Journal of Trace Elements in Medicine and Biology 28 (2014) 80–86

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.de/jtemb

Physiology

Effect of copper on pro- and antioxidative reactions in radish (*Raphanus sativus* L.) *in vitro* and *in vivo*

Alexander Lukatkin[a], Irina Egorova[a], Irina Michailova[a], Przemysław Malec[b,*], Kazimierz Strzałka[b]

[a] *Department of Botany and Plant Physiology, N.P. Ogarjov Mordovia State University, Bolshevistskaja Str. 68, 430005 Saransk, Russia*
[b] *Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, ul. Gronostajowa 7, 30-387 Kraków, Poland*

ARTICLE INFO

Article history:
Received 17 January 2013
Accepted 9 November 2013

Keywords:
Raphanus sativus
Callus
Copper
Oxidative stress
Antioxidant enzymes

ABSTRACT

The generation of superoxide radicals, lipid peroxidation (as measured by malone dialdehyde formation) and the activity of selected antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase) were assessed in radish (*Raphanus sativus* L.), in response to elevated concentrations of copper ions in the culture medium *in vitro* and *in vivo*. Experiments were performed on 7-day-old seedlings and 5-week-old calluses grown on media supplemented with $CuSO_4$ in concentrations of 10, 100 and 1000 μM. The exposure to elevated Cu concentrations in the medium significantly reduced both callogenesis and the proliferation of radish calluses *in vitro*. Cu treatment resulted in the increased generation of the superoxide radical ($O_2^{\bullet -}$) in radish seedlings and calluses indicating the occurrence of oxidative stress in radish cells, whereas the level of lipid peroxidation (LPO) remained unchanged. Both in calluses and in radish seedlings *in vivo*, the relative level of oxidative stress was maximal at micromolar Cu concentrations and became attenuated with increasing Cu concentrations. Stronger oxidative stress occurred in the radish seedlings *in vivo*, compared with radish calluses *in vitro*. The observed lower sensitivity of calluses to Cu-induced oxidative stress and their ability to proliferate upon exposure to Cu concentrations of up to 1000 μM demonstrate the potential of *in vitro* cell-selection to obtain metal-tolerant radish plant lines.

Introduction

Copper (Cu) is an essential micronutrient for plants [1,2]. Due to its redox properties, copper is an indispensable component of many of the metalloenzymes that are involved in a variety of metabolic pathways, predominantly those accompanied by redox processes [1,3,4]. In the past decades, Cu has emerged as one of the major environmental pollutants because of its extensive use in many industrial and agricultural applications [5–7]. The exposure to elevated Cu concentrations causes a range of deleterious effects in plants, such as inhibition of photosynthetic and respiratory activity, inhibition of pigment biosynthesis and damage to membrane permeability [3,8,9]. In consequence, Cu exposure results in a profound reduction in growth rates and biomass production [10–12].

It is commonly accepted that oxidative stress plays an important role in copper toxicity to plants [13–15]. The synthesis of reactive oxygen species (ROS), comprising both free radical ($O_2^{\bullet -}$; $OH^{\bullet}$; $OH_2^{\bullet}$) and non-radical (H_2O_2) forms, is observed in plants under Cu-induced stress [16]. ROS formation leads eventually to cellular damage, metabolic disorders and accelerated senescence processes [17]. Particularly, the ROS – induced oxidation of lipids is destructive for biological membranes. This process generates large quantities of MDA (malone dialdehyde), which is commonly used as an indicator of lipid peroxidation (LPO) [18].

In order to overcome oxidative stress, plants have developed two main antioxidant defense mechanisms, which can be classified as non-enzymatic and enzymatic systems. Among the plant enzymes involved in antioxidant cell-defense responses the superoxide dismutase (SOD), catalase (CAT) and peroxidases (POX) were found to be specifically activated by exposure to elevated Cu concentrations [19].

