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KS: concept development and study design, conducting experiments, data acquisition and analysis, drafting of manuscript; JS: measurement and interpretation of UV-VIS spectra and reflectance spectra, analysis of lead content in samples; AS: interpretation of results, critical revision of manuscript; EG: revision and critical evaluation of manuscript for intellectual content; EK: responsibility for integrity of data and accuracy of data analysis, critical revision of manuscript; all authors discussed the results and implications, and commented on the manuscript at all stages

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Competing interests

No competing interests have been declared.

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SHORT COMMUNICATION

Critical factors besides treatment dose and duration need to be controlled in Pb toxicity tests in plant cell suspension cultures

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Abstract

The study was designed to determine the proper conditions for suspension culture of *Viola tricolor* cells in toxicity studies of Pb at different concentrations (0, 200, 500, 1000, 2000 μM) and exposure times (24, 48, 72 h). By forming insoluble salts with ions from the medium, lead (II) nitrate added to the medium decreased the initial 5.7–5.8 pH of the medium, depending on the Pb salt concentration and light intensity.

In alamarBlue assays, we found no dose- or time-dependent effect of Pb on cell viability when we did not adjust pH and did not standardize the illumination conditions to correct the effect of lead-salt-induced turbidity.

When effective illumination was adjusted to correct for turbidity at the highest lead concentration and pH was adjusted to 5.7–5.8, cell viability decreased with the increase of $\text{Pb}(\text{NO}_3)_2$ concentration and with treatment time. These experiments demonstrate that the toxic action of lead on cells in suspension depends strongly on culture conditions, and not only on the metal concentration and duration of treatment.

Keywords

Viola tricolor; lead (II) nitrate; cell suspension culture; cell viability; alamarBlue assay; reflectance

Introduction

Lead is a dangerous element and a pollutant; it is toxic to plants, humans and other animals, and is not biodegradable or only barely so [1,2]. Elevated concentrations (>650 ppm) of Pb in soil affect physiological processes by reducing the availability of various macro- and microelements, changing the water balance and thereby inhibiting photosynthesis, and altering phytohormone status, cell membrane permeation, and cell membrane structure [3]. Lead is difficult to remediate because it forms sparingly soluble salts in soil [4]. The solubility of lead depends on soil pH [5] and ligand binding [6]. Cell suspension culture is a model used for studying cells' tolerance and survival under exposure to heavy metals. It has been employed to estimate lead accumulation and toxicity in cells of the metal hyperaccumulator *Jatropha curcas* [7] and the non-metallophytes *Catharantus roseus* [8] and *Prosopis laevigata* [9]. Usually, the level of cells' tolerance under treatment with heavy metals is evaluated by biomass measurements or by establishing the frequency of viable cells with the use of different staining techniques [10,11].

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In media, lead forms insoluble salts with elements or compounds such as sulfur, phosphates, iodides, chlorides, borates, or insoluble hydroxides [12]. The formation of insoluble compounds changes the conditions of culture dramatically, altering the pH of a liquid medium and also its turbidity; the latter reduces the intensity of light supplied to cells in suspension.

Viola tricolor L. is a metal-tolerant species colonizing old zinc-lead waste heaps containing high concentrations of Zn, Pb, and Cd in southern Poland [13,14]. The present experiments using this species were designed to examine whether plant cell viability in suspension depends not only on the toxic effect of Pb ions but also on the changes in pH and effective illumination that are caused by adding lead salt to the medium.

Material and methods

The *Viola tricolor* L. plants used in these experiments originated from seeds harvested from natural populations in Zakopane (Tatra Mts, southern Poland, N 49°20'09", E 20°00'05"). The seeds were germinated and then callus was induced from seedling leaves on MS medium [15] solidified with agar and supplemented with 2,4-D (2 mg L⁻¹) combined with BAP (2 mg L⁻¹). A gram of fragmented 3-month-old callus was transferred to an Erlenmeyer flask filled with 25 mL MS liquid medium containing the same plant growth regulators as for callus induction. Medium pH was adjusted to 5.7–5.8 using 0.1 M HCl or 0.1 M NaOH. The cultures with callus and derived cells were shaken constantly on a rotary shaker (WL-972; JW ELECTRONIC, Poland). Suspension culture in exponential growth phase was used for the Pb toxicity tests. For *V. tricolor*, this phase occurred 10 days after culture initiation.

