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Bisphenol A disrupts microtubules and induces multipolar spindles in dividing root tip cells of the gymnosperm *Abies cephalonica*

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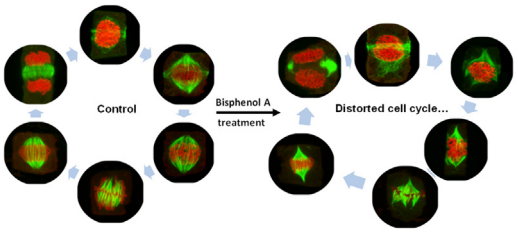
HIGHLIGHTS

- BPA disrupted microtubules in all mitotic stages of *Abies cephalonica*.
- Chromosomal aberrations were underlain by microtubule derangement under BPA stress.
- Endoplasmic reticulum malformations reflected those of microtubules.
- Microtubule recovery after oryzalin treatment was more effective in BPA than in water.
- BPA-induced multipolar spindles are attributed to the centrosomal properties of gymnosperms.

GRAPHICAL ABSTRACT



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ABSTRACT

The effects of bisphenol A (BPA), an endocrine chemical disruptor extensively used in the plastic and epoxy resin industry, on dividing root tip cells of the gymnosperm *Abies cephalonica* Loudon were investigated by confocal laser scanning microscopy after tubulin and endoplasmic reticulum immunolocalization and DNA staining. Microtubule arrays of all mitotic stages were disrupted within a few hours of treatment: preprophase bands exhibited asymmetric width; prometaphase, metaphase and anaphase spindles appeared sharply pointed, sigmoid or multipolar; phragmoplast microtubules were elongated and occasionally bended toward the daughter nuclei. Depending on the mitotic stage, the chromosomes appeared condensed at prophase, as a compact mass at metaphase and anaphase, unsegregated or bridged at telophase. Endoplasmic reticulum patterns were also affected, reflecting those of the respective microtubule arrays. Recovery of the microtubules after oryzalin treatment was more effective in a BPA solution than in water. It is concluded that the plant mitotic apparatus microtubules are very sensitive to BPA, the effect of which depends on the specific cell cycle stage. The formation of multipolar spindles is reminiscent of animal cells and is ascribed to the induction of multiple microtubule nucleation sites, deriving from the centrosomal properties of gymnosperms.

1. Introduction

Microtubules (MTs) are usually nucleated at certain cellular loci, known as microtubule organizing centres (MTOCs), a term coined by Pickett-Heaps (1969) for a cellular site postulated to organize specialized MT systems. The best defined MTOC is the centrosome, an organelle occurring in animal and lower plant cells, consisting of two centrioles. The spindle MTs emanate from the pericentriolar

Abbreviations: BPA, bisphenol A; ER, endoplasmic reticulum; CLSM, confocal laser scanning microscopy; PPB, preprophase band; MT, microtubule.

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material, which creates the spindle poles and establishes spindle bipolarity (Merdes and Cleveland, 1997). In higher plants centrioles are absent and MTOCs are dispersed and pleiomorphic (Schmit, 2002; Brown and Lemmon, 2007). Though not morphologically distinct, the plant MTOCs are extremely important as they are responsible for the proper organization of the mitotic and cytokinetic apparatus. MT turnover from one array to the other requires MT depolymerization and repolymerization at the new site, taking advantage of their dynamic nature.

Environmental pollutants are notorious for adversely affecting the MT arrays and consequently plant cell division. One of the emerging organic pollutants is bisphenol A [BPA, 2,2-bis-(4-hydroxyphenyl)propane] (Clarke and Smith, 2011). BPA is a profusely manufactured organic chemical, extensively used in the production of numerous household and every-day products (Staples et al., 1998; Cooper et al., 2011). As a result, BPA is bulkily discharged in the environment, being traced in municipal wastewaters, sewage sludge, inland and marine waters (Gatidou et al., 2007; Pothitou and Voutsa, 2008; Fu and Kawamura, 2010). Humans may be exposed to BPA through consumption of BPA-polluted food such as fish (Mita et al., 2011) or *via* handling BPA-containing thermal paper of supermarket receipts (Lu et al., 2013). Moreover, BPA may leach from food containers through hydrolysis of the plastic polymers into foodstuffs and beverages, thus entering the consumers and raising concerns for human health risks (Le et al., 2008; Wagner and Oehlmann, 2009; Cooper et al., 2011).

Consequently, the presumed harmful effects of BPA on living organisms have been extensively investigated in animals and human cell lines and BPA has been characterized as an endocrine disrupting chemical (Staples et al., 1998; Le et al., 2008; Clarke and Smith, 2011). BPA was shown to destroy the mitotic spindle arresting mitosis at metaphase and inducing the formation of micronuclei (Pfeiffer et al., 1997; Šutiaková et al., 2014), to disorganize the cytoplasmic MTs and generate rings and loops of tubulin (Lehmann and Metzler, 2004), to cause aneuploidy (Ochi, 1999; Tsutsui et al., 2000; George et al., 2008), genotoxicity (Tiwari et al., 2012), meiotic arrest (Can et al., 2005) and the formation of multipolar spindles (Ochi, 1999; Can et al., 2005; George et al., 2008).

