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Microtubule integrity and cell viability under metal (Cu, Ni and Cr) stress in the seagrass *Cymodocea nodosa*

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HIGHLIGHTS

- Metal effect on microtubules and viability of seagrass leaf cells was assessed.
- Cu and Ni depolymerized MTs after 3–7 d of exposure depending on metal dose.
- Cr effect on MT cytoskeleton organization differed compared to that of Cu and Ni.
- Cell death at later time than MT disturbance was observed at most treatments.
- MTs in seagrass leaf cells could be used as a biomarker of metal-induced stress.

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ABSTRACT

The effects of increasing Cu, Ni and Cr concentrations (0.5, 5, 10, 20 and 40 mg L^{-1}) on microtubule organization and the viability of leaf cells of the seagrass *Cymodocea nodosa* for 13 consecutive days were investigated under laboratory conditions. Increased oblique microtubule orientation, microtubule depolymerization at the 5–40 mg L^{-1} Ni treatments after 3 d of exposure, and a complete microtubule depolymerization at all Ni treatments after 5 d were observed. Cu depolymerised microtubules after three to 7 d of exposure, while Cr caused an extensive microtubule bundling after 9 or 11 d of exposure, depending on metal dosage. Fluorescence intensity measurements further consolidated the above phenomena. Cell death, occurring at later time than microtubule disturbance, was also observed at all Cu and Ni treatments and at the 10–40 mg L^{-1} Cr treatments and adding to the above quantification of the number of dead cells clearly showed that only a portion of the cell population studied died. The data presented, being the first assessment of microtubule disturbance in seagrasses, indicate that microtubules in seagrass leaf cells could be used as a valuable and early marker of metal-induced stress in biomonitoring programmes.

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1. Introduction

Seagrasses occur in most shallow coastal waters throughout the world playing a key multifunctional role. They are important primary producers, provide critical habitat for a variety of organisms, stabilize sediments and fix nutrients (Beal and Schmit, 2000). However, human population growth along coastal environments has resulted in a worldwide deterioration of seagrass meadows (Hemminga and Duarte, 2000). Many factors such as eutrophication, siltation, mechanical damage, thermal stress and invasions by exotic species are among the threats. Toxic chemicals such metals are also considered as a contributing factor to seagrass losses (Hemminga and Duarte, 2000; Ralph et al., 2007).

Most of the available information is focused on the accumulation of metals on seagrasses; scientific knowledge about the effects of heavy metals on seagrasses is limited, thus restricting a realistic assessment of their role in seagrass decline (see review in Lewis and Devereux (2009)). The toxicity database consists of results for about ten species, but most commonly for *Zostera* and *Halophila* species; copper, cadmium, lead and zinc have been mainly used as test substances in toxicity tests, and photosynthetic activity, photosynthetic pigment concentration, growth rate and leaf cell mortality as response parameters (Lewis and Devereux, 2009). Information on the effects of metals on seagrass species which display extensive distribution and notable ecological importance is currently missing (Lewis and Devereux, 2009). This also applies to *Cymodocea nodosa* (Ucria) Ascherson, which along with *Posidonia oceanica* (L.) Delile are the most important and widespread seagrasses in the Mediterranean Sea. In addition, we are not aware of any published data concerning the effects of metals on the microtubule (MT) cytoskeleton in seagrasses, despite the fact that MTs play an essential role in higher plant morphogenesis and growth (Hasezawa and Kumagai, 2002) and represent one of the

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intra-cellular targets of metal ions (review by Adamakis et al. (2012) and references therein).

Potential disturbances induced by metals to MT cytoskeleton in seagrass cells could be used as a biomarker for the evaluation of metal contamination in coastal environments and could provide evidence about the role of metals in seagrass decline. Biomarkers, defined as "cellular, molecular and biochemical changes induced by chemical pollutants, measurable in biological systems such tissues, cells and biological fluids" (Depledge et al., 1995), are increasingly utilized in monitoring programmes; they can provide information on the potential impact of toxic pollutants on the health of organisms and can be used as early warning signals for general or particular stress (see synthesis in Ferrat et al., 2003).

The present study aims to provide information on the toxic effects of metals on seagrass condition. We investigated under laboratory conditions the effects of increasing concentrations of copper (Cu), nickel (Ni) and hexavalent chromium (Cr) on the microtubule cytoskeleton organization in leaf meristematic cells and the viability of leaf cells of the seagrass *C. nodosa* for 13 consecutive days. Notable changes in microtubule organization occurring at earlier time than cell death are checked and the suitability of microtubule integrity as a biomarker of metal-induced stress is discussed.

2. Materials and methods

2.1. Plant collection

C. nodosa was collected from the eastern coast of the Gulf of Thessaloniki, Northern Aegean Sea at Viamyl site (site V, 40°33′N, 22°58′E) at 0.7–1.0 m depth in July 2011 with a 20 cm diameter acrylic corer, which penetrated to a depth of 30 cm. All plants were rinsed in seawater at the collection site and transported to the laboratory in plastic containers containing seawater (Malea and Zikidou, 2011; unpublished data).