All cultures were maintained in stable growth-chamber conditions at 25 ± 3°C with a 16-h photoperiod under cool-white fluorescent lamps (flux 70–100 μmol m⁻² s⁻¹).

Different concentrations of Pb (200, 500, 1000, 2000 μM) in the form of Pb(NO₃)₂ salt were applied to test cell viability under Pb exposure for 72 h.

Experiment No. 1: Pb(NO₃)₂ at the different concentrations was added directly to 25 mL of cells in liquid medium at pH 5.7–5.8. The pH of each medium was established after adding Pb salt.

Experiment No. 2: Pb(NO₃)₂ at the different concentrations was added directly to liquid MS media. Medium pH in this treatment was adjusted with 0.1 M NaOH to pH 5.7–5.8. In parallel with pH adjustment, the effective illumination of the same flasks was adjusted to correct for turbidity at the highest concentration of lead, by using a black marker to block enough light to make its intensity among all flasks equal to that in the treatment with the highest concentration of Pb(NO₃)₂. To achieve this, ~1.5 mL of each medium dosed with Pb(NO₃)₂ at concentrations of 0 (control), 200, 500, 1000, and 2000 μM was measured by reflection-absorption infrared spectroscopy (RAIRS). The percentage of marker-obscured surface was established using average reflectance at 370–700 nm. The UV-VIS reflectance spectra were recorded with a Shimadzu UV-2101PC spectrophotometer fitted with an ISR-260 integrating sphere attachment. BaSO₄ was used as the internal reflectance standard. The Erlenmeyer flasks without pH adjustment were not obscured (experiment No. 1). In both experiments, medium without Pb ions served as the control. The experiments were done in triplicate.

Cell viability was estimated every 24 h in alamarBlue assays: 200 μL suspension was pipetted in three replicates per flask to a 96-well plate, after which alamarBlue stain was added to each well and the plates were incubated for 3 h in darkness on a rotary shaker. Then the number of living cells was measured with a FLUORO-microplate reader (BIO-TECH Instruments, USA). Cell viability at each time interval was estimated with respect to time zero of each treatment.

Results and discussion

In this work, we wanted to answer a number of questions: (i) does adding Pb salt change the medium pH; (ii) does the change in effective illumination caused by clouding of

Tab. 1 Average reflectance and total obscured surface of Erlenmeyer flask used for *Viola tricolor* suspension culture.

Concentration of Pb(NO ₃) ₂ (μM)	Average reflectance (% of light reflection)	Percent of darkness caused by lead compounds	Percent of obscured surface	Total obscured surface in mm ² /100 mL Erlenmeyer flask
2000	9.40	8.49	0.00	0.00
1000	5.46	4.55	3.94	509.99
500	3.09	2.18	6.31	816.77
200	2.52	1.61	6.88	890.55
0	0.91	0.00	8.49	1098.95

the medium after adding Pb salt affect the cells in culture; and *(iii)* do the suspended insoluble salts that form after adding Pb salt reduce its toxicity (estimated with alamarBlue assays) in a dose-dependent and/or duration-dependent manner.

Lead salt changed pH of the medium due to binding of lead ions to anions in the medium. Comparing pH of control media, adding Pb(NO₃)₂ decreased the pH of liquid MS medium from its initial value: 5.70 → 4.82 at 200 μM, 5.76 → 4.74 at 500 μM, 5.69 → 4.44 at 1000 μM, 5.72 → 3.45 at 2000 μM. Despite the formation of suspended insoluble

salts, lead was bioavailable in the medium, as found by AAS analysis of medium containing 2000 μM Pb and EDTA solution (data not shown). Another effect of adding Pb salt was reduction of effective illumination due to clouding of the medium by insoluble salts, which were formed by lead bound to components of the medium. Reflectance was ~1% for the control medium (Pb-salt-free); this critical parameter of cell culture conditions increased to ~9% in medium containing 2000 μM Pb (Tab. 1). The most important conclusion from these experiments is that these conditions dramatically influence the effect of Pb on cell viability. We found no significant effect of Pb in the experimental series in which the effect of Pb dosing on medium pH and effective illumination were not corrected (experiment No. 1). The viability curves oscillated around 100% at the highest (2000 μM) Pb concentration. Slight hormesis (increase of viable cells vs. control) was noted at 200 μM, the lowest Pb concentration (Fig. 1a). The negative effects of Pb was observed in the experimental series in which constant pH 5.7 was maintained and effective illumination was adjusted to the value in the treatment with the highest Pb concentration (experiment No. 2). The frequency of cells surviving at the highest lead concentration (2000 μM) decreased to 34% at 48 h and 10.5% at 72 h (Fig. 1b). In the

