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Nitric Oxide Biosynthesis in White Poplar (*Populus alba* L.) Suspension Cultures Challenged with Heavy Metals

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ABSTRACT

The present work reports on the generation of nitric oxide (NO) in white poplar (*Populus alba* L., cv. 'Villafranca') cell suspension cultures exposed to copper (150 µM $CuCl_2$), zinc (2 mM $ZnSO_4$) and cadmium (200 µM $CdSO_4$). Since it is currently believed that at least two distinct enzymatic pathways are responsible for NO production in plants, the response of 'Villafranca' cells to heavy metals was monitored using specific inhibitors of the nitrate-dependent pathway and a mammalian inhibitor of the L-arginine-dependent pathway. Production of nitrite (NO_2^-), as a measure of NO released in the culture medium, was quantified using the Griess reaction. Copper treatment resulted into a 3.2-fold enhancement of NO production in white poplar cell cultures. A lower increase (2-fold) was observed with the cadmium treatment. In contrast, NO production did not change in the zinc-treated cells. The use of 100 µM sodium azide and 200 µM sodium tungstate resulted into complete inhibition of NO production while in cells exposed to 500 µM N^G-monomethyl-L-arginine the rate of NO generation was only partially affected. The white poplar cultures exposed to heavy metals showed the morphological hallmarks of both Programmed Cell Death and necrosis, as evidenced by Evans Blue staining. The nuclear morphology was also investigated.

Keywords: inhibitor, necrosis, programmed cell death, oxidative stress
Abbreviations: **L-NMMA**, N^G-monomethyl-L-arginine; **NO**, nitric oxide; **NOS**, nitric oxide synthase; **NR**, nitrate reductase; **PCD**, programmed cell death

INTRODUCTION

According to the recent literature, nitric oxide (NO) production in plants follows different routes, involving the cytosolic nitrate reductase (NR) (Yamasaki and Sakihama 2000), a root-specific plasma membrane nitrite-NO reductase (Ni-NOR) (Stohr *et al.* 2001) and a nitric oxide synthase (NOS)-like enzyme whose identity is still controversial (Neill *et al.* 2003; Besson-Bard *et al.* 2008). It has been reported that NR, a key enzyme of nitrate assimilation in higher plants (Pattanayak and Chatterjee 1989), might be responsible for the basal level of NO production in leaves and roots of several plants (Rockel *et al.* 2002; Vanin *et al.* 2004) while there is evidence that the NR-derived NO acts also in signal transduction processes (Bright *et al.* 2006).

Notwithstanding the efforts documented by the increasing number of studies, the biosynthetic origin of NO in plants has not been completely clarified (Besson-Bard *et al.* 2008). As an antioxidant, NO is involved in the response to heavy metals (Laspina *et al.* 2005; Yu *et al.* 2005; Tewari *et al.* 2007). The ability to withstand heavy-metal induced oxidative stress has been intensively investigated in poplar trees, considered useful tools in phytoremediation projects (Peuke and Rennenberg 2006). However, since several aspects of the molecular mechanisms involved in heavy-metal tolerance are still unexplored, the availability of a plant model system facilitating such a study is desirable.

Cell cultures, represented by rapidly dividing and relatively homogeneous populations, seem to address this requirement (McCabe and Leaver 2000). Cell suspension cultures have been obtained from internodal explants of the white poplar (*Populus alba* L.) cv. 'Villafranca' and tested in previous studies (Zelasco *et al.* 2006; Balestrazzi *et al.* 2008a). 'Villafranca' has been engineered with relevant agronomic traits such as herbicide tolerance (Confalonieri *et al.* 2000), disease and insect pest resistance (Giorcelli *et al.* 2004; Balestrazzi *et al.* 2006), tested with MAT (Multi-Auto-Transformation) vectors for marker-free gene-transfer (Zelasco *et al.* 2007), used in investigation related to the biosafety of GM trees (Balestrazzi *et al.* 2007, 2008b) and for phytoremediation purposes (Castiglione *et al.* 2007; Lingua *et al.* 2007). In the present work, NO production in 'Villafranca' cell suspension cultures, challenged with heavy metals, was monitored. The inhibitory effect of sodium azide, sodium tungstate and N^G-monomethyl-L-arginine (L-NMMA) was tested in order to investigate the possible involvement of the two most relevant biosynthetic pathways and quantify the relative proportion of NR- and NOS-derived NO.

