Journal of Biological Research-Thessaloniki **13**: 59 – 66, 2010
J. Biol. Res.-Thessalon. is available online at http://www.jbr.gr
Indexed in: WoS (Web of Science, ISI Thomson), SCOPUS, CAS (Chemical Abstracts Service) and DOAJ (Directory of Open Access Journals)

The cortical microtubules are a universal target of tungsten toxicity among land plant taxa

Ioannis-Dimosthenis S. ADAMAKIS*,
Emmanuel PANTERIS and Eleftherios P. ELEFTHERIOU

Department of Botany, *School of Biology*, *Aristotle University of Thessaloniki*, *54124*, *Thessaloniki*, *Greece*

Received: 9 September 2009 Accepted after revision: 25 November 2009

Despite the increasing concern about the potential environmental toxicity of tungsten (W) for living organisms, its specific subcellular effects remain largely unknown. In plants, the cortical microtubules of *Pisum sativum* root cells have recently been identified as a subcellular target of W. In order to examine the possible universality of this phyto-effect, treatments with tungstate were applied on taxonomically diverse land plant taxa representing monocots, dicots, gymnosperms, pterophytes, and bryophytes, and the microtubules were examined by tubulin immunofluorescence. In all plant species studied, the cortical microtubule array was affected by W, but the W-affected microtubules differed in appearance among the plant taxa. Differences in the susceptibility of several plant organs as well as in the resistance of the various plant species to W toxicity were also recorded. It is concluded that the cortical microtubule array seems to be a universal target of W toxicity among land plants, a feature that might be a useful tool for monitoring W presence in the soil, in natural, and agricultural ecosystems.

Key words: microtubules, land plants, tungsten, toxicity.

Abbreviations: BCD: moss routine basal medium components B, C, D; CLSM: Confocal Laser Scanning Microscope; GFP: Green Fluorescent Protein; TUA: α-tubulin.

INTRODUCTION

Tungsten (W) is a rare heavy metal, useful in a wide range of industrial, military, and consumer applications due to its unique properties (Harper & Graedel, 2008). However, awareness about W as environmental pollutant is only recently increasing. Industrial areas, mines, battlefields, and military firing ranges are among the most expected sites for W accumulation, in addition to its occurrence in some urban environments. Consequently, W has become a subject of intensive research to clarify its possible beneficial, damaging or toxic influence for humans, animals, plants, and microorganisms (Dermatas *et al.*, 2004; Strigul *et al.*, 2005; Koutsospyros *et al.*, 2006; Wilson & Pyatt, 2006; Sheppard *et al.*, 2007; Steinberg *et al.*, 2007; Adamakis *et al.*, 2008, 2010; McInturf *et al.*, 2008; Clausen & Korte, 2009; Ringelberg *et al.*, 2009).

* Corresponding author: tel.: +30 2310 998385, fax: +30 2310 998389, e-mail: iadamaki@bio.auth.gr

In plants, W toxicity is alarming as it may interfere with the food chain and deteriorate natural ecosystems. W has been shown to disturb plant development (Strigul *et al.*, 2005; Jiang *et al.*, 2007; Adamakis *et al.*, 2008) and have negative effect on plant metabolism, mainly due to its relevance and antagonism to molybdenum (Mo) (Deng *et al.*, 1989; Jiang *et al.*, 2004; Llamas *et al.*, 2006). However, little is known about the possible subcellular targets of W toxicity. Recently, Adamakis *et al.* (2010) showed that W had a negative effect on the cortical microtubule array of *Pisum sativum* root cells through an indirect, yet unclear mechanism. Cortical microtubules of W-affected cells were fewer, short, not uniformly arranged, and resistant to anti-microtubule drugs.

As the integrity and patterning of cortical microtubules is crucial for plant cell and tissue development and morphogenesis (reviews by Panteris & Galatis, 2005; Smith & Oppenheimer, 2005; Paradez *et al.*, 2006), it is quite important to determine the ex-

tent and presumed universality of this novel finding among the plant kingdom. Accordingly, we investigated the effects of W on the cortical microtubules of diverse plant species from major land plant taxa. Like other heavy metals, W is known to be accumulated by plants (Koutsospyros *et al*., 2006; Johnson *et al*., 2009). Extending our knowledge on the potential toxicity of this emerging environmental pollutant to many taxonomically remote plant species, might also be useful in developing strategies for phytoremediation of contaminated soils.

MATERIALS AND METHODS

Plant material

Representatives of major land plant taxa were investigated. *Arabidopsis thaliana* (ecotype Columbia, expressing GFP-TUA) was selected to represent dicots, *Zea mays* cv. Aris and *Allium cepa* for monocots, *Pinus brutia* for gymnosperms, *Adiantum capillus-veneris* for pterophytes, and *Physcomitrella patens* and *Tortula muralis* for bryophytes.