Radish (*Raphanus sativus* L.) from *Cruciferae* is an important crop, cultivated worldwide for its nutritional values and also as a source of the peroxidases and isothiocyanates used in medicine [20]. Twenty years ago, radish was proposed as a model plant for studying responses to environmental pollutants [21]. More recently, there have been an increasing number of studies on HM

Abbreviations: 2,4-D, 2,4-dichlorophenoxy acetic acid; 6-BAP, 6-benzylaminopurine; APOX, ascorbate peroxidase; CAT, catalase; HM, heavy metals; LPO, lipid peroxidation; MDA, malone dialdehyde; MS, Murashige and Skoog medium; NBT, nitroblue tetrazolium; $O_2^{\bullet -}$, superoxide radical; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid.

* Corresponding author. Tel.: +48 12 664 65 20; fax: +48 12 664 69 02.
E-mail addresses: aslukatkin@yandex.ru (A. Lukatkin), przemyslaw.malec@uj.edu.pl (P. Malec).

0946-672X/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.jtemb.2013.11.002

effects in radish both *in vivo* [22–26] and *in vitro* [27]. The determination of physiological responses and tolerance mechanisms of this plant to HM is of particular interest, because radish is grown often in urban/suburban areas, where crops may be exposed to elevated metal pollution [28–30]. Due to its ability to bioaccumulate and concentrate HM in hypocotyls and shoots, radish is also investigated as a species potentially useful for the phytoextraction of metals from contaminated soils [29,31,32]. In particular, radish has been used as a model plant to study Ni and Cu stress effects on the activities of antioxidant enzymes (ascorbate peroxidase, superoxide dismutase, catalase, monodehydroascorbate reductase, dehydroascorbate reductase, guaiacol peroxidase and glutathione reductase), lipid peroxidation, proline and protein content [25,26], as well as Cd-induced stress leading to changes in the anatomical and morphological characteristics of roots, stems and leaves [23]. However, there is limited information on HM effects in radish callus cultures.

In vitro tissue cultures constitute an important tool to study the physiological and biochemical mechanisms that operate in response to stress at the cellular level. Furthermore, a plant tissue culture allows the control of stress homogeneity and a characterization of cell behavior under stress, independently of the regulatory systems that operate at the whole plant level [33]. Also, the cell culture method can be useful for improving metal tolerance through the *in vitro* selection of tolerant cell lines able to regenerate plants. The selection and characterization of various cell cultures tolerant to heavy metals have been documented [34,35].

In this study we compare selected oxidative and antioxidative processes induced by elevated concentrations of Cu ions in seedlings and tissue cultures of radish. The Cu concentration range included concentrations from supra-optimal to sublethal: 10 μM being a supra-optimal (not destructive) concentration, 1000 μM a sublethal concentration, and 100 μM an intermediate concentration. The differences both in the efficiency of ROS generation and in the activation of antioxidative enzymes *in vitro* and *in vivo* are compared.

Materials and methods

Plant material and growth conditions in vivo

Radish seeds (*R. sativus* L. cv. Red Giant) were commercially purchased. The seeds were placed on filter paper bridges in test tubes on sterilized distilled water (5 ml per seed) with increasing concentrations of $CuSO_4 \times 5H_2O$ (10 μM; 100 μM; 1000 μM). Seedlings were grown for 7 days in stable conditions at a temperature of 22–25 °C, and at an illumination of c.a. 80 μmol photons m^{-2} s^{-1}, photoperiod 12/12 h (day/night). The control seedlings were grown up on distilled water without copper ions.

Induction and growth of radish callus

Seeds of *R. sativus* L. were surface sterilized with 0.5% $KMnO_4$ (20 min), 6% chloramine (w/v, 10 min) and 70% ethanol (v/v, 1 min). Subsequently, they were rinsed three times with sterile distilled water. Then the seeds were grown on bridges of filter paper in sterile test-tubes. Explants from 7-day old sterile plants were cultured on an agarized MS medium. Standard macro- and microsalt MS media [36] were supplemented with 30 g L^{-1} of sucrose, vitamins (0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine, 0.1 mg L^{-1} thiamine), 2 mg L^{-1} glycine, plant growth regulators: 2,4-dichlorophenoxyacetic acid (2,4-D; 2–5 mg L^{-1}) and 6-benzylaminopurine (6-BAP; 1–2 mg L^{-1}), the gelling agent (agar 7.5 g L^{-1}), and different concentrations of $CuSO_4 \times 5H_2O$ (10, 100 and 1000 μM), and adjusted pH 5.7–5.8. The concentration of Cu in the control MS medium was in the range of 0.1 μM. Explants for callus induction were taken from the upper (hypocotyls and cotyledons) and lower (root) parts of seedlings. The cultures were maintained in a thermostat at a temperature of 25 °C in darkness. The callus culture was grown in 5 weeks.