MATERIALS AND METHODS

Cell cultures and treatments

The white poplar (*Populus alba* L.) cv. 'Villafranca' used in this study was kindly supplied by Dr. Stefano Bisoffi (C.R.A. – Research Unit for Wood Production outside Forest, Casale Monferrato, Alessandria, Italy). Cell suspension cultures of 'Villafranca' were obtained and maintained *in vitro* as previously described (Zelasco *et al.* 2006). Exponentially growing (4-day old) cell suspension cultures were exposed to 150 µM $CuCl_2$ ($CuCl_2 \cdot 2H_2O$, reagent grade 97%; Sigma-Aldrich S.r.l., Milan, Italy), 2 mM $ZnSO_4$ ($ZnSO_4 \cdot 7H_2O$ ACS reagent, 99%; Sigma-Aldrich S.r.l., Milan, Italy) and 200 µM $CdSO_4$ ($CdSO_4$ ACS reagent, ≥ 99%; Sigma-Aldrich S.r.l., Milan, Italy) and subsequently monitored at the indicated times (0, 15, 30 and 45 min, 1, 2, 4 and 6 h). Sodium azide (NaN_3, SigmaUltra; Sigma-Aldrich S.r.l., Milan, Italy), sodium tungstate ($Na_2WO_4 \cdot 2H_2O$, ACS reagent, 99%; Sigma-Aldrich S.r.l., Milan, Italy) and L-NMMA, Fluka BioChemika, ≥ 95%; Sigma-Aldrich S.r.l., Milan, Italy) were added to cell cultures (final concentration: 100 µM NaN_3; 50, 100 and 200 µM $Na_2WO_4 \cdot 2H_2O$; 500 µM L-NMMA) 24 h before the heavy-metal

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treatment. Three independent experiments were performed and three replicated samples were used for each treatment combination.

Determination of extracellular NO content

Aliquots (1 mL) of cell suspension culture were collected, centrifuged and 0.5 mL of the culture medium were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid) (Sigma-Aldrich) (Green *et al.* 1982). Samples were incubated at room temperature for 15 min and nitrite was measured by spectrophotometric analysis at 540 nm, using a V-530 spectrophotometer (Jasco Europe S.r.l., Cremella, Italy). NO content was calculated by comparison to a standard curve of $NaNO_2$. Standard solutions of $NaNO_2$ were prepared in cell culture medium.

Cell viability

Cell viability was evaluated by Evans Blue assay as described by Carimi *et al.* (2003). Cells were collected by centrifugation and incubated in 0.25% Evans Blue (dye content ≥ 75%, Sigma-Aldrich S.r.l., Milan, Italy) for 10 min and then washed extensively with distilled water to remove excess dye. The dye bound to dead cells was solubilized with elution buffer (1% SDS, 50% methanol) for 30 min at 50°C and subsequently quantified by measuring the absorbance at 600 nm. The percentage cell death of a sample was determined based on the absorbance of intact healthy cells and that of dead cells obtained by heat shock treatment (65°C, 10 min), as reported by Carimi *et al*. (2003). Whole cell morphology was analyzed on Evans Blue stained cells just after treatment, while nuclear appearance was visualized after DAPI staining (BioChemika, ≥ 95%, Sigma-Aldrich, S.r.l., Milan, Italy) (Callard *et al.* 1996). The screenings for cell death and nuclear morphologies were carried out under a ZEISS Axioplan fluorescence microscope equipped with a CCD (Computer Coupled Device) camera (Photometrics). Images were acquired, pseudocoloured and merged using IPLab software (Digital Pixel Advanced Imaging System, Brighton). Three independent experiments were carried out and 500 cells were scored for each treatment combination.

Statistical analyses

Experiments were repeated three times. For each treatment combination, three independent replications were tested. Data were subjected to Analysis of Variance (ANOVA) and statistical significance of mean differences was determined using Student's *t*-test (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

RESULTS

Heavy metal-induced NO generation in white poplar suspension cultures

NO production in response to heavy-metal treatment was investigated in exponentially growing cells, monitored at different times (0, 15, 30 and 45 min, 1, 2, 4 and 6 h), following the heavy-metal treatment. The response to heavy metal was also analysed in the presence of 500 µM L-NMMA and 100 µM NaN_3, respectively, and using both compounds. Cells were exposed to inhibitors 24 h before starting the heavy-metal treatment.

NO production in untreated cells did not change over the tested period, even in the presence of inhibitors (**Fig. 1A**). When 150 µM $CuCl_2$ was added to white poplar cell suspensions, a 3.2-fold increase (from 6.70 ± 0.04 µmol L^{-1}, recorded at time 0, up to 22.03 ± 0.40 µmol L^{-1}) was observed 30 min after the treatment (**Fig. 1B**, 150 µM $CuCl_2$). Then the rate of NO production decreased at 2 h and it further lowered to 4.86 ± 0.15 µmol L^{-1} at the end of the experiment (**Fig. 1B**, 150 µM $CuCl_2$, 6 h). NO production was significantly ($p<0.001$) affected when copper (Cu) was added to cells treated with 500 µM L-NMMA. After 30 min, the recorded value was 11.74 ± 0.13 µmol L^{-1}, approximately 50% less compared to the value observed in cells exposed only to Cu (**Fig. 1B**, 150 µM $CuCl_2$ + 500 µM L-NMMA). Finally, when the Cu treatment was carried out in the presence of sodium azide or using both inhibitors, the amount of NO released in the culture medium significantly dropped ($p<0.001$) (**Fig. 1B**, 150 µM $CuCl_2$ + 100 µM NaN_3 and 150 µM $CuCl_2$ + 100 µM NaN_3 + 500 µM L-NMMA, respectively).