Seeds of *Arabidopsis thaliana* were germinated and grown in Petri dishes on an agar nutrient substrate (Bannigan *et al*., 2006). *Zea mays*, *Allium cepa*, and *Pinus brutia* seeds were germinated on filter paper soaked with tap water at 25˚C in the dark. *Adiantum capillus-veneris* plants were collected from the waterfalls of the city of Edessa (Western Macedonia, Greece) and conditioned in the laboratory at 25˚C and high humidity for about one month before use. Chloronemata of *Physcomitrella patens* were cultured in moss routine basal medium (BCD) supplemented with 1 mM $CaCl_2$ (Ashton & Cove, 1977) in order to induce the production of gametophytes. Wild growing *Tortula muralis* plants were collected from the park of the Aristotle University of Thessaloniki, Greece.

Treatment with tungstate

A standard 1 μM aqueous solution of sodium tungstate (Na_2WO_4), pH 5.7 (adjusted with 0.1 M KOH and 0.1 M HCl solutions), was used for all materials, unless otherwise stated. This solution strength and pH were selected based on previous experiments on *P. sativum* and *Gossypium hirsutum* (Adamakis *et al.*, 2008, 2010). Three day-old *A. thaliana* seedlings were transferred in new dishes supplemented with tungstate solution, where they remained for 24 hrs; roots, hypocotyls, and leaves were investigated. *Zea mays* and *A. cepa* seedlings were treated for 24, 48 and 72 hrs and both roots and leaves were studied. *Pinus brutia* and *A. capillus-veneris* roots were immersed into tungstate solution for 24 hrs. As soon as a small number of leaflets emerged, gametophytes of *P. patens* were placed in liquid BCD medium containing 1 μM tungstate solution for 24 hrs before they were examined. Whole *T. muralis* plants were immersed in 1 μM or 5 μM

TABLE 1. Plant material and cell wall digesting enzyme combinations

Plant material	Enzyme treatment
Arabidopsis thaliana: root tips, hypocotyls, and leaves	0.5% (w/v) Cellulase (Sigma) + 1% (w/v) macerozyme R-10 (Yalkult-Honsha or Serva), 40 min
Zea mays: root tips	2% (w/v) Cellulase (Sigma) + 2% (w/v) macerozyme R-10 (Yakult-Honsha or Serva), 40 min
Zea mays: leaves	2% (w/v) Macerozyme R-10 (Serva), 40 min
Allium cepa: root tips	2% (w/v) Cellulase (Sigma) + 2% (w/v) macerozyme R-10 (Yakult-Honsha or Serva), 40 min
Pinus brutia: root tips	5% (w/v) Cellulase (Sigma) + 5% (w/v) macerozyme R-10 (Yakult-Honsha or Serva) + 2% (w/v) driselase (Sigma), 40 min
Adiantum capillus-veneris: root tips	2% (w/v) Cellulase (Sigma) + 2% (w/v) macerozyme R-10 (Yakult-Honsha or Serva) + 2% (v/v) β-glucuronidase (type H-2, Sigma), 40 min
Physcomitrella patens: leaflets	3% (w/v) Cellulase (Sigma) + 3% (w/v) macerozyme R-10 (Yakult-Honsha or Serva) + 2% (v/v) β-glucuronidase (type H-2, Sigma), 40 min
Tortula muralis: leaflets	3% (w/v) Cellulase (Sigma) + 3% (w/v) macerozyme R-10 (Yakult-Honsha or Serva) + 3% (v/v) β-glucuronidase (type H-2, Sigma), 40 min

aqueous tungstate solution, where they remained for 24 or 48 hrs and the leaflets were used further. For all materials, appropriate controls in the same media and conditions but without tungstate were processed.

Microtubule imaging

Arabidopsis thaliana seedlings expressing GFP-tubulin were gently transferred to microscope slides, moistened with a small drop of Hoagland solution with or without (control) tungstate. Afterwards, a coverslip was placed on top and the material was examined with a Nikon D-Eclipse C1 Confocal Laser Scanning Microscope (CLSM). In the other plant materials (and in some cases in root tips of *A. thaliana*), tubulin immunofluorescence was conducted according to the protocol described by Adamakis *et al*. (2010), with different enzyme combinations for each material (Table 1). The immunofluorescent specimens were examined with a Zeiss IM35 inverted microscope equipped with epi-fluorescence or with a Nikon D-Eclipse C1 CLSM.

