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Evaluation of phytotoxicity and cytotoxicity of industrial catalyst components (Fe, Cu, Ni, Rh and Pd): A case of lethal toxicity of a rhodium salt in terrestrial plants

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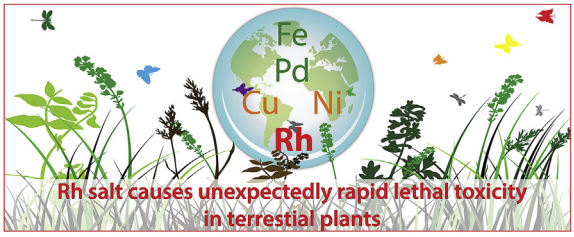
HIGHLIGHTS

- Toxicity of metal components of industrial catalysts (Fe, Cu, Ni, Rh and Pd) was evaluated.
- $RhCl_3$ caused extraordinarily rapid lethal toxicity in *Pisum sativum*.
- This effect considerably exceeded effects of other common metal pollutants.
- In contrast, $RhCl_3$ was relatively low-toxic in human fibroblasts.

GRAPHICAL ABSTRACT



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ABSTRACT

Until recently, chemical derivatives of platinum group metals have not been in a systematic direct contact with living organisms. The situation has changed dramatically due to anthropogenic activity, which has led to significant redistribution of these metals in the biosphere. Millions of modern cars are equipped with automotive catalytic converters, which contain rhodium, palladium and platinum as active elements. Everyday usage of catalytic technologies promotes the propagation of catalyst components in the environment. Nevertheless, we still have not accumulated profound information on possible ecotoxic effects of these metal pollutants. In this study, we report a case of an extraordinarily rapid development of lethal toxicity of a rhodium (III) salt in the terrestrial plants *Pisum sativum*, *Lupinus angustifolius* and *Cucumis sativus*. The growth stage, at which the exposure occurred, had a crucial impact on the toxicity manifestation: at earlier stages, $RhCl_3$ killed the plants within 24 h. In contrast, the salt was relatively low-toxic in human fibroblasts. We also address phytotoxicity of other common metal pollutants, such as palladium, iron, nickel and copper, together with their cytotoxicity. None of the tested compounds exhibited phytotoxic effects comparable with that of $RhCl_3$. These results evidence the crucial deficiency in our knowledge on environmental dangers of newly widespread metal pollutants.

1. Introduction

Biological activity of various metals and their influence on the

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living world is the central topic of Green chemistry and sustainable development (Giller and McGrath, 1988; Nriagu and Pacyna, 1988; Nel et al., 2006; Egorova and Ananikov, 2016; Martínez-Calvo and Mascareñas, 2018; Stephan, 2018). The probability for an organism to meet a certain metal is related to the content and distribution of the latter in the environment. The Earth's crust is rich in iron and also contains substantial amounts of nickel and copper (Kabata-Pendias, 2011). Thus, living organisms have been in direct contact with these widespread metals for a long time under natural conditions and have developed the necessary tolerance as a protective evolutionary mechanism (Fig. 1a). Moreover, some metals have acquired various functional roles in organisms (Finney and O'Halloran, 2003; Lu et al., 2009; Cvetkovic et al., 2010). The effect of these metals on the environment has been addressed relatively well in a number of studies (Sigel et al., 2011; Egorova and Ananikov, 2016, 2017).

In contrast, the quantities of platinum group metals (PGMs), such as Rh, Pd and Pt, are at the trace level in the Earth's crust. Distribution of these rare metals is not uniform and they are usually localized in some particular, relatively small areas. Therefore, living organisms have had no direct contacts with bioavailable forms of PGMs and have developed no evolutionary resistance mechanisms (Fig. 1b).