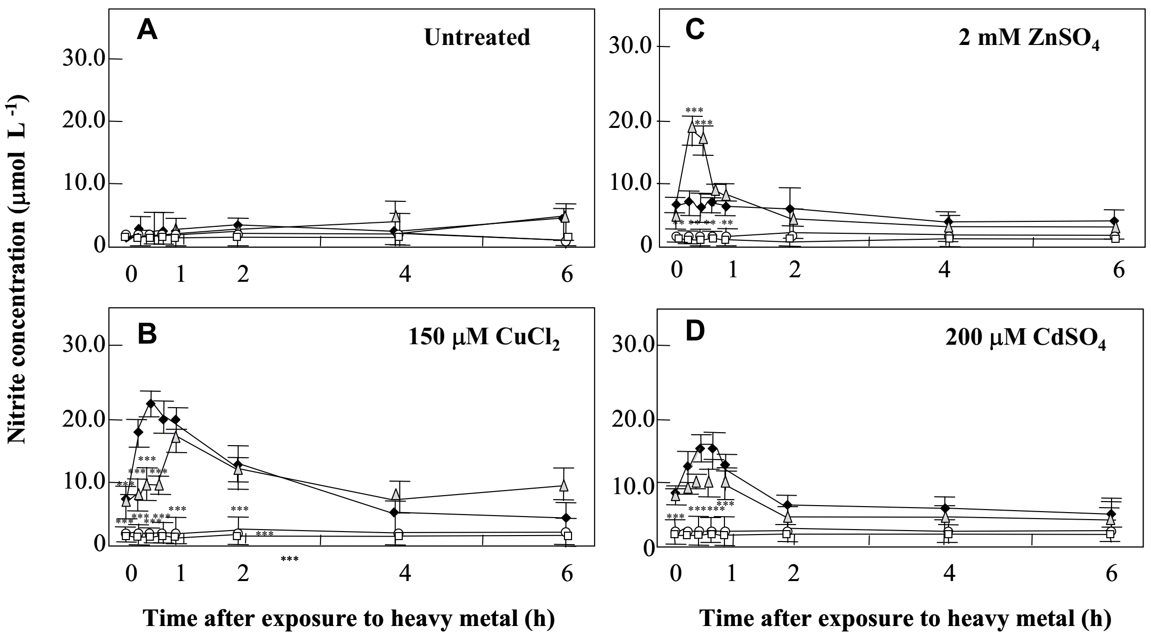

Fig. 1 NO generation in white poplar 4-day old cell suspension cultures challenged with heavy metals and inhibitors. (**A**) Untreated culture. (♦) NT, non treated cells. (□) 100 µM NaN_3. (▲) 500 µM L-NMMA. (○) 100 µM NaN_3 + 500 µM L-NMMA. (**B**) Cu treatment. (♦) 150 µM $CuCl_2$. (□) 150 µM $CuCl_2$ + 100 µM NaN_3. (▲) 150 µM $CuCl_2$ + 500 µM L-NMMA. (○) 150 µM $CuCl_2$ + 100 µM NaN_3 + 500 µM L-NMMA. (**C**) Zn treatment. (♦) 2 mM $ZnSO_4$. (□) 2 mM $ZnSO_4$ + 100 µM NaN_3. (▲) 2 mM $ZnSO_4$ + 500 µM L-NMMA. (○) 2 mM $ZnSO_4$ + 100 µM NaN_3 + 500 µM L-NMMA. (**D**) Cd treatment. (♦) 200 µM $CdSO_4$. (□) 200 µM $CdSO_4$ + 100 µM NaN_3. (▲) 200 mM $CdSO_4$ + 500 µM L-NMMA. (○) 200 µM $CdSO_4$ + 100 µM NaN_3 + 500 µM L-NMMA. Values are expressed as means ± SD of three independent experiments. Statistical significance was determined (** $p<0.01$; *** $p<0.001$ compared to heavy-metal treated cells not exposed to inhibitors).

Subsequently, the response to zinc (Zn) was also examined. NO production in the untreated cells, with or without inhibitors, did not vary (**Fig. 1A**). The response to Zn treatment was different, since apparently no enhancement in NO production was observed during the tested time, compared to the untreated control (**Fig. 1C**, 2 mM $ZnSO_4$). In contrast, the exposure to both Zn and the NOS inhibitor caused a significant increase (p<0.001) in the amount of NO released into the culture medium (**Fig. 1C**, 2 mM $ZnSO_4$ + 500 µM L-NMMA). The highest values (18.02 ± 0.10 and 17.46 ± 0.20 µmol L^{-1}, respectively) were recorded 15 and 30 min following treatment. As already shown in the case of Cu, a significant drop (p<0.001) in NO production was observed in cells exposed to sodium azide (**Fig. 1C**, 2 mM $ZnSO_4$ + 100 µM $NaNa_3$) and to both inhibitors (**Fig. 1C**, 2 mM $ZnSO_4$ + 100 µM $NaNa_3$ + 500 µM L-NMMA).

