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

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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).

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 CaCl₂ (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 (Na₂WO₄), 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

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<th>TABLE 1. Plant material and cell wall digesting enzyme combinations</th>
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<td><strong>Plant material</strong></td>
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<tr>
<td><em>Arabidopsis thaliana</em>: root tips, hypocotyls, and leaves</td>
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<td><em>Zea mays</em>: root tips</td>
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<td><em>Zea mays</em>: leaves</td>
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<td><em>Allium cepa</em>: root tips</td>
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<td><em>Pinus brutia</em>: root tips</td>
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<td><em>Adiantum capillus-veneris</em>: root tips</td>
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<td><em>Physcomitrella patens</em>: leaflets</td>
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<td><em>Tortula muralis</em>: leaflets</td>
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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. 1A; cf. Fig. 4A), 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).
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 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. protone-mata, 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 (Baluska 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. 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).

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 Adiantum capillus-veneris W-microtubules appeared disoriented and in thick threads (D) (bar = 10 μm).
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

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**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).
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.

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REFERENCES


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


