Microchemical Journal 128 (2016) 305–311

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Direct analysis of elemental biodistribution in pea seedlings by LA-ICP-MS, EDX and confocal microscopy: Imaging and quantification

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ARTICLE INFO

Article history:
Received 3 April 2016
Received in revised form 11 May 2016
Accepted 12 May 2016
Available online 13 May 2016

Keywords:
LA-ICP-MS
EDX
Confocal microscopy
Elemental distribution
Phytoremediation
Metal transport

ABSTRACT

Qualitative and quantitative methods were developed for cadmium, lead, cooper and zinc mapping and quantification in root of pea *Pisum sativum* L. Plants were cultivated hydroponically in a Hoagland medium with the addition of 50 μM of: $CdCl_2$, $Pb(NO_3)_2$, $CuSO_4$, or $ZnSO_4$. After 48 h, parts of roots were cut and analysed. LA-ICP-MS was used for *in vivo* quantitative imaging of the distribution of metals in thin sections of plant tissues. Calibration curve was obtained using standard addition method NIST SRM 1515 Apple Leaves spiked with standard and application of sulfur isotope as an internal standard. Energy Dispersive X-ray microanalysis Spectroscopy (EDX) was used in order to confirm results obtained by LA-ICP-MS and identify the exact localization of metals deposits in root tissue. In the electron-microscopic photographs we observed deposits of metals in the cell wall, cell membrane, vacuoles, cytoplasm and organelles, such as: mitochondria and peroxisomes. Formation and localization of O_2^- and H_2O_2 in cross-sections of root tips was confirmed by confocal microscopy. The presence of metals induced ROS (reactive oxygen species: O^{2-}, H_2O_2) production in areas corresponding to patterns of metals accumulation.

1. Introduction

Trace element contamination of soil due to natural or anthropogenic activity (mainly urbanization and industry) threatens ecosystems, surface, and ground waters, food safety, and human health, as well as decrease the plant yield [1]. Elements can be divided into: indispensable (essential) for the proper functioning of the plant cell or unnecessary — not used in cellular metabolism and toxic even in small quantities (*e.g.* Cd, Pb, Hg, Al) [2]. Essential elements also induce toxicity symptoms if they are present in excess [3,4]. These elements – physiological but toxic in excess – are commonly called "metal(loid)s" according to proposition made in an interesting review by Duffus, 2002 [5]. Mn, Zn, Cu, Mo are examples of essential metals in plants, *e.g.*: copper is a cofactor in enzymes such as Cu-SOD, laccase and plastocyanin, participant in cell wall metabolism and stress response, and also acts as component of the electron transport chain [6]. Zinc is an enzyme cofactor in electron transport and antioxidant metabolism, and also a component of transcription factors [7].

In recent years, major scientific progress has been made in understanding the physiological mechanisms of elemental uptake and transport in plants [8–10]. A much larger portion of the metal(loid)s after uptake to plants is accumulated in the roots [11]. Part of the metal(loid)s is transported then from the roots to the shoots by xylem [12–14]. In plant cells, the metal(loid)s are transported in a bound form to decrease their reactivity; they can be bound by amino acids, peptides, proteins, organic acids, and often deposited in vacuoles and cell wall [14].

A lot of studies provide quantitative information on uptake of metal (loid)s after acid digestion of the ground samples, which can be determined by methods such as ICP-OES or ICP-MS. Much less is known regarding the distribution of the metal(loid)s in the different cells and tissues of the plant, especially *in vivo*. Laser ablation connected with ICP-MS or ICP-OES is an excellent method for direct *in vivo* determination of elemental distribution in plant samples. Additionally, this technique allows to pinpoint the metal pathway in a plant tissue [15–21]. For example, different spatial distribution of Fe was found in transgenic and non-transgenic soybean leaves by Oliveira and Arruda [16]. Fe is homogenously distributed in the whole transgenic soybean leaf, while in the non-transgenic leaf this element is greatly concentrated in the main vein and nerves. In another study Basnet et al. [17] applied laser ablation for trace metal spatial distribution in rice (*Oryza sativa*) seeds from the active Xikuangshan Sb mine area in China. They concluded that bioimages of elements suggest that both Sb and Cd may be competing with Zn for binding sites. Wu et al. [18] showed that the embryo accumulated high concentrations of nutrient elements, such as Fe, K, Cu,

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http://dx.doi.org/10.1016/j.microc.2016.05.008
0026-265X/

and Zn. However, Ca was accumulated in the bran of the wheat which might be attributed to its function in structural maintenance. In the endosperm, the majority of the nutrients were located in the aleurone layer. However, in order to improve knowledge about uptake, translocation, and detoxification of metals in plants LA-ICP-MS must be supported by the use of electron microscopy with X-ray microanalysis (EDX) and confocal microscopy. The aim of the study was to show and quantify the distribution of metals (Cu, Zn, Cd, Pb) in plant tissues, at the organ, cellular and subcellular level, with respect to the negative impact caused by these elements. For this purpose we have used complementary methods: LA-ICP-MS for the direct *in vivo* analysis, EDX for the metals measurement from the fixed samples and confocal microscopy for the *in vivo* imaging of the generation of reactive oxygen species (superoxide anion and hydrogen peroxide).

2. Materials and methods

2.1. Plant material

Pea seedlings (*Pisum sativum* L., cv. Bohun) were grown hydroponically on the Hoagland medium for 72 h in a growth chamber with 16/8 h photoperiod, day/night at temperature of 25 °C and light intensity of 82 μmol m^2 s^{-1}. Then, the medium was changed to 100×-diluted Hoagland medium and the $Pb(NO_3)_2$, $CuSO_4$, $CdCl_2$, $ZnSO_4$ solutions were added so that the final the concentration of 50 μM of metal ion was achieved. The roots were harvested after 0, 24, 48 and 72 h of cultivation. Heavy metals accumulated on the surface of roots were washed with 10 mM of $CaCl_2$ and 10 mM EDTA.