Finally, the white poplar cell suspension cultures were exposed to cadmium (Cd). As previously reported, NO production in the untreated cells did not change over the tested period, even in the presence of inhibitors (**Fig. 1A**). When 200 µM $CdSO_4$ was added, at 30 min the amount of NO increased up to 15.54 ± 0.15 µmol L^{-1} (**Fig. 1D**, 200 µM $CdSO_4$) while the values detected in cells exposed to 500 µM L-NMMA were significantly (p<0.001) reduced by 35% (**Fig. 1D**, 200 µM $CdSO_4$ + 500 µM L-NMMA). Sodium azide blocked NO synthesis also in Cd-treated cells (**Fig. 1D**, 200 µM $CdSO_4$ + 100 µM NaN_3) and also when both inhibitors were used, NO production dropped to almost zero.

Cell viability in heavy-metal treated white poplar suspension cultures

In the absence of heavy metal the percentage of dead cells did not significantly change after 24 h of exposure to the inhibitors sodium azide and L-NMMA, compared to untreated cells (**Fig. 2A**, 1, 2, 3 and 4). Thus, no residual toxic effects, related to both types of inhibitors were present in the heavy-metal treated cells. In contrast, the rate of cell death reached 82.00 ± 0.15% after 24 h of incubation with 150 µM $CuCl_2$ (**Fig. 2B**, 1). Significant differences in the rate of cell death were recorded for the samples containing 150 µM $CuCl_2$ + 500 µM L-NMMA (p<0.001), 150 µM $CuCl_2$ + 100 µM NaN_3 (p<0.01), and with both inhibitors (p<0.001) (**Fig. 2B**, 2, 3 and 4). The rate of cell death was approximately 60% after 24 h of exposure to 2 mM $ZnSO_4$ (**Fig. 2C**, 1). A significant reduction in the percentage of dead cells was observed in presence of 2 mM $ZnSO_4$ + 500 µM L-NMMA (p<0.001), 2 mM $ZnSO_4$ + 100 µM NaN_3 (p<0.01) and when both inhibitors were tested (p<0.001) (**Fig. 2C**, 2, 3 and 4).

Mortality was 67.00 ± 10.31% in samples exposed to 200 µM $CdSO_4$ (**Fig. 2D**, 1). The values significantly (p<0.01) increased in presence of 200 µM $CdSO_4$ + 100 µM NaN_3 (**Fig. 2D**, 2). Finally, the treatments with 200 µM $CdSO_4$ + 500 µM L-NMMA, and by using both inhibitors, resulted into lower values, respectively (**Fig. 2D**, 3 and 4).

Morphology of dead cells and chromatin condensation

The untreated healthy cells, which were not stained by Evans Blue, showed an intact protoplast with dense cytosol (**Fig. 3A**). Twenty four h following treatment with 150 µM $CuCl_2$, two different cell death morphologies were evidenced by Evans Blue staining. A subpopulation was detected which included cells with evident protoplast shrinkage (**Fig. 3B**) while other cells did not show retraction of the protoplast from the cell wall and displayed a uniformly blue cytosol (**Fig. 3C**). The latter is considered a hallmark of necrosis. Similar morphologies were observed in cells treated with 200 µM $CdSO_4$ (data not shown). Exposure to Zn resulted into highly vacuolated cells (**Fig. 3E**) which could be easily distinguished from the healthy cells (**Fig. 3D**). Protoplast shrinkage was also detected in the Zn-treated white poplar cultures (**Fig. 3F**).

The frequencies of PCD and necrosis morphology respectively are reported in **Table 1**. The coefficient of variation (CV) of cell counts was ≤ 19.04%. In white poplar cells exposed to 150 µM $CuCl_2$, the cells without protoplast shrinkage and uniform staining of cytosol corresponded to 25.30 ± 1.00% of the total Evans Blue positive population while the percentage of cells showing protoplast shrinkage was significantly higher (p<0.001) (75.30 ± 0.00). In the Zn-treated cells stained by Evans Blue, PCD morphology was observed in 90.00 ± 0.00% of the cells while the necrotic event was recorded in 10.00 ± 0.00% of the population.