RESULTS

In all plant species studied, the cortical microtubule array was affected by tungstate, as compared with the untreated specimens. However, the tungsten-affected microtubules (W-microtubules, for brevity) differed in appearance among the plant species. In *A. thaliana*, W-microtubules appeared short and misoriented and could be observed in root cells only (Fig. 1B; *cf.* Fig. 1A), while those of hypocotyl and leaf cells remained rather unaffected (Fig. 1D, F; *cf.* Fig. 1C, E). In *Z. mays* root cells, cortical W-microtubules appeared after 72 hrs of treatment; they were fewer than those of untreated cells, transversely oriented and highly bundled (Fig. 2B; *cf.* Fig. 2A). However, the cortical microtubules of *Z. mays* leaf protodermal cells appeared to be unaffected by W (Fig. 2D; *cf.* Fig. 2C). In W-treated *A. cepa* root cells the cortical microtubules appeared fewer and disoriented (Fig. 2F), in contrast to those of untreated cells (Fig. 2E). In the root cells of *P. brutia*, W-microtubules formed cortical ring-shaped configurations (Fig. 3B; *cf.* Fig. 3A). After treatment with tungstate, root cells of *Adiantum capillus-veneris* displayed a network of bundled and/or undulating cortical W-microtubules (Fig. 3D; *cf.* Fig. 3C).

In tungstate-treated *P. patens* leaflets only fragments of cortical microtubules could be observed (Fig. 4B; *cf.* Fig. 4A), while the endoplasmic microtubule network seemed to be less affected (Fig. 4D; *cf.* Fig. 4C). In *T. muralis* leaflets, treatment with 1 μM tungstate had no effect (Fig. 4F) compared to the control (Fig. 4E). It was necessary to use 5 μM of tungstate solution for 48 hrs to obtain a complete disorganization of cortical microtubules in this species (Fig. 4G).

DISCUSSION

Tungsten (W) toxicity has been reported in several plant species, such as rye grass, *Brassica* spp., *Pisum sativum*, *Gossypium hirsutum, Hordeum vulgare, Zea mays*, and *Helianthus annuus* (Hale *et al*., 2002; Jiang *et al*., 2004; Strigul *et al*., 2005; Jiang *et al*., 2007; Adamakis *et al*., 2008; Johnson *et al*., 2009). In the present study, we show that W affects the cortical microtubules in terrestrial plant representatives of monocots, dicots, gymnosperms, pterophytes, and bryophytes, thus extending significantly the list of plant species studied for W toxicity. It is then concluded that the cortical microtubule array appears to be a common subcellular target in the species examined.

Previous studies in *Z. mays* have shown that most of the W taken up from the nutrient medium is deposited in the root system, while only a very small proportion reaches the leaf sheath and lamina (Jiang *et al*., 2007). This is in agreement with our findings in *Zea mays* and *A. thaliana*. Depending on the degree of cortical microtubule disturbance, it seems that the root is the main organ affected by W. The microtubules of *A. thaliana* hypocotyls were only slightly affected, while leaf cells of both species (*Z. mays* and *A. thaliana*) did not appear to be influenced. Motility of the metal inside plant tissues and the mechanism by which it is trapped in the root is an interesting question to be further addressed.

The two species of bryophytes studied showed different response to W treatment. *Tortula muralis* appeared quite resistant, while *P. patens* was equally sensitive to most higher plant species studied. This is not surprising, as the two species were grown under quite different conditions. Since *T. muralis* grew outdoors, the cuticle and surface cell walls of its leaflets would be expected to be thicker and harder in comparison to those of *P. patens*, which was cultured in a liquid medium under controlled conditions. Consequently, the difference in susceptibility towards W between the two bryophytes should rather be considered as a difference in W penetration. In general, bryophytes are known to withstand pollution, especially high