Very recently, the situation has changed dramatically and compounds of PGMs have come in direct contact with the biosphere due to anthropogenic factors (Egorova and Ananikov, 2016, 2017; Shahid et al., 2017). Automobiles can be listed among the most influential human inventions impacting the modern environment, and their widespread usage causes significant ecological concerns (Fig. 1c) (Battke et al., 2008; Šebek et al., 2011). PGMs have been spreading steadily in the environment via leaching from the automotive catalytic converters with exhaust gases, and the level of PGM contamination has reached up to 2 mg kg^{-1} in highway areas (Zereini and Wiseman, 2015). Leaching of palladium, platinum and rhodium from automotive catalytic converters is called among the main environmental dangers, together with emission of harmful gases (Fig. 1c). These metals are predominantly emitted as nanoparticles in the metallic or oxide forms, which dissolve readily and can be easily converted to bioavailable complexes (Colombo et al., 2008; Šebek et al., 2011; Pawlak et al., 2014; Wagner et al., 2014; Egorova and Ananikov, 2017). PGMs are prone to form complexes with various compounds found in the environment which can modulate and enhance their bioavailability (Colombo et al., 2008; Kabata-Pendias, 2011; Egorova and Ananikov, 2017; Zereini et al., 2017). Wiseman and co-workers have recently published an important study assessing the bioavailability of PGMs in road dust to reveal potential dangers relevant to human respiratory health (Wiseman et al., 2018). Apart from automotive converters, PGMs are also actively involved in various industrial applications due to advantages of catalytic technologies (Delidovich and Palkovits, 2016; Murzin, 2017).

Immobile organisms, such as plants, are first to be affected by pollutant metals and can serve as reliable indicators of the pollution (Gartside and McNeilly, 1974; Walley et al., 1974; Wu et al., 1975; Jamali et al., 2009). Plants growing on soils rich in heavy metals generally suffer from growth inhibition, and even essential microelements can be dangerous when in excess (DalCorso et al., 2013; Ovečka and Takáč, 2014; Singh et al., 2015; Egorova and Ananikov, 2017). In spite of several studies on possible toxic effects of PGMs in various ecosystems, the data relevant to the environmental exposure are limited (Borgmann et al., 2005; Battke et al., 2008; Zereini and Wiseman, 2015). Whereas toxicity of essential metals, such as nickel, copper and iron, has been actively explored, ecological effects of non-essential metals have not been comprehensively described so far (Palmer and Guerinot, 2009; Nagajyoti et al., 2010; Egorova and Ananikov, 2016). In particular, rhodium supposedly manifests the lowest phytotoxicity among PGMs (Kalavrouziotis and Koukoulakis, 2009; Sobrova et al., 2012). Only a few studies on rhodium bioaccumulation and toxic effects in plants are available due to low abundance of this metal in the

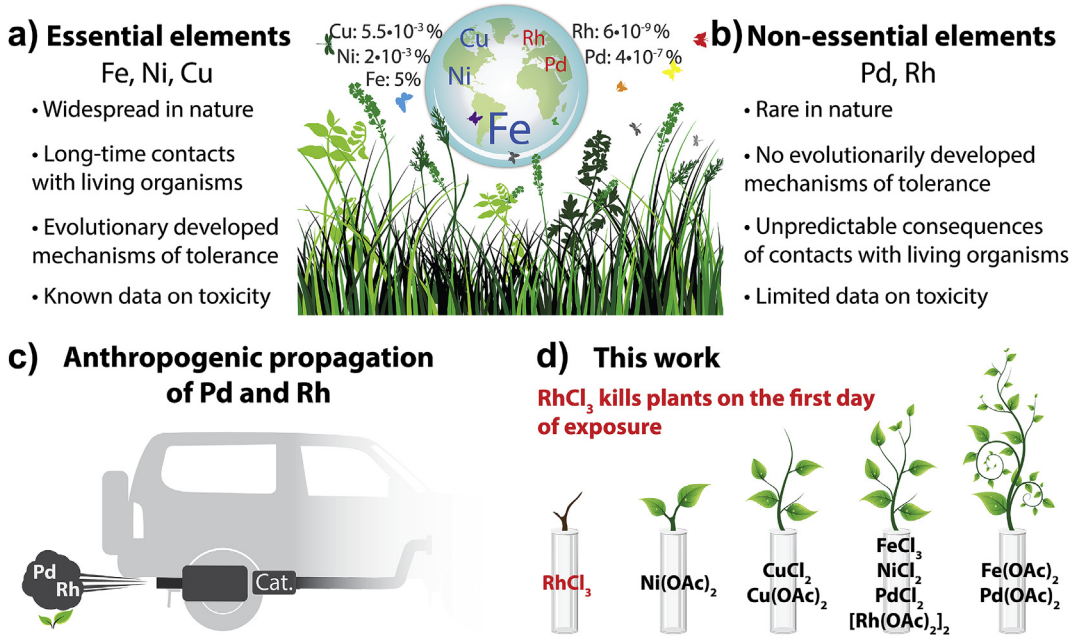


Fig. 1. Abundance of Fe, Ni, Cu, Pd and Rh on Earth. Essential (a) and non-essential (b) metals differ significantly in their abundance in nature and contacts with living organisms. The contents of iron, nickel, copper, palladium and rhodium in the Earth's crust are provided (based on data from ref. (Kabata-Pendias, 2011)). (c) In recent years, anthropogenic activity has led to propagation of rhodium and palladium in the environment. (d) A case of an extraordinarily rapid development of lethal phytotoxicity of $RhCl_3$ is reported in this work. (Images from www.all-free-download.com are used in the figure.)