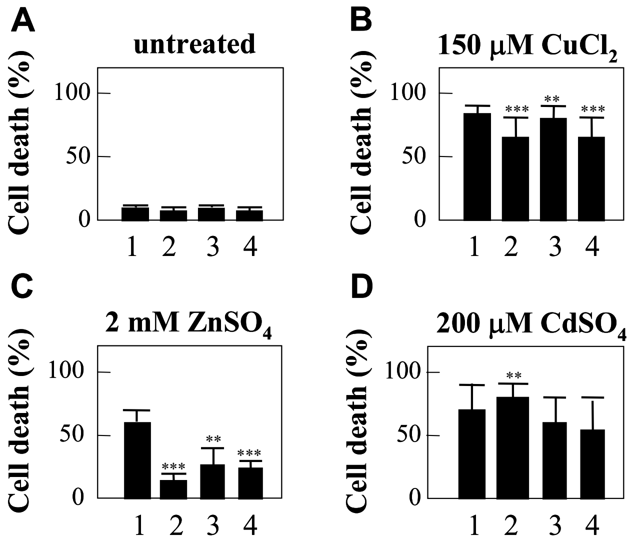

Fig. 2 Cell death quantified by Evans Blue staining and spectrophotometric assay. (**A**) Untreated culture. 1, NT, non treated cells. 2, 100 µM NaN_3. 3, 500 µM L-NMMA. 4, 100 µM NaN_3 + 500 µM L-NMMA. (**B**) Cu treatment. 1, 150 µM $CuCl_2$. 2, 150 µM $CuCl_2$ + 100 µM NaN_3. 3, 150 µM $CuCl_2$ + 500 µM L-NMMA. 4, 150 µM $CuCl_2$ + 100 µM NaN_3 + 500 µM L-NMMA. (**C**) Zn treatment. 1, 2 mM $ZnSO_4$. 2, 2 mM $ZnSO_4$ + 100 µM NaN_3. 3, 2 mM $ZnSO_4$ + 500 µM L-NMMA. 4, 2 mM $ZnSO_4$ + 100 µM NaN_3 + 500 µM L-NMMA. (**D**) Cd treatment. 1, 200 µM $CdSO_4$. 2, 200 µM $CdSO_4$ + 100 µM NaN_3. 3, 200 µM $CdSO_4$ + 500 µM L-NMMA. 4, 200 µM $CdSO_4$ + 100 µM NaN_3 + 500 µM L-NMMA. Values are expressed as means ± SD of three independent experiments. Statistical significance was determined (** p<0.01; *** p<0.001 compared to heavy-metal treated cells not exposed to inhibitors).

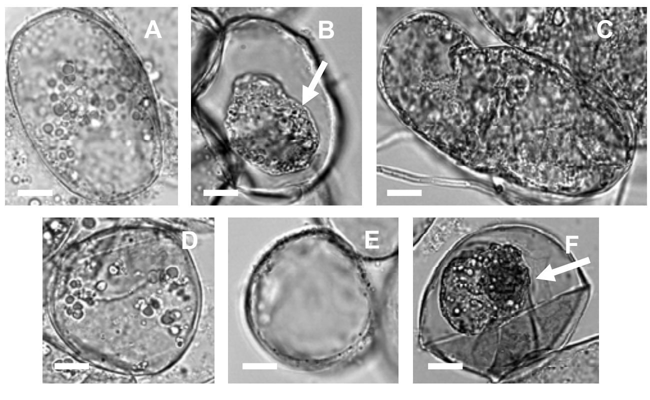

Fig. 3 Dead cell morphology after Evans Blue staining. (**A**) Untreated healthy cell. (**B**) Cell death in response to copper (150 µM $CuCl_2$), 24 h following treatment. Protoplast shrinkage, a typical PCD hallmark evidenced by Evans Blue staining, is indicated by arrow. (**C**) A necrotic event characterized by lack of protoplast shrinkage and evident uniform cytosol staining is shown. (**D**) Nuclear morphology in cells treated with heat shock. (**D, E**) An untreated healthy cell and a highly vacuolated cell (visible 24 h following treatment with 2 mM $ZnSO_4$) are shown. (**F**) Cell death in response to zinc. Protoplast shrinkage, a typical PCD morphological hallmark evidenced by Evans Blue staining, is indicated by arrow. Bar, 20 µm.

Table 1 Frequency of cell death morphologies which are typical hallmarks of PCD and necrosis, respectively, in white poplar suspension cultures exposed to heavy-metal treatment. At 24 h following treatments, the cell population positive to Evans Blue staining underwent the microscopy-based screening in order to detect the subpopulations with PCD and necrosis morphologies. Values are expressed as means ± SD of three independent experiments. Statistical significance was determined (*** p<0.001 compared to untreated cells).

