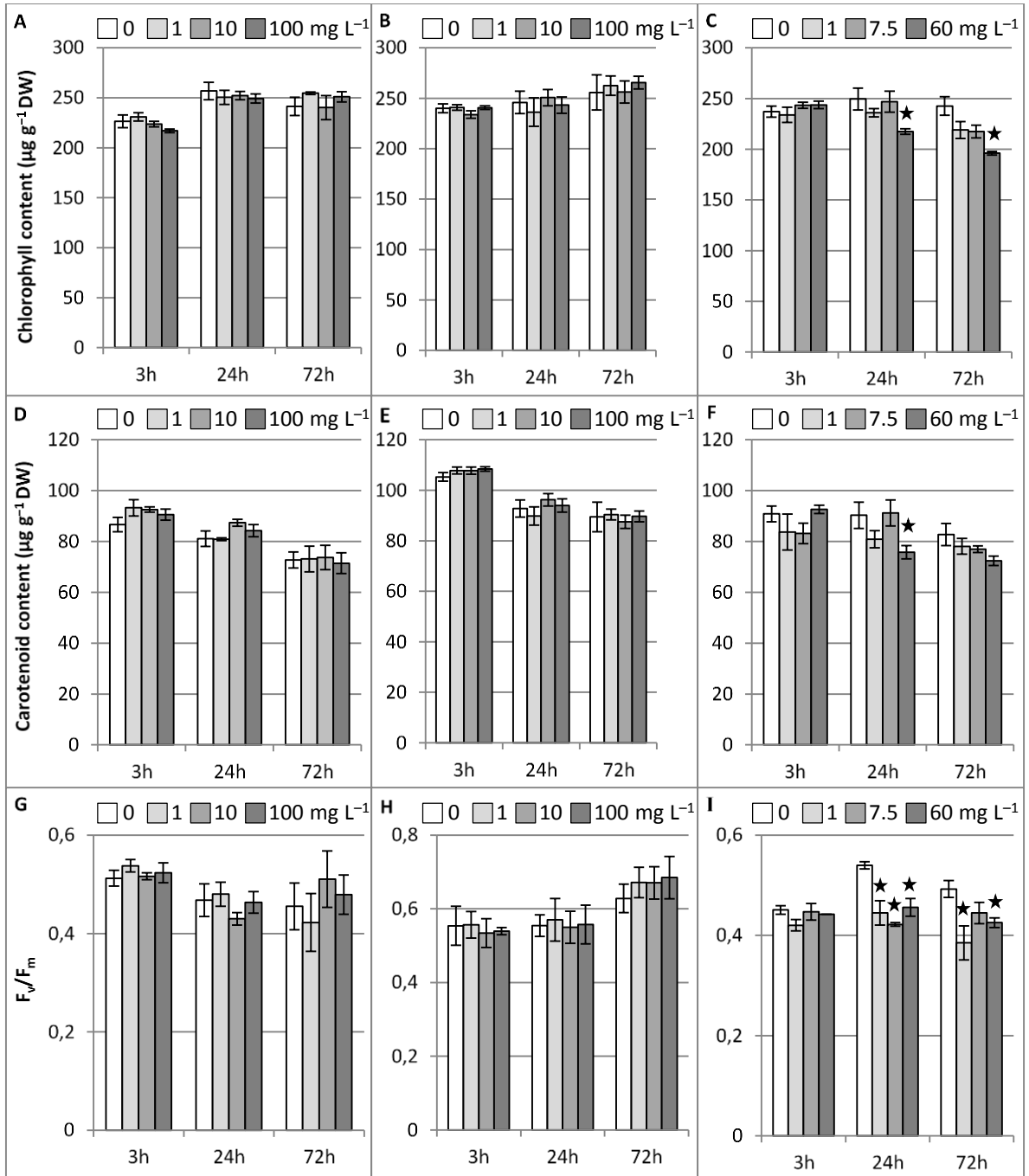


Figure 3