environment (Fig. 1b) (Zereini and Wiseman, 2015). In soil experiments, rhodium demonstrated the highest solubility among PGMs; in spite of its lower amounts, its uptake rates are suggested to be higher than those of palladium and platinum (Zereini and Wiseman, 2015). Moreover, rhodium placental concentrations were found to correlate negatively with various infant parameters (Mikelson et al., 2019). However, rhodium showed significantly lower toxicity than palladium and platinum in experiments with *Caenorhabditis elegans* and *Daphnia magna* (Schertzinger et al., 2017; Zimmermann et al., 2017).

In this study, we report a case of an extraordinarily rapid development of lethal toxicity of a water-soluble rhodium (III) salt in the pea plant (*Pisum sativum*), as well as in two other terrestrial plants, lupine (*Lupinus angustifolius*) and cucumber (*Cucumis sativus*) (Fig. 1d). We also address phytotoxicity of other common metal pollutants (Pd, Fe, Ni, Cu) and compare it with the data on their cytotoxicity.

2. Materials and methods

2.1. Reagents and instruments

Metal salts used in the study (see Table S1) were obtained from 'Sigma-Aldrich', 'Acros', or 'Alfa Aesar'. $RhCl_3 \cdot xH_2O$ was obtained from 'Sigma-Aldrich', 'Alfa Aesar' and 'Krastsvetmet' (Russia). The possibility of contamination of the used batch of $RhCl_3 \cdot xH_2O$ by some highly toxic pollutant was ruled out by elemental analysis. All other chemical reagents were purchased from commercial suppliers and were tested by NMR before use. pH measurements were carried out using a Hanna Instruments HI2211-02 pH-meter. ^1H and ^{13}C NMR spectra were recorded using NMR spectrometers Bruker DRX 500 and Bruker Avance 400 with the residual solvent peak as an internal standard. The NMR spectra were processed using TopSpin 3.5. Mass spectra were measured on a high-resolution Time-of-Flight Bruker maXis instrument using electrospray ionization (ESI-MS). Measurements were performed in the positive ion mode, interface capillary voltage at 4.5 kV, effective scan range at *m/z* 100–1200, external calibration (ESI-L Low Concentration Tuning Mix, Agilent Technologies), direct syringe injection at flow rate of 3 $\mu L\,min^{-1}$, nitrogen as dry gas at 4 $L\,min^{-1}$, interface temperature at 180 °C. The spectra were processed using the Bruker Data Analysis 4.0 software package.

2.2. $Rh(acac)_3$ synthesis and solubility measurements

$Rh(acac)_3$ was synthesized according to the following procedure (Collins et al., 1995). Hydrated rhodium (III) chloride (0.209 g, 3.8 mmol) was dissolved in distilled H_2O (8 mL). pH of the solution was adjusted to the range 4.00–5.00 by dropwise addition of aqueous 10% $NaHCO_3$. If necessary, 0.1 M HCl was used as a back-titrating reagent. Then the deep purple solution was treated with 2,4-pentanedione (3.6 mL, 35.3 mmol) and was refluxed for 30 min. After allowing the solution to cool to room temperature, pH of the system was readjusted to the range 4.00–5.00. Precipitation of $Rh(acac)_3$ occurred during this process. This mixture (without filtering) was refluxed for 24 h, during which time the color of the reaction solution lightened considerably. The orange-yellow solid was collected by vacuum filtration and was recrystallized from boiling methanol. The hot methanol solution was slowly cooled, followed by further cooling in an ice-water bath to produce the orange-yellow crystalline product (0.136 g, 88%).

$Rh(acac)_3$. ^1H NMR ($CDCl_3$; δ, ppm): 2.16 (18H, s), 5.49 (3H, s). $^{13}C\{^1H\}$ NMR ($CDCl_3$; δ, ppm): 188.82, 99.39, 26.71. ESI-MS: calculated for $C_{15}H_{21}O_6RhNa$ $[M + Na]^+$ *m/z* 423.0285, found *m/z* 423.0279 ($\Delta = 1.4$ ppm). Anal. calcd for $C_{15}H_{21}O_6Rh$ (%): C, 45.02; H, 5.29. Found (%): C, 45.13; H, 5.33.