Treatment	PCD [a]	Necrosis [b]
NT	9.45 ± 1.80	2.75 ± 0.50
150 µM $CuCl_2$	75.30 ± 0.00***	25.30 ± 1.00***
2 mM $ZnSO_4$	90.00 ± 0.00***	10.00 ± 0.00***
200 µM $CdSO_4$	72.00 ± 0.00***	28.00 ± 0.00***
Heat shock[c]	12.00 ± 0.00 n.s.	82.00 ± 0.00***

[a] Presence of protoplast shrinkage
[b] absence of protoplast shrinkage and presence of cytosol with uniform Evans Blue staining
[c] 65°C, 10 min
n.s., non significant

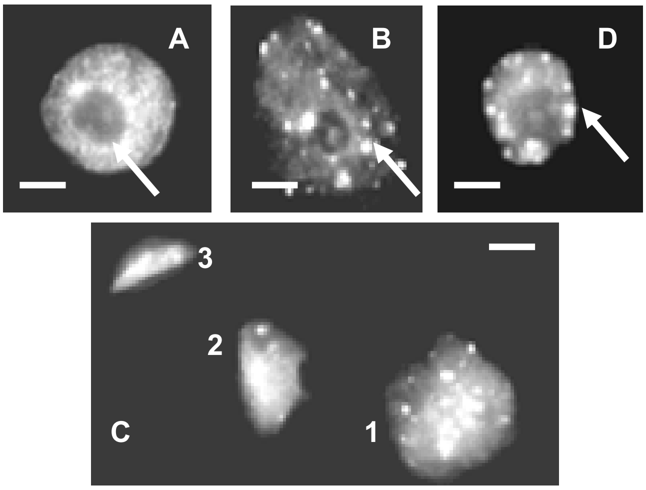

Fig. 4 Nuclear morphology evaluated by DAPI staining. (**A**) Nucleus of a healthy cell with homogeneous chromatin and a large nucleolus (arrow). (**B**) Nuclear morphology in cells exposed to 150 µM $CuCl_2$. Chromatin condensation resulted into irregular granuli (arrow) dispersed within the nuclear compartment. (**C**) Nuclear morphology in cells exposed to 2 mM $ZnSO_4$. Chromatin condensation is visible (1). Collapsed nuclei in highly vacuolated cells are also shown (2, 3). Bar, 5 µm.

All the reported values were still significantly different (p<0.001) from those observed with the untreated cultures. As for Cd treatment (200 µM $CdSO_4$), values similar to those reported for Cu (72.00 and 28.00% for the PCD and necrosis morphology, respectively) were observed. As expected, when the white poplar cultures underwent thermal treatment (heat shock, 65°C, 10 min), necrosis (82.00%) (p<0.001) was the predominant event (**Table 1**).

The nuclear morphology of white poplar cells exposed to heavy metals was evaluated by DAPI staining. The nucleus of untreated cells was uniformly stained and a large central nucleolus was clearly evident (**Fig. 4A**). In cells treated with 150 µM $CuCl_2$ the predominant morphology was characterized by chromatin condensation in the form of granuli randomly distributed within the nuclear compartment which displayed also an irregular shape (**Fig. 4B**). In the Zn-treated white poplar cultures an altered nuclear morphology was also detected (**Fig. 4C**, 1) while in the highly vacuolated cells the nuclei appeared as collapsed on the cell wall (**Fig. 4C**, 2 and 3). Finally, when the nuclear morphology of necrotic cells resulting from heat shock was examined, the absence of the central nucleolus and chromatin condensation were evident. Interestingly, the chromatin granuli were predominantly located at the periphery of the nuclear compartment (**Fig. 4D**).

Effects of tungstenum on NO generation in white poplar suspension cultures

Sodium tungstate, a well known NR inhibitor, was used to investigate the involvement of this enzyme in NO production in heavy-metal treated and untreated cultures. In the absence of heavy metal, no significant fluctuactions in the amount of NO released in the culture medium were observed when increasing concentrations of $NaWO_4{\cdot}2H_2O$ were tested (**Fig. 5A**). Due to the ability of Cu in stimulating NO production, previously observed, the treatment with this specific heavy metal was chosen to assess the effects of sodium tungstate. NO production in response to Cu was significantly enhanced (24.21 ± 1.00 µmol L^{-1}) at 45 min (**Fig. 5B**; 150 µM $CuCl_2$) and subsequently decreased until the end of the experiment. When 50 and 100 µM $NaWO_4{\cdot}2H_2O$ were added, NO production was lowered of approximately 50% (p<0.001) and with the highest dose (200 µM) it was completely inhibited (p<0.001) (**Fig. 5B**; 150 µM $CuCl_2$ + 200 µM $NaWO_4{\cdot}2H_2O$). Exposure to Cu and molybdenum, an essential cofactor of NR, did not cause inhibition of NO production (data not shown). Cell viability was not affected by 50 µM $NaWO_4{\cdot}2H_2O$ (**Fig. 6A**, 2) while a significant increase (p<0.001) in cell death (up to 10.33 ± 0.57%) was evident with the 100 and 200 µM doses (**Fig. 6A**, 3 and 4). As expected, the Cu treatment induced significantly (p<0.01) high rates of cell death (**Fig. 6B**, 1) which were further enhanced (up to 88.66 ± 1.52%) with the highest $NaWO_4{\cdot}2H_2O$ concentrations (**Fig. 6B**, 3 and 4).