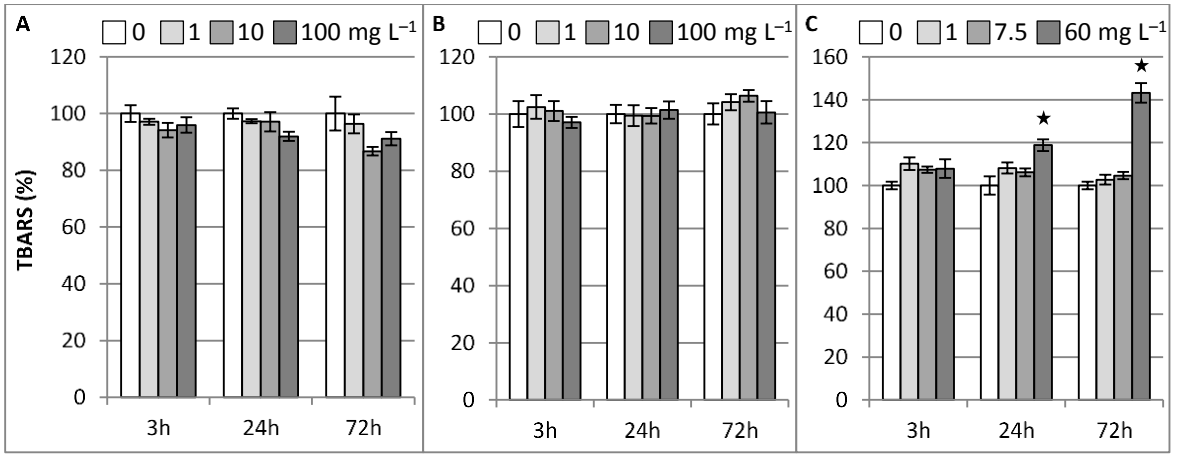


Figure 4

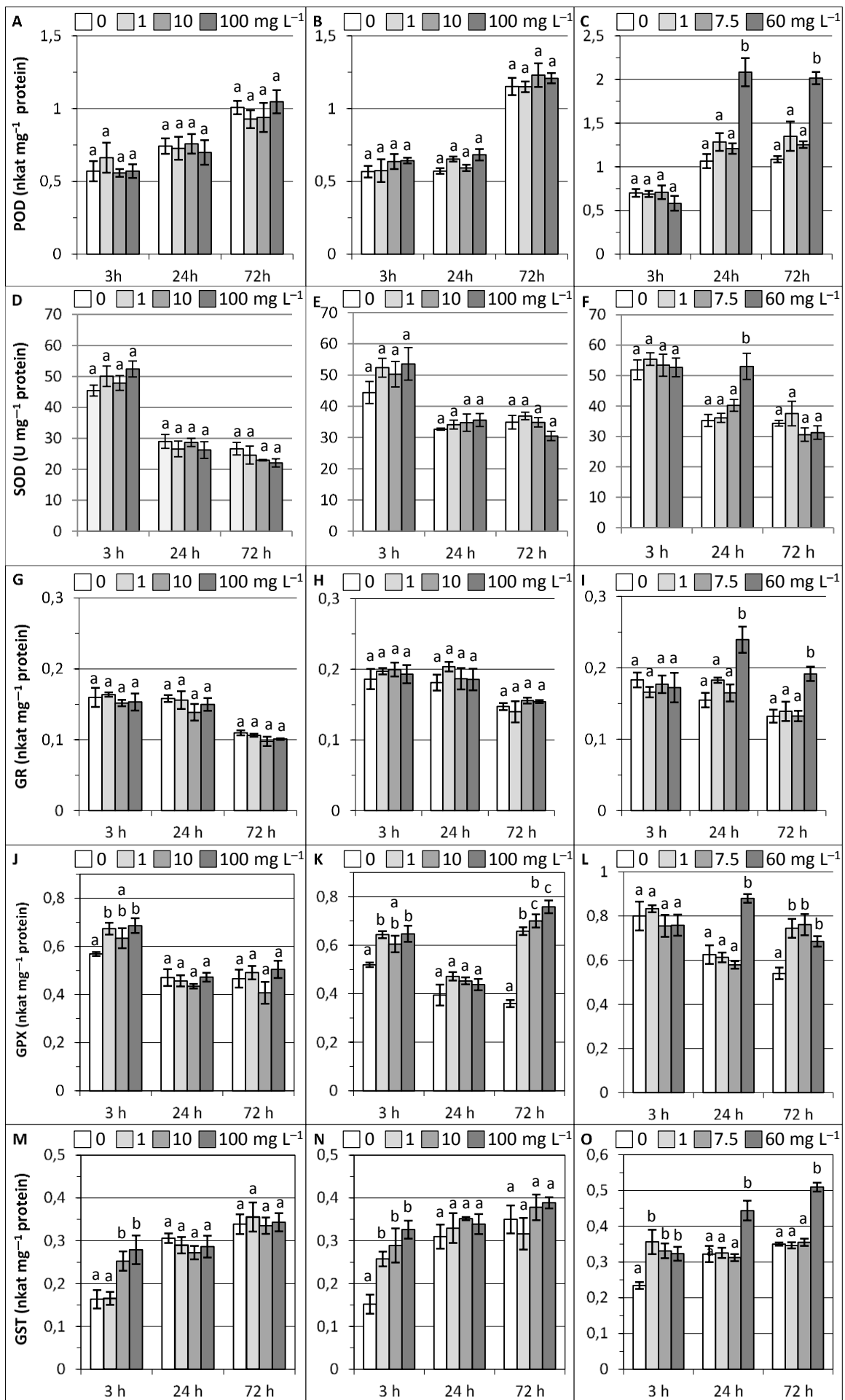