The water solubility of $Rh(acac)_3$ was measured as follows. A saturated solution of $Rh(acac)_3$ (~100 mL) was prepared at r.t. (23 °C) and then was filtered. Aliquots of 25 mL were taken and evaporated under reduced pressure. The procedure was repeated thrice. By the mass difference of the evaporating flask the desired value was measured. The $Rh(acac)_3$ solubility in water was 165 mg/L.

2.3. Elemental analysis by inductively coupled plasma emission spectrometry (ICP-ES) and inductively coupled plasma mass spectrometry (ICP-MS)

ICP-ES measurements were carried out in the Central Research Institute of Chemistry and Mechanics (Moscow, Russia). Dried plant tissues were used for the analysis. Samples were placed into ceramic bowls and were incinerated in a muffle furnace (3 h at 500 °C). The bowls were cooled and filled with a 1: 3 (v/v) mixture of HNO_3 and HCl. The resulting solutions were placed into graduated flasks and were diluted 2.5 times with bidistilled water. Then the solutions were filtered, and the measurements were conducted on an iCAP 6300 Duo emission spectrometer. Calibration plots for Rh are provided in the Supplementary data (Fig. S1).

ICP-MS measurements were performed using equipment of CKP FMI IPCE RAS (Moscow, Russia). The detection limits of the method were 10^{-4}-10^{-7}%, depending on the element; the accuracy of detection was 10% (relative).

Grinded samples with exact masses up to 100 mg were placed into glasses, into which 5 mL of 70% HNO_3 and 2 mL of 35% HCl were added. The mixtures were stirred thoroughly and were left for 20 min. Then the mixtures were brought to boil, were supplemented with 1 mL of 70% $HClO_4$ and were boiled down to the volume of 2 mL. The samples were transferred into graduated flasks and were diluted to the volume of 10 mL. Blank samples without test materials were prepared similarly to exclude the influence of the reagents used for the sample preparation on the analysis. The element contents were measured by a standard method of quantitative analysis on an Agilent 7500ce mass spectrometer. Calibration plots for ^{57}Fe, ^{60}Ni, ^{63}Cu, ^{103}Rh and ^{105}Pd are provided in the Supplementary data (Fig. S2).

2.4. Scanning electron microscopy

For SEM-EDX (scanning electron microscopy – energy-dispersive X-ray spectroscopy) measurements, samples of pea root cross-sections were mounted on a 15 mm aluminum specimen stub, were fixed by a conductive water-based carbon adhesive and dried under reduced pressure. Coating with a 15 nm carbon film was performed by using a Cressington 208 carbon coater. Observations were carried out using a Hitachi SU8000 field-emission scanning electron microscope (FE-SEM). Images were acquired in a secondary electron mode at 2 kV and 20 kV accelerating voltages. SEM-EDX studies were carried out using an Oxford Instruments X-max 80 EDX system at 20 kV accelerating voltage.

2.5. Phytotoxicity studies

The following plant species were used in the studies: *Pisum sativum* L. cv. Viola, *Lupinus angustifolius* L. cv. Nemchinovskii 846, and *Cucumis sativus* L. cv. Izyaschny. To reconstruct an actual event of pollution, we used the hydroponic cultivation method, in which a soluble metal salt was added to the growth medium only once, before the experiment (water was added as necessary to compensate absorption and evaporation). Therefore, the total amount of the salt changed only due to absorption of the ions by the plant or

due to salt transformations in the water solution. We avoided soil experiments on purpose due to a large impact of the chemical composition of soils on toxic effects of metals (Clemens et al., 2002; Nagajyoti et al., 2010; Antoniadis et al., 2017). Similarly, distilled water was used as a medium to avoid interactions of metal salts with nutrients. Application of any standard growth media, such as the Hoagland solution, might cause chemical reactions between the components of the medium and the test metal salts. Moreover, none of the observed phenotypic effects (necrosis, etc.) were detected in control plants grown in distilled water, so all these changes could be attributed exclusively to the action of the test substances. Therefore, we considered the seedlings grown in distilled water as a relevant control. Significantly higher metal concentrations (200 μM and 1000 μM) than those commonly mentioned in the literature for places of pollution (Pawlak et al., 2014) were employed in the experiments for simulating an accidental spillage. It should be noted that the content of PGMs in soil grows rapidly (Zereini et al., 2007; Zereini and Wiseman, 2015), and in some cases, the accumulation of significant amounts of traffic-related metals has been reported (Ruchter and Sures, 2015; Zereini and Wiseman, 2015).