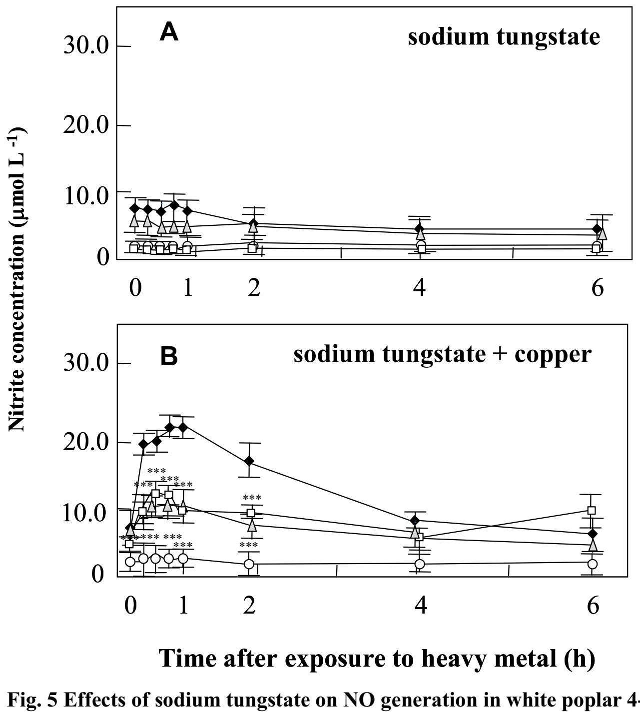

Fig. 5 Effects of sodium tungstate on NO generation in white poplar 4-day old cell suspension cultures. (**A**) Untreated culture. (♦) NT, non treated cells. (□) 50 µM $NaWO_4{\cdot}2H_2O$. (▲) 100 µM $NaWO_4.2H_2O$. (○) 200 µM $NaWO_4{\cdot}2H_2O$. (**B**) Cu treatment. (♦) 150 µM $CuCl_2$. (□) 150 µM $CuCl_2$ + 50 µM $NaWO_4{\cdot}2H_2O$. (▲) 150 µM $CuCl_2$ + 100 µM $NaWO_4{\cdot}2H_2O$. (○) 150 µM $CuCl_2$ + 200 µM $NaWO_4{\cdot}2H_2O$. Values are expressed as means ± SD of three independent experiments. Statistical significance was determined (*** p<0.001 compared to heavy-metal treated cells not exposed to sodium tungstate).

DISCUSSION

The reported data are in agreement with the current literature which, however, includes only *in planta* studies. In *Brassica* and *Pisum* plantlets treated with Cu, it has been demonstrated that a biphasic reaction takes place, characterized by a burst in NO production, occurring within two

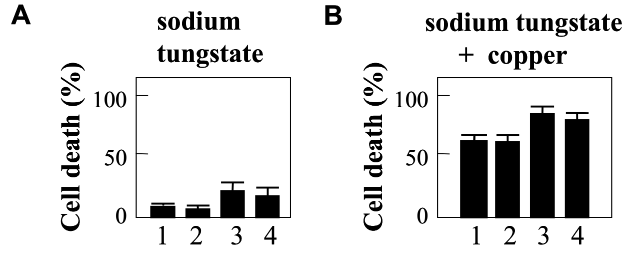

Fig. 6 Cell death induced by heavy-metal, in presence/absence of sodium tungstate, quantified by Evans Blue staining and spectrophotometric assay. (A) Sodium tungstate. 1, NT (non treated cells). 2, 50 μM $NaWO_4 \cdot 2H_2O$. 3, 100 μM $NaWO_4 \cdot 2H_2O$. 4, 200 μM $NaWO_4 \cdot 2H_2O$. **(B)** Sodium tungstate + copper. 1, 150 μM $CuCl_2$. 2, 150 μM $CuCl_2$ + 50 μM $NaWO_4 \cdot 2H_2O$. 3, 150 μM $CuCl_2$ + 100 μM $NaWO_4 \cdot 2H_2O$. 4, 150 μM $CuCl_2$ + 200 μM $NaWO_4.2H_2O$. Values are expressed as means ± SD of three independent experiments. Statistical significance was determined (** $p<0.01$; *** $p<0.001$ compared to heavy-metal treated cells not exposed to sodium tungstate).

hours, followed by a slow increase (Bartha *et al.* 2005). In the same plants, NO production in response to Cu was 7-8 times higher in the first three hours compared to Cd treatment which induced only a slow response. The time-dependent response observed in white poplar cells exposed to Cu, characterized by an early peak, resembled the temporal pattern of NO production previously described by Bartha *et al.* (2005). As reported by these authors, also in white poplar cultures the NO level subsequently decreased but it was still consistently high, compared to the untreated samples. In the white poplar cells the amount of NO detected 30 min following Cd treatment was approximately 30% lower than in the case of Cu. Interestingly, Majumder *et al.* (2008) demonstrated that in human cells Cd impaired NO production by affecting phosphorylation of endothelial NO synthase. Information concerning the specific effects of Cd on the enzymes belonging to the NO biosynthetic pathways are lacking in plants.