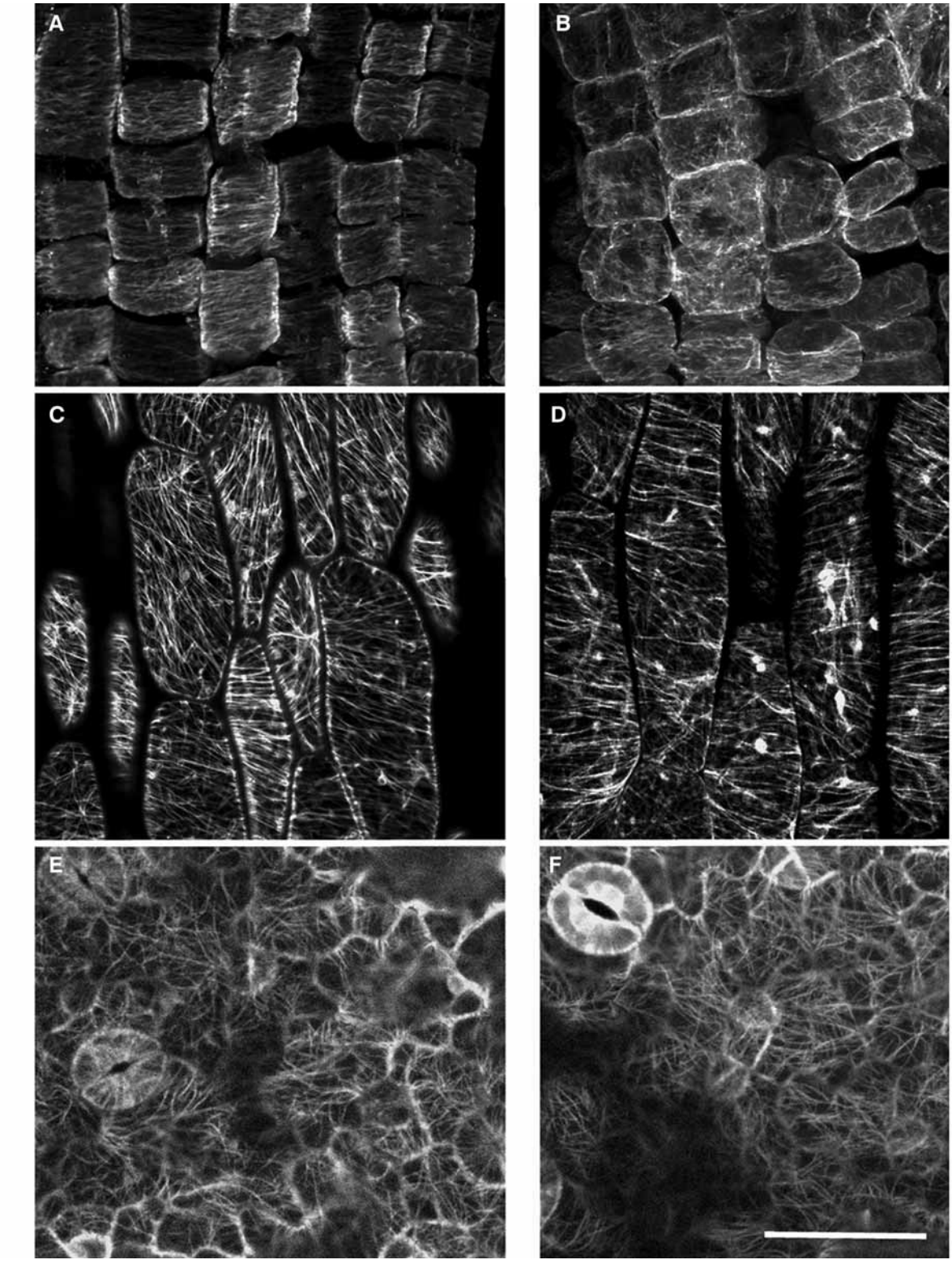

FIG. 1. CLSM micrographs of immunolabeled microtubules (A, B) and GFP-tubulin (C-F) in control (A, C, E) and W-treated (B, D, F) *Arabidopsis thaliana* seedlings. While the cortical array of untreated root cells consists of parallel, uniformly arranged microtubules (A), in W-treated root cells cortical microtubules appear fragmented and disoriented (B). The cortical microtubules of W-treated hypocotyl (D) and leaf (F) cells do not exhibit much difference in comparison to the counterparts of untreated cells (C and E, respectively). The images in A and B are projections of 30 CLSM sections, while C-F represent single CLSM sections of the cortical cytoplasm (bar = 50 μm).