2.2. Treatments

Fresh green plants without epiphytes were kept for 24 h in seawater under laboratory conditions in order to equilibrate. Seagrass ramets (roots, horizontal rhizomes, vertical rhizomes, leaves) were incubated in plastic aquaria containing 10 L solutions of $K_2Cr_2O_7$, $CuSO_4{\cdot}5H_2O$ or $Ni(NO_3)_2{\cdot}6H_2O$ (Merck) in filtered seawater (Whatman GF/C) in the following dissolved metal concentrations: 0.5, 5, 10, 20 and 40 mg L^{-1}. A control treatment, with no added metal, was included in the experiments. At least 21 seagrass shoots were contained in each aquarium. The seawater used for the experiments was also collected from the site V. The characteristics of the seawater used in the experiments were: salinity 36.7 psu, pH 7.9, dissolved O_2 5.88 mg L^{-1}, $N{-}NO_2^-$ 0.02 μM, $N{-}NO_3^-$ 0.37 μM and $N{-}NH_4^+$ 0.43 μM. The solutions in the aquaria were changed every 2 d in order to adjust to the original levels. The aquaria were aerated constantly using aquarium pumps and covered with plastic foil in order to prevent evaporation. The experiments were conducted under a constant 16 h d/8 h night regime at an ambient temperature of 21 ± 1 °C with light intensity set at 120 μmol m^{-2} - s^{-1}. After 0, 3, 5, 7, 9, 11 and 13 d at least three seagrass shoots from each aquarium were randomly removed. Similar procedures have been also used in previous studies (e.g. Malea, 1994; Malea et al., 1995a,b).

2.3. Tubulin immunostaining

Tubulin immunostaining was applied on either the smallest juvenile leaf or the meristematic region of the next smallest juvenile leaf of each collected shoot. As juveniles, leaves with no visible sheath were classed (Orfanidis et al., 2010). All chemicals and reagents were purchased from Sigma (Taufkirchen, Germany), Merck (Darmstadt, Germany) and Applichem (Darmstadt, Germany), unless otherwise stated. Whole mound tubulin immunostaining was conducted in leaf pieces, following the protocol of Katsaros and Galatis (1992) with both anti-α-tubulin (YOL1/34, AbD Serotec, Kidlington, UK) and FITC-anti-rat secondary antibody (Invitrogen, Carlsbad, CA) diluted at 1:80. DNA was counterstained with 3 μg mL^{-1} propidium iodide in PBS and the leaf pieces were finally mounted in an anti-fade solution. Then the leaf pieces were examined with a Nikon D-Eclipse C1 confocal laser scanning microscope (CLSM) and image recording was done with proper software (EZ-C1 3.20) according to the manufacturer's instructions. Special care was taken in order to retain the laser beam gain equal among the different treatments.

2.4. Fluorescence intensity measurements

Intensity fluorescence measurements were done in single cortical CLSM sections using the Image J (http://rsbweb.nih.gov/ij/) software. Linear adjustments in pixel values were made when measuring signal intensities and the corrected total cell fluorescence (CTCF) was calculated using this formula: CTCF = Integrated Density − (Area of selected cell X Mean fluorescence of background readings). From each treatment, fluorescence intensity from 15 individual cells was measured and the mean values were calculated. Control values were considered as 100% and every other value obtained were plotted against the control values expressed as a percentage.

2.5. Evans Blue staining

In order to check the viability of the variously treated leaf cells, an Evans Blue staining was conducted. Evans Blue staining was done following the protocol by Chen et al. (2008). In short, leaves of the randomly selected shoots were incubated in a 0.25% aqueous Evans Blue solution done in seawater collected from the collection site V for 15 min at room temperature. After several washes with seawater the leaf segments were observed under a Zeiss Axiostar-Plus light microscope equipped with a Canon PowerShot A640 camera and photographed. From a total number of 500 cells in each treatment the dead cells (stained blue) were measured and the measurements were expressed as percentages.

3. Results

3.1. Microtubule distribution

3.1.1. Control

The cortical microtubule array of interphase leaf cells of untreated plants typically consisted of a dense network with variously oriented microtubules, either transverse or oblique to long axis (Fig. 1a). Moreover numerous cells were found in phases of the cell division in which distinct MT systems were observed: young as well as mature preprophase MT bands (PMBs); preprophase perinuclear MT arrays, prophase and metaphase spindles (Fig. 1b), similar to what is typically observed in higher land plants (Smirnova, 2012).

3.1.2. Metal effect

Generally all three metals applied, affected the MT arrays. Their effect was concentration and time depended (Table 1). One of the first effects observed was the loss of cells in division. Moreover, leaf cells responded differentially to the different metals applied as it will be further on discussed.

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