As for Zn, most of the studies currently available deal with Zn deficiency while knowledge concerning Zn toxicity in plants is still limited. This metal was demonstrated to improve the stress response in plant cells (Kawano *et al.* 2002) but other authors observed that excess Zn has toxic effects leading to oxidative stress (Fang and Kao 2000; Cuypers *et al.* 2001). In our system, the response to Zn treatment was different compared to the other heavy metals since apparently no enhancement in NO production was observed during the tested time.

There is general agreement that, in plants, the mammalian NOS inhibitors not always can efficiently block NO production. These compounds did not affect NO synthesis in *Arabidopsis* cell cultures exposed to bacteria (Clarke *et al.* 2000) while Carimi *et al.* (2005) showed that, in *Arabidopsis* cell suspension cultures exposed to cytokinin, NO synthesis was only partially dependent on a NOS enzyme. In addition, Wang *et al.* (2006) demonstrated that the ultrasound-induced NO production in *Taxus yunnanensis* cell cultures was not completely blocked by mammalian NOS inhibitors. The same authors suggested that the extremely high level of endogenous L-arginine might have been responsible for the partial effects exerted by the mammalian inhibitors.

The limited specificity of mammalian NOS inhibitors in plant cells has been also discussed in a recent review by Besson-Bard *et al.* (2008). As concerns forest tree species, *Populus euphratica* has been the subject of a recent investigation, which demonstrated the occurrence of NOS-dependent NO release in calluses in presence/absence of salt stress (Zhang *et al.* 2007).

The increase in NO generation observed in cells exposed to both Zn and L-NMMA and the fact that the rate of cell death was lowered in the Zn-treated cultures exposed to inhibitors remain difficult to explain. Francis *et al.* (1995) reported that Zn can act in a cell-cycle-specific manner, with a predominant effect in late G1 phase. The same authors demonstrated that in tobacco suspension cultures, toxic Zn concentrations cause the cell cycle block rather than cell death and, once the cells have overcome the late G1 check-points, they divide despite the excess heavy metal. In view of this, the protective effects on cell viability exerted by inhibitors in presence of toxic Zn concentrations deserve further investigation.

It has been reported that a critical mechanism of Zn homeostasis is sequestration in the vacuole and that, in root meristematic cells, Zn treatment strongly increases the vacuolar volume (Davies *et al.* 1990). This response was also evident in the Zn-treated white poplar cells.

The cell death morphology of white poplar cultures exposed to heavy metals and stained with Evans Blue were screened in order to assess the PCD/necrosis ratio (Reape *et al.* 2008), based on the presence/absence of protoplast shrinkage and uniform cytosol staining. The PCD morphology was predominant in all the treatments and this finding confirmed our previous work on Cd (Balestrazzi *et al.* 2008a). The relevance of the PCD/necrosis ratio has been recently highlighted by Reape *et al.* (2008) who demonstrated that this specific parameter might represent an useful indicator of the level of stress imposed to cells and that it can help assessing the optimal stress conditions required to induce high PCD rates.

In the present work the PCD/necrosis ratio, evaluated by means of morphological parameters, was significantly higher (9.0) in cultures treated with Zn than in samples exposed to Cu (2.97) and Cd (2.57), respectively. This suggests that other Cu and Cd doses might be tested to further enhance the PCD frequency.

When considering the possible source of NO production in white poplar cells challenged with heavy metals, the inhibitory action of sodium azide is outstanding, since no NO has been detected when this inhibitor was added to the suspension cultures. NR can be inhibited by sodium azide (Yamasaki and Sakihama 2000), although some authors believe that this compound can also inhibit other enzymes (Neill *et al.* 2002). In white poplar cells, the possible involvement of NR in NO biosynthesis is supported by the fact that sodium tungstate was also able to block NO generation. NR activity can be modulated by tungstate, a molybdenum analogue responsible for the synthesis of an inactive tungstoprotein (Notton and Hewitt 1971). In our experimental conditions, the inhibitory effect of 200 μM sodium tungstate on NO generation in cells exposed to Cu was clearly evidenced. In contrast, 100 μM sodium tungstate did not affect the NO-dependent taxol production in *Taxus brevifolia* cell cultures, thus indicating that there was no significant contribution from NR (Durzan 2005).

The experimental work carried out on cv. 'Villafranca' adds novel and useful information concerning the cellular responses to heavy metals in forest tree species, confirming the reliability of the white poplar suspension cultures as model system. The latter will be used in future to afford issues related to other toxic pollutants for which the field research is already started or foreseen in short times.

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