Determination of the rate of superoxide anion radical generation

Superoxide generation was analyzed as described elsewhere [37]. Seedling or callus tissue (300 mg) were homogenized in 15 ml of distilled water. Then the homogenate was centrifuged 15 min at 4000 × *g*. 100 μl of 0.01% (w/v) adrenaline (epinephrine) solution was added to 3 ml of the supernatant, and incubated for 45 min at room temperature and 80 μmol photons m^{-2} s^{-1} illumination. The optical density of the adrenochrom formed was measured immediately after incubation against homogenate supplemented with 100 μl of water at a wavelength of 480 nm. The rate of generation $O_2^{\bullet -}$ was calculated in μM g^{-1} FW min^{-1} ($\varepsilon = 4020\ M^{-1}\ cm^{-1}$).

Determination of lipid peroxidation (LPO)

LPO was analyzed according to Lukatkin and Golovanova [38] with modifications. Samples of seedling or callus tissue (300 mg) were homogenized in a 10 ml isolation medium (0.1 M Tris–HCl buffer pH 7.6, containing 0.35 M NaCl). 2 ml of thiobarbituric acid (TBA) in 20% trichloracetic acid was added to 3 ml of homogenate, heated in a boiling water bath for 30 min and filtered through white BFS filter paper. Optical density was recorded at 532 nm against an analogously prepared isolation medium with reagents. The concentration of malone dialdehyde (MDA) was calculated using the molar extinction coefficient ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$) and the quantity of MDA in the leaves and calluses was calculated in micromoles per gram of fresh weight of tissue [37].

Determination of superoxide dismutase (SOD; EC 1.15.1.1) activity

SOD activity was analyzed according to Kumar and Knowles [39] with modifications. 300 mg of seedling or callus tissue were homogenized in 20 ml of a phosphate buffer (0.067 M KH_2PO_4 and 0.067 M Na_2HPO_4) pH 7.8, then centrifuged 15 min at 7000 × *g* and supernatant was used as crude extract of cytosolic SOD. 2.6 ml of this fraction was supplemented with the following components of the reaction medium: 0.1 ml of 13 mM L-methionine, 0.05 ml of 75 mM NBT, 0.1 ml of 0.1 mM EDTA, and 0.1 ml of 2 mM riboflavin. The reaction was started by adding riboflavin, followed by incubation for 20 min on a white light (fluence rate of 160 μmol photons m^{-2} s^{-1}). Measurements were taken at 560 nm, against control tubes kept in the dark. The SOD activity was estimated using the difference in concentration of formazan formed in the absence and in the presence of the plant material. A unit of SOD activity was defined as a half-maximal inhibition [40].

Determination of catalase (CAT; EC 1.11.1.6) activity

CAT activity was analyzed according to Kumar and Knowles [39] with modifications. 1 g of seedling or callus tissue were ground in a mortar with 10 ml of 50 mM phosphate buffer (pH 7.0). The resulting homogenate was filtered and centrifuged 10 min at 8000 × *g*. The reaction mixture consisted of 2.2 ml of phosphate buffer, 100 μl of obtained enzyme extract and 70 μl of 3% H_2O_2. The control consisted of a mixture of 2.3 ml of phosphate buffer (pH 7.0) and 70 μl 3% H_2O_2. Hydrogen peroxide was poured directly before the measurement of optical density at a wavelength of 240 nm. Enzyme activity was calculated in mM g^{-1} FW min^{-1}, based on the fall of