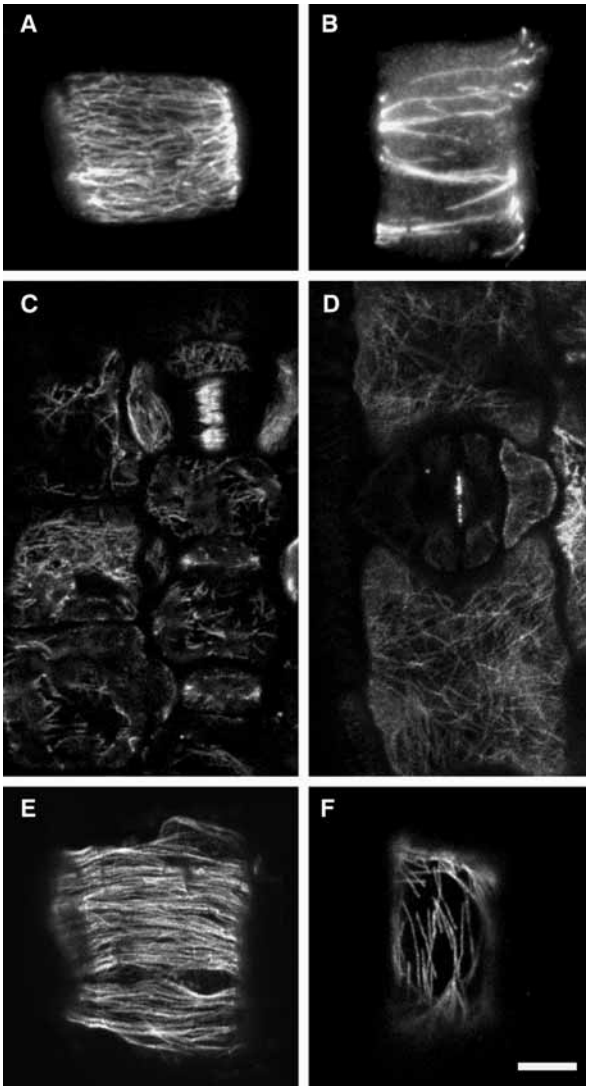

FIG. 2. Effect of W on monocots. A-D. Tubulin immunofluorescence micrographs of control (A) and 72 hrs W-treated (B) root cells, and CLSM micrographs of immunolabeled microtubules in control (C) and W-treated (D) leaf protodermal tissue of *Zea mays*. In the root cells, W-microtubules appear fewer and bundled (B) as compared to the typical appearance in the untreated cell (A). No significant difference in microtubule appearance is evident between untreated (C) and W-treated (D) protodermal tissue. E, F. Tubulin immunofluorescence micrographs of control (E) and 72 hrs W-treated (F) root cells of *Allium cepa*. In W-treated root cells cortical microtubules are fewer and disoriented (bar = 10 μm).

concentrations of heavy metals (Pearson *et al*., 2000). A main tolerance mechanism of bryophytes against heavy metals is the efficiency of their cell walls to immobilize heavy metal ions [reviewed by Tyler (1990)]. Therefore, it could be concluded that the resistance of *T. muralis* represents the rule for land mosses, while the exceptional susceptibility of *P. patens* may

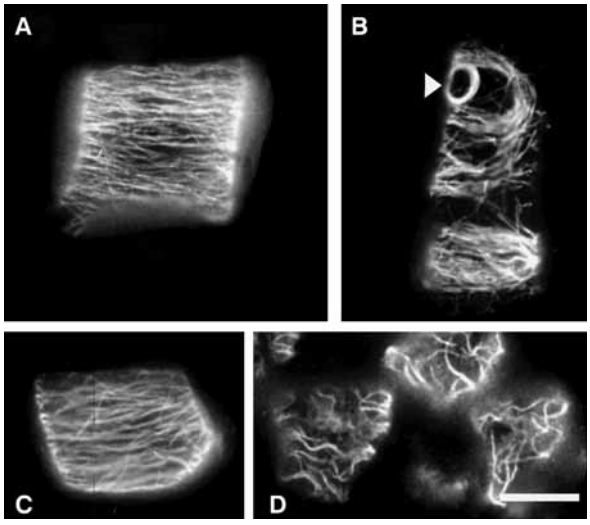

FIG. 3. Tubulin immunofluorescence micrographs of untreated (A, C) and W-treated (B, D) interphase root cells of *Pinus brutia* (A, B) and *Adiantum capillus-veneris* (C, D). In *Pinus brutia* cortical ring-shaped configurations of W-microtubules are observed (B) (arrowhead), while in *Adiantun capillus-veneris* W-microtubules appeared disoriented and in thick threads (D) (bar = 10 μm).

be attributed to the culture conditions.

Interestingly, while the cortical microtubules of *P. patens* are severely affected by W, the endoplasmic ones seem to be more resistant. Endoplasmic microtubules are characteristic features of specialized cell types in mosses, having a major role in organelle alignment and shaping (Ligrone & Duckett, 1994). Moreover, many cell types of mosses (e.g. protonemata, chloronemata, food-conducting cells) contain endoplasmic microtubules, which have been shown to be resistant to anti-microtubule drugs (Ligrone & Duckett, 1996), but not the cortical ones. On the contrary, endoplasmic microtubules of tracheophyte cells are more sensitive than cortical ones, as evaluated by their response to chemical and physical anti-microtubule agents, high levels of extracellular calcium, and short-term exposure to low temperature (Baluška *et al*., 1992, 1993; Eleftheriou, 1993). Accordingly, it seems that there is a different functional significance between the cortical *vs* endoplasmic microtubules among bryophytes and tracheophytes, reflected in a differential susceptibility to W.

Another interesting finding is that the two monocot species, *Z. mays* and *A. cepa*, seem to be more resistant to W, at least in terms of cortical microtubule organization, than the rest of the tracheophytes tested. In both monocot species, 24 hrs of W treatment was not enough to affect the cortical microtubules of root