On the other hand, much less information is available on the effects of BPA to plants. Nevertheless, a number of crop species hydroponically grown in 10 and 50 mg L^{-1} BPA solutions displayed species-specific phytotoxicity, morphological anomalies, bioaccumulation, clastogenicity and formation of micronuclei (Ferrara et al., 2006). BPA affected germination, elicited stress response and altered steroid hormone production in the pollen of kiwifruit (*Actinidia deliciosa* var. *deliciosa*) (Speranza et al., 2011). Onion (*Allium cepa*) root tip cells exposed to BPA suffered chromosomal and mitotic aberrations (Jadhav et al., 2012). Moreover, BPA decreased the growth indices and photosynthetic parameters of soybean seedlings (Qiu et al., 2013), while in *Arabidopsis thaliana* it influenced leaf blade differentiation significantly (Pan et al., 2013). In pea (*Pisum sativum*), 0.044–0.44 mM BPA prevented chromosome segregation, hampered the completion of cytokinesis, deranged interphase and mitotic MT arrays and induced the formation of macrotubules (Adamakis et al., 2013).

Gymnosperms are a peculiar plant group as the spindle poles of their cells are known to display special centrosomal properties, similar to animal MTOCs (Wang et al., 1991; Fowke, 1993; Zachariadis et al., 2004). Since BPA induced the formation of multipolar spindles in animal and human cells (Ochi, 1999; Can et al., 2005; George et al., 2008), the objective of the present study was to investigate whether BPA might cause similar effects to gymnosperms. In particular, the impact of BPA on the MT arrays of dividing root tip cells of the cephalonian fir (*Abies cephalonica*) was studied with confocal laser scanning microscopy (CLSM) after immunolocalization of MTs and fluorescent staining of chromosomes. The effects of BPA on the organization of the endoplasmic reticulum (ER) in dividing cells and recovery of oryzalin-treated cells in the presence and absence of BPA were also studied.

2. Materials and methods

2.1. Plant material and preliminary BPA experiments

Seeds of the Greek endemic cephalonian fir (*A. cephalonica* Loudon) were germinated in commercial peat at room temperature (~21 ± 1 °C) for about 20–25 days. For experimentation, seedlings were removed from the peat medium, washed gently in tap water and transferred in Petri dishes containing the desired concentration of BPA. In a preliminary experiment, seedlings were exposed to 0.088, 0.22 and 0.44 mM BPA (20, 50 and 100 mg/L BPA respectively) for 8, 12, 24 and 48 h, while others were placed in water and used as controls. The root length of each individual seedling was recorded and compared with that of control seedlings. Experimentation was conducted in three replicates, in a sample size n = 7 in each replicate.

For the germination tests seedlings were germinated in petri dishes on filter paper soaked with distilled water (control) and 0.44 mM BPA. Experimentation was conducted in three replicates, in a sample size n = 6 for each replicate. The filter papers were replaced weekly with fresh ones. The germination index was calculated on the 20th day in both control and 0.44 mM BPA-treated seedlings [$SGI = \sum(Gt/Tt)$, where Gt is the number of the seeds on Day t, and Tt is time corresponding to Gt in days]. Afterwards the vigor index ($VI = GI \times Rs$, where Rs is root length) was calculated (Zhang et al., 2007; Kaya et al., 2008).

All chemicals and reagents used were purchased from Sigma–Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany) and Applichem (Darmstadt, Germany), unless otherwise stated. A 0.88 mM stock BPA solution was prepared in double distilled water [solubility in water = 120–300 mg L^{-1} (0.528–1.320 mM) at 21.5 °C, pH 7.0] (Staples et al., 1998).

2.2. Imaging of MTs and chromosomes

Samples of all combinations were prepared and tentatively examined by CLSM to determine the most suitable experimental conditions. Judging from the microscopic images of dividing and interphase cells, we considered that the best BPA concentration at which a quick and clearly visible effect could be observed was 0.44 mM at 3, 6 and 12 h. This was supported by the BPA impact on root growth of treated seedlings (Fig. 1). All experimentation was conducted at three replicates.

After the preliminary trials, seedlings were exposed to 0.44 mM BPA for 3, 6 and 12 h, while other seedlings were placed on distilled water as control. Then, the root tips were excised and prepared for tubulin immunolabelling for MT imaging and DNA staining for chromatin or chromosome visualization as previously described by Adamakis et al. (2013). Cell preparations were examined with a Nikon D-Eclipse C1 CLSM with the appropriate filters, at an optical sectioning step of 0.30 μm. Special care was taken in order to retain the laser beam gain equal among the different treatments. Image recording was done with proper software (EZ-*C1* 3.20) according to the manufacturer's instructions.

2.3. Imaging of endoplasmic reticulum

To observe the ER, the same experiments and protocol as above were conducted, except for the antibodies: the monoclonal mouse anti-HDEL (the tetrapeptide histidine, aspartic acid, glutamic acid, leucine) antibody 2E7 (Santa Cruz) was used in dilution 1:40,