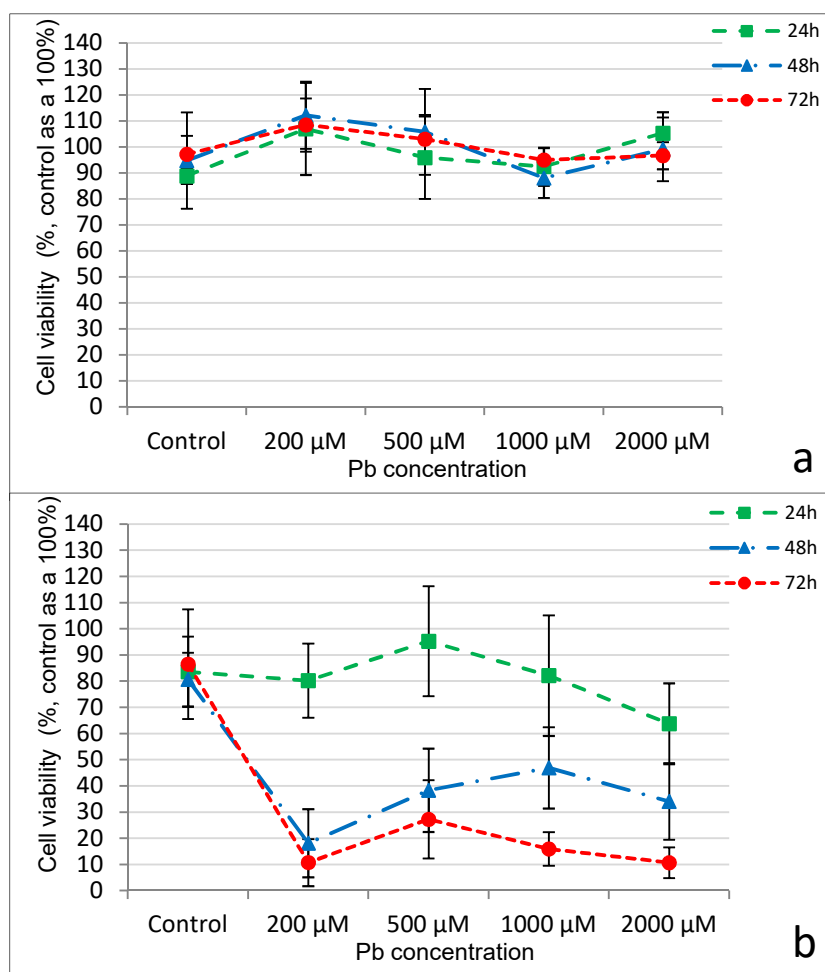


Fig. 1 Frequency of viable cells of *Viola tricolor* in suspension culture after treatment with different concentrations of Pb(NO₃)₂; without adjustment of culture conditions (pH, effective illumination) (a); after adjustment of culture conditions (pH, effective illumination) (b).

control conditions (0 μM) of both series, the frequency of viable cells was 80–90%, indicating that light intensity, the only differentiating factor, had no effect under stable 5.7 pH. At the highest $\text{Pb}(\text{NO}_3)_2$ concentration (2000 μM), the percentage of reflectance was the same in both experimental series but pH differed; medium pH influenced the effect of lead, as shown by the lack of a negative effect of Pb on cell viability at pH 3.4 and the drastic drop in the frequency of living cells at pH 5.7. Acidity was the factor affecting the toxicity of Pb, rather than changes in light intensity. As suggested in studies on *Oryza sativa* [16], low pH can affect the cell membrane, which forms an effective barrier to transport of ions into the cell via high H^+ activity, which inhibits Ca^{2+} -ATPase activity, reducing Pb^{2+} transport into the cell. Hydrogen ions may compete with metal cations for ligand binding sites on the cell and lessen the metal's interaction with cells [17]. Low pH reduces the kinetics of Na^+ , K^+ -ATPase in oocytes [18]. These factors block metal uptake by plant cells, limiting the influence of Pb on cell viability.

As demonstrated here, for lead toxicity studies of plant cells cultured in suspension, it is not enough to control the treatment concentrations and duration of exposure. Other culture conditions, if wrongly set or adjusted, can skew the results and lead to erroneous conclusions.

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