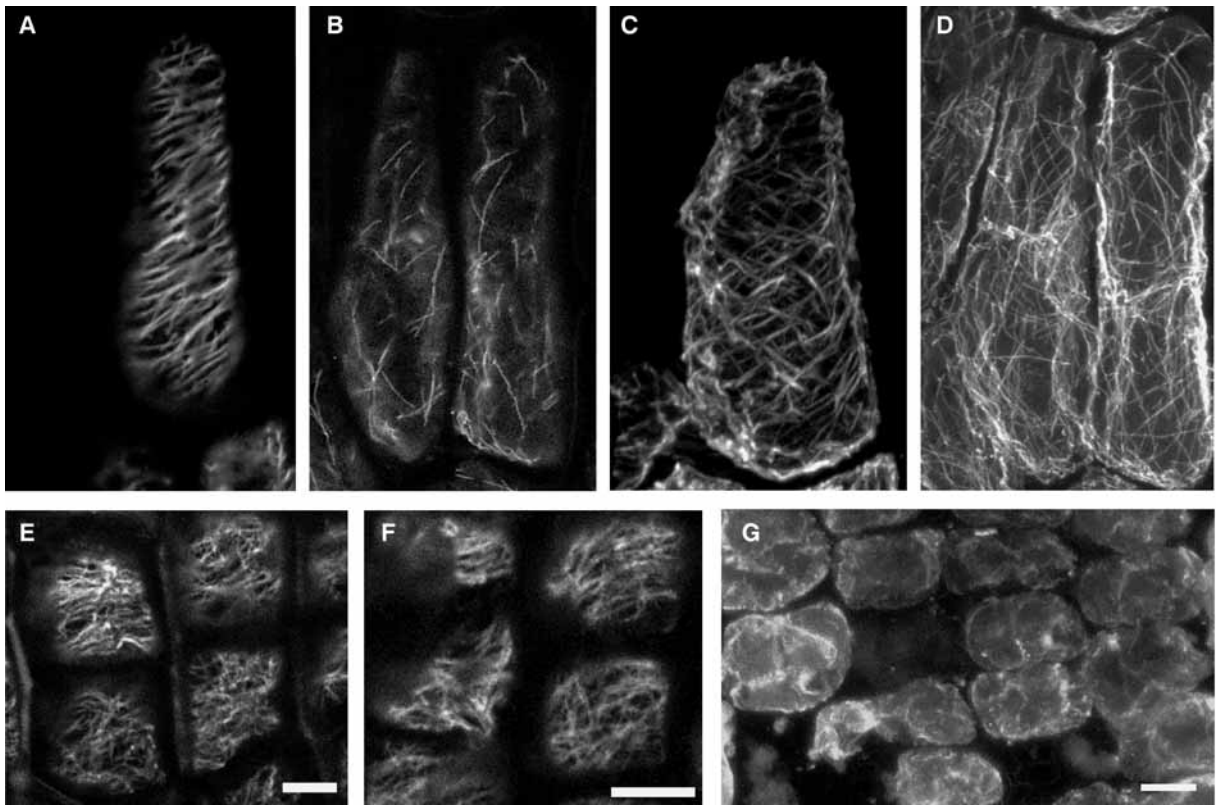

FIG. 4. Effect of W on bryophytes. A-D. CLSM micrographs of immunolabeled microtubules in control (A, C) and W-treated (B, D) *Physcomitrella patens* leaflets. Plates A and B represent single CLSM sections through the cortical cytoplasm, while C and D are projections of 25 CLSM sections, revealing the endoplasmic microtubules. In W-affected leaflets only remnants of cortical microtubules can be observed (B, *cf.* A). The endoplasmic microtubule network (D) seems to be less affected (compare with C) than the cortical one. E-G. CLSM micrographs of control (E), 1 μM for 24 hrs (F) and 5 μM for 48 hrs (G) W-treated cells of *Tortula muralis* leaflets. No disturbance can be observed in microtubule features in F, but in G the microtubule network appears highly disrupted (bars = 10 μm; for A-D the corresponding bar is that of E).

cells, which were disturbed only after 72 hrs of treatment. This might be attributed to several factors such as the difference in W penetration or the higher resistance of the microtubules themselves. Another plausible explanation is that monocots might be less dependent on Mo than other plants. For example, it has been stated that grasses, including *Z. mays*, accumulate Mo at low levels (Williams & Gogna, 1981). In accordance, if W affects cortical microtubules through the Cnx1 pathway of Mo (Adamakis *et al*., 2010), the resistance of monocots to W may reflect the lower need for Mo.

Plants are suitable organisms for *in vitro* screening and monitoring the presence of soil contaminants and are already used as standard indicators for the effects of various chemicals in the environment, as heavy metals (Wilson & Pyatt, 2007; Monteiro *et al*., 2009, and references therein). Land plants could be used as bioindicators and/or bioaccumulators of W in polluted environments, since it is known that W can be accumulated by various plant taxa (Pratas *et al*., 2005; review by Koutsospyros *et al*., 2006). The already recommended tests focus only on non-specific responses of seedling emergence and plant growth (ISO, 1993, 1995; OECD, 2006). However, microtubule pattern changes were suggested as a suitable specific marker of cadmium pollution stress (Fusconi *et al*., 2007). Similarly, the profile of cortical W-microtubules might be used in combination with standard procedures for W monitoring and screening.