Figure 5

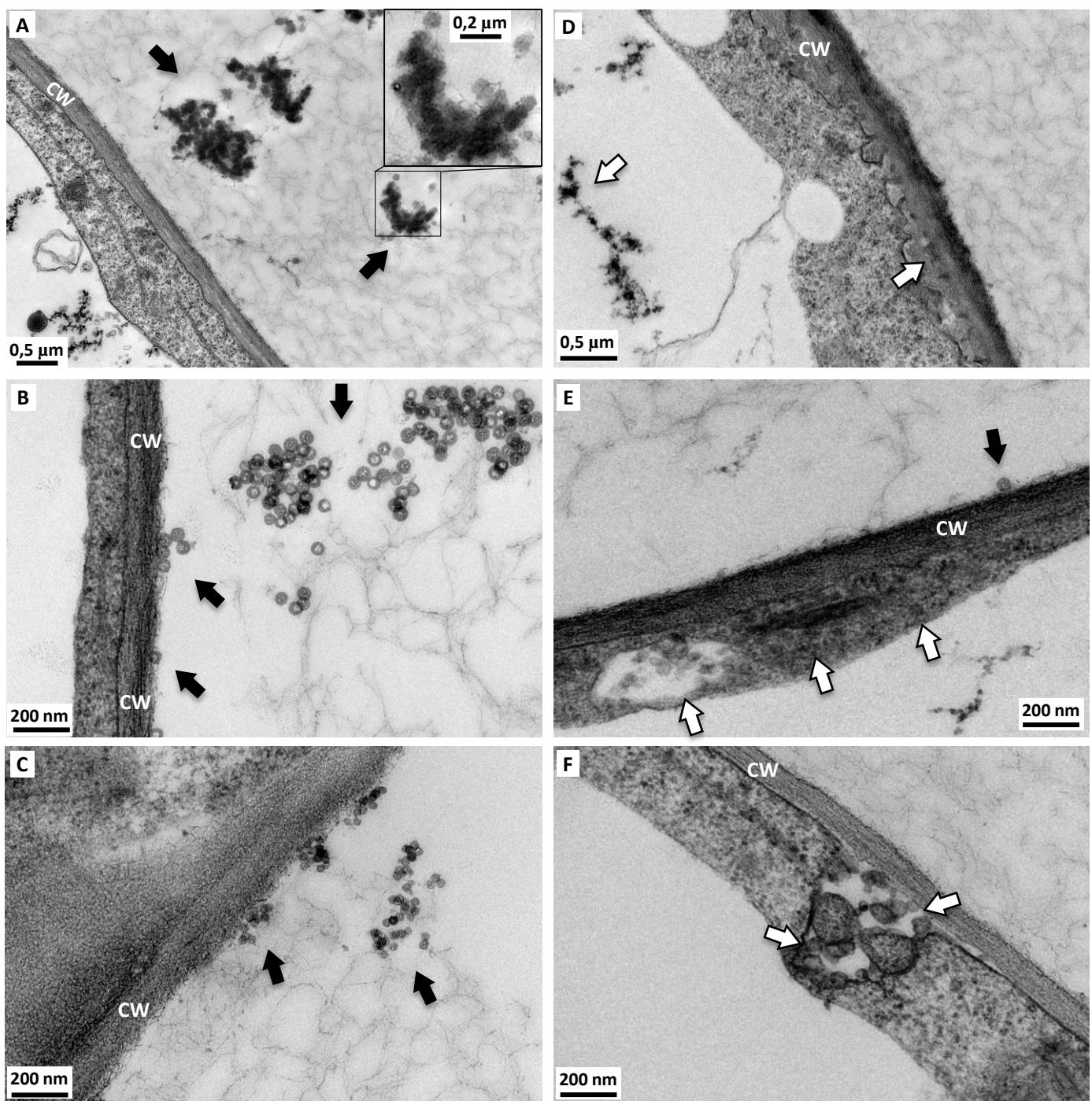


Fig. 5