2.2. Sample preparation

Samples of roots were collected and prepared according to the measurement technology applied subsequently. To ensure statistical significance, plant material was collected and analysed separately from three different cultures grown during one vegetation season.

2.2.1. Laser ablation

Treated and control plant material was prepared with two methods: 1) 3 mm thick cross-sections were cut from roots and stems and were analysed *in vivo*; 2) roots and stems were ground, sieved, divided into 200 mg equal portions and pressed into ~12 mm diameter discs of ~2 mm thickness.

2.2.2. Energy Dispersive X-ray microanalysis (EDX)

The root tips of pea plants, 4–6 mm long, were fixed with 2% glutaraldehyde and with 2% formaldehyde in 0,05 M cacodylate buffer of pH 6.8 at 0 °C for 4 h. After fixation, the samples were rinsed with 0.05 M cacodylate buffer, then, the samples were postfixed in 1% (v/v) OsO4 and dehydrated in a series of alcohols and acetones at increasing concentration up to 90% alcohol at 0 °C, and then at room temperature. The samples were embedded in Spurr's resin and cut to ultrathin (c. 100 nm) sections using the Reichert Ultra 5 microtome (Wien, Austria). In order to enhance electric conductivity, the slides were dusted with coal. The surveyed material was stained with osmium tetroxide during processing to improve image contrast, except for samples treated with Pb, because Os and Pb spectra overlap at certain energies.

2.2.3. Confocal microscopy

After 24-h cultivation with Pb, Cu, Cd, Zn ions pea roots were submerged for 12 h in 100 μM solution of $CaCl_2$ containing 20 μM of dihydroethidium (DHE), pH 4.75 and in 4 μM dichlorodihydrofluorescein diacetate (DCFH-DA) solution with 5 mM dimethyl sulfoxide (DMSO). After rinsing with 100 μM of $CaCl_2$ or 50 mM phosphate buffer (pH 7.4) the roots were cut, placed on 20–30 μm slides and observed under a confocal microscope.

2.3. Instrumentation

2.3.1. Laser ablation

We used ICP-MS spectrometer model Elan DRC II (Perkin-Elmer Sciex, Canada) equipped with an Nd:YAG laser ablation system (LSX-500, CETAC Technologies, Omaha, NE, USA) operating at wavelength of 266 nm. The optimization of the LA-ICP-MS conditions was performed by ablating a standard reference glass material NIST SRM610 to obtain maximum signal intensity for $^{7}Li^{+}$, $^{115}In^{+}$ and $^{238}U^{+}$, while keeping $^{232}Th^{16}O^{+}/^{232}Th^{+} < 0.2\%$ and the ratio of $^{42}Ca^{2+}/^{42}Ca^{+} < 0.5\%$.

The accuracy of the results obtained with LA-ICP-MS method depends on: the distribution of the analyte on a sample's surface, homogeneity of the matrix and geometry of the sample. The exact description of laser parameters optimization has been described previously by Hanć et al. [22]. Table 1 shows the operating parameters used for the EDX and LA-ICP-MS and confocal microscopy measurements.

2.3.2. Energy Dispersive X-ray microanalysis (EDX)

For the X-ray analysis of elements we used transmission electron microscope JEM 1200EX of the JEOL company, with a scanning attachment and energy dispersion system (EDX) Link AN 10000 of the Link company. The point analyses of 10–20 nm in diameter or of the areas of changing value were conducted at an accelerating voltage of 80 keV. The time of registration of the signals forming the spectrum was 400 s for each point analysis.

Table 1
Optimized experimental parameters used for Cd, Cu, Pb and Zn determination and imagining by LA-ICP-MS, EDX and confocal microscopy.

Laser ablation	
Laser ablation system	CETAC LSX-500, Nd:YAG
Wavelength, nm	266
Pulse duration, ns	5
Ablation frequency, Hz	10
Spot size, μm	50
Laser energy, mJ	8.1
Scan rate, μm/s	25
Scan method	single line scan; peak hoping
ICP-MS	
Instrument	PE Sciex ELAN 6100 DRC II
Nebulizer gas flow, L/min	0.85–0.9
Auxiliary gas flow, L/min	0.8
Plasma gas flow, L/min	15
RF power, W	1250
Lens setting	autolens calibrated
Detector mode	dual (pulse counting and analogue mode)
Sweeps/Reading/Replicate	2/180/1
Measured mass	^{111}Cd, ^{65}Cu, ^{208}Pb, ^{66}Zn
Internal standard	^{34}S
EDAX	
Instrument	transmission electrone microscope JEM 1200EX (JEOL company, Japan)
Scanning attachment and energy dispersion system	(EDX) Link AN 10000 (Link company)
Camera	CCD MORADA (SiS-Olympus)
Point analysis	10–20 nm diameter
Voltage	80 keV
Time of registration	400 s
Confocal microscopy	
Instrument	model Zeiss LSM 510, Axiovert 200 M, Jena, Germany
Filter	no. 10
Excitation	450–490 nm
Emission	520 nm or more
Gas laser	argon
Instrument	transmission electrone microscope JEM 1200EX (JEOL company, Japan)
Scanning attachment and energy dispersion system	(EDS) Link AN 10000 (Link company)
Camera	CCD MORADA (SiS-Olympus)
Point analysis	10–20 nm diameter