Moreover, it has been reported recently that some plants (e.g. sunflower) might be used for phytoremediation of W from polluted soils (Johnson *et al*., 2009, and references therein). Consequently, resistance to W, as estimated by the occurrence of cortical W-microtubules, could be used for determining the most

suitable plant species for phytoremediation. In accordance, more plant species should be investigated, thus a more extensive study of W toxicity to plants remains vital.

ACKNOWLEDGEMENTS

Seeds of *Arabidopsis thaliana* expressing GFP-tubulin were a generous gift from Prof. Tobias Baskin (Biology Department, University of Massachusetts, Amherst, MA, USA), *Zea mays* cv. Aris seeds were offered by the Institute of Cereals, Thessaloniki, and *Physcomitrella patens* was cordially supplied by Dr. Hartmut Quader (Biocenter, University of Hamburg, Germany). The authors thank Prof. Thomas Rost (Section of Plant Biology, University of California, Davis, CA, 95616, USA) for critically reading the manuscript. Access to the Nikon D-Eclipse C1 CLSM of the Faculty of Veterinary Medicine of our University was kindly provided by Dr. Anastasia Tsingotjidou, while the identification of *Tortula muralis* was carried out by Dr. Eudoxia Tsakiri (Department of Botany, School of Biology, Aristotle University of Thessaloniki).

REFERENCES

Adamakis I-DS, Eleftheriou EP, Rost TL, 2008. Effects of sodium tungstate on the ultrastructure and growth of pea (*Pisum sativum*) and cotton (*Gossypium hirsutum*) seedlings. *Environmental and experimental botany*, 63: 416-425.

Adamakis I-DS, Panteris E, Eleftheriou EP, 2010. Tungsten affects the cortical microtubules of *Pisum sativum* root cells: Experiments on tungsten-molybdenum antagonism. *Plant biology*, 12: 114-124.

Ashton NW, Cove DJ, 1977. The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants of the moss *Physcomitrella patens*. *Molecular genetics*, 154: 87-95.

Baluška F, Parker JS, Barlow PW, 1992. Specific patterns of cortical and endoplasmic microtubules associated with cell growth and tissue differentiation in roots of maize (*Zea mays* L.). *Journal of cell science*, 103: 191-200.

Baluška F, Barlow PW, Parker JS, 1993. The microtubular cytoskeleton in cells of cold-treated roots of maize (*Zea mays* L.) shows tissue-specific responses. *Protoplasma*, 172: 84-96.

Bannigan A, Wiedemeier AM, Williamson RE, Overall RL, Baskin TI, 2006. Cortical microtubule arrays lose uniform alignment between cells and are oryzalin resistant in the *Arabidopsis* mutant, *radially swollen 6*. *Plant and cell physiology*, 47: 949-958.

Clausen JL, Korte N, 2009. Environmental fate of tungsten from military use. *Science of the total environment*, 407: 2887-2893.

Deng M, Moureaux T, Caboche M, 1989. Tungstate, a molybdate analog inactivating nitrate reductase, deregulates the expression of the nitrate reductase structural gene. *Journal of plant physiology*, 91: 304-309.

Dermatas D, Braida W, Christodoulatos C, Strigul N, Panikov N, Los M, Larson S, 2004. Solubility, sorption, and soil respiration effects of tungsten and tungsten alloys. *Environmental forensics*, 5: 5-13.

Eleftheriou EP, 1993. Differentiation of abnormal sieve elements in roots of wheat (*Triticum aestivum* L.) affected by colchicine. *New phytologist*, 125: 813-827.

Fusconi A, Gallo C, Camusso W, 2007. Effects of cadmium on root apical meristems of *Pisum sativum* L.: Cell viability, cell proliferation and microtubule pattern as suitable markers for assessment of stress pollution. *Mutation research*, 632: 9-19.

Hale KL, Tufan HA, Pickering IJ, George GN, Terry N, Pilon M, Pilon-Smits EAH, 2002. Anthocyanins facilitate tungsten accumulation in *Brassica*. *Physiologia plantarum*, 116: 351-358.

Harper EM, Graedel ET, 2008. Illuminating tungsten's life cycle in the United States: 1975-2000. *Environmental science and technology*, 42: 3835-3842.

ISO, 1993. *Soil quality-determination of the effects of pollutants on soil flora. Part 1: methods for the measurement of inhibition of root growth*. ISO (International Organization for Standardization), Genéve.

ISO, 1995. *Soil quality determination of the effects of pollutants on soil flora. Part 2: effects of chemicals on the emergence of higher plants*. ISO (International Organization for Standardization), Genéve.

Jiang F, Heilmeir H, Hartung W, 2007. Abscisic acid relations of plants grown on tungsten enriched substrates. *Plant and soil*, 301: 37-49.

Jiang XY, Omarov T, Yesbergenova SZ, Sagi M, 2004. The effect of molydbate and tungstate in the growth medium on abscisic acid content and the Mo-hydroxylases activities in barley (*Hordeum vulgare* L.). *Plant science*, 167: 297-300.

Johnson DR, Inouye LS, Bednar AJ, Clarke JU, Winfield LE, Boyd RE, Ang CY, Goss J, 2009. Tungsten bioavailability and toxicity in sunflowers (*Helianthus annuus* L.). *Land contamination and reclamation*, 17: 141-151.

Koutsospyros A, Braida W, Christodoulatos C, Dermatas D, Strigul N, 2006. A review of tungsten: from environmental obscurity to scrutiny. *Journal of hazardous materials*, 136: 1-19.

Ligrone R, Duckett JG, 1994. Cytoplasmic polarity and endoplasmic microtubules associated with the nucleus and organelles are ubiquitous features of food-conducting cells in bryoid mosses. *New phytologist*, 127:

601-614.

Ligrone R, Duckett JG, 1996. Polarity and endoplasmic microtubules in food-conducting cells of mosses: an experimental study. *New phytologist*, 134: 503-516.

Llamas A, Otte T, Multhaup G, Mendel RR, Schwarz G, 2006. The mechanism of nucleotide-assisted molybdenum insertion into molybdopterin. A novel route toward metal cofactor assembly. *The journal of biological chemistry*, 281: 18343-18350.

McInturf SM, Bekkedal MYV, B, Wilfong E, Arfsten D, Gunasekar PG, Chapman GD, 2008. Neurobehavioral effects of sodium tungstate exposure on rats and their progeny. *Neurotoxicology and teratology*, 30: 455-461.

Monteiro MS, Santos C, Soares AMVM, Mann RM, 2009. Assessment of biomarkers of cadmium stress in lettuce. *Ecotoxicology and environmental safety*, 72: 811-818.

OECD, 2006. *Terrestrial plant test: seedling emergence and seedling growth test.* OECD (Organisation for Economic Co-operation and Development), Paris.

Panteris E, Galatis B, 2005. The morphogenesis of lobed plant cells in the mesophyll and epidermis: organization and distinct roles of cortical microtubules and actin filaments. *New phytologist*, 167: 721-732.

Paradez A, Wright A, Ehrhardt DW, 2006. Microtubule cortical array organization and plant cell morphogenesis. *Current opinion in plant biology*, 9: 571-578.

Pearson J, Wells DM, Seller KJ, Bennett A, Soares A, Woodall J, Ingrouille MJ, 2000. Traffic exposure increases natural and heavy metal concentrations in mosses. *New phytologist*, 147: 317-326.

Pratas J, Prasad MNV, Freitas H, Conde L, 2005. Plants growing in abandoned mines of Portugal are useful for biogeochemical exploration of arsenic, antimony, tungsten and mine reclamation. *Journal of geochemical exploration*, 85: 99-107.

Ringelberg DB, Reynolds CM, Winfield LE, Inouye LS, Johnson DR, Bednar AJ, 2009. Tungsten effects on microbial community structure and activity in a soil. *Journal of environmental quality*, 38: 103-110.

Sheppard PR, Speakman RJ, Ridenour G, Witten ML, 2007. Temporal variability of tungsten and cobalt in Fallon, Nevada. *Environmental health perspectives*, 115: 715-719.

Smith LG, Oppenheimer DG, 2005. Spatial control of cell expansion by the plant cytoskeleton. *Annual review of cell and developmental biology*, 21: 271-295.

Steinberg KK, Relling MV, Gallagher ML, Greene CN, Rubin CS, French D, Holmes AK, Carroll WL, Koontz DA, Sampson EJ, Satten GA, 2007. Genetic studies of a cluster of acute lymphoblastic leukemia cases in Churchill County, Nevada. *Environmental health perspectives*, 115: 158-164.

Strigul N, Koutsospyros A, Arienti P, Christodoulatos C, Dermatas D, Braida W, 2005. Effects of tungsten on environmental systems. *Chemosphere*, 61: 248-258.

Tyler G, 1990. Bryophytes and heavy metals: a literature review. *Botanical journal of the Linnean society*, 104: 231-253.

Williams JH, Gogna JC, 1981. Molybdenum uptake from sewage sludge treated soil. In: *Proceedings of the Third International Conference on Heavy Metals in the Environment*. Amsterdam, 15-18 September 1981. CEP Consultants, Edinburgh: 189-193.

Wilson B, Pyatt FB, 2006. Bio-availability of tungsten in the vicinity of an abandoned mine in the English Lake District and some potential health implications. *Science of the total environment*, 370: 401-408.

Wilson B, Pyatt FB, 2007. Heavy metal bioaccumulation by the important food plant, *Olea europaea* L., in an ancient metalliferous polluted area of Cyprus. *Bulletin of environmental contamination and toxicology*, 78: 390-394.