In the experiments, 20 plants per point were used. The experiments with Rh salts were repeated at least 5 times to attest the inhibitory effects. Before the planting, seeds were soaked in distilled water and left to sprout for 4–5 days on wet filter paper in Petri dishes. Conical glass tubes (15 mL) were filled with salt solutions in two concentrations (200 μM and 1000 μM) (experiment) or distilled water (control), and the necks of the tubes were wrapped tightly with the plastic film Parafilm M (Bemis NA). Seedlings were weighed and measured; then the film layer on the tubes was pierced through and the root of a seedling was inserted into each solution. The tubes with the plants were placed on a rack under fluorescent lamps (two ULI-P16-10W/SPLE IP20 white 570 mm LED lamps, one ULI-P10-18W/SPFR IP40 white 550 mm LED lamp; long day conditions). The inhibitory influence of the media was assessed on the tenth day of the plant growth. The plants were weighed and measured again, and their biomass gain (the difference between the final weight and the weight of the seedling) and root elongation (the difference between the final root length and the length of the root of the seedling) were calculated. In the case of 1000 μM $RhCl_3$, the plant death was detected visually by drying of roots and shoots. To confirm that there was no detectable root elongation, the length of the plant roots was assessed on day 10, similarly to other samples. Statistical data processing was carried out using Microsoft Excel 2010 (Microsoft). The significance of differences between samples was assessed by the two-tailed Mann-Whitney test (Statistica 8.0, StatSoft).

To alleviate possible environmental effects on the plant growth, each experiment was supplemented with its own control group. For illustrative purposes, Δ between test and control samples was calculated on the basis of medians by using the following formula:

$$\Delta = \frac{GAIN(control) - GAIN(test)}{GAIN(control)}$$

Thus, Δ reflects the normalized difference between the biomass gain or root elongation in the control and test plants. In this system, 0 corresponds to zero effect, and higher values correspond to higher inhibition.

2.6. Cytotoxicity studies (MTS assay)

3215 LS cells (human fibroblasts; courtesy of E. Kopantsev, M.M. Shemyakin and Yu.A. Ovchinnikov Institute of Bioorganic Chemistry RAS) were cultured in clear plastic TC-treated dishes or multiple-well plates (Corning Inc., USA) in a HeraCell 150 incubator (Thermo Electron Corp., USA) at 37 °C, 95% humidity, and 5% CO_2. The DMEM/F-12 media (1:1) with 2.5 mM L-glutamine and 1.5 mM HEPES (HyClone, USA) supplemented with 10% fetal bovine serum (HyClone, USA), 100 un. mL^{-1} penicillin (OAO Sintez, Russia), and 100 μg mL^{-1} streptomycin (OAO Biokhimik, Russia), was used.

The MTS assay was employed for evaluating cytotoxicity of metal salts. Before the test, cells were released by trypsinization and seeded into 96-well flat-bottomed plates, 10,000 cells per well. The boundary wells were filled with 200 μL phosphate-buffered saline (HyClone, USA). Cells were cultivated until reaching 70% monolayer (40–48 h), and then were incubated with solutions of metal salts in the concentration range from 1–50 mM to 2–100 μM, ten points in total. The same concentration ranges were applied to an empty plate to allow an adjustment for the influence of the salt solutions on light absorption. 1% Triton (HyClone, USA) in DMEM/F-12 was used as a positive control, and culture medium was used as a negative one. All test points were measured in 3–4 replicas. Cells were incubated with the metal salt solutions for 24 h; then 20 μL of the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, USA) were added into each well, and the plates were incubated for additional 4 h. Optical density was measured at 492 and 650 nm using Original Multiskan EX (Lab Systems, USA); optical density values obtained at 650 nm were subtracted from the ones obtained at 492 nm to exclude background absorption. Statistical processing of the obtained data was carried out using Microsoft Excel 2010 (Microsoft) and Prism 5 (GraphPad); IC_{50} (half maximal inhibitory concentration) was calculated for each metal salt (data are expressed as the mean and its 95% confidence interval). Representative concentration-response curves are provided in Fig. S3.

3. Results and discussion

3.1. Exposure to $RhCl_3$ leads to very rapid development of lethal phytotoxicity

The initial goal of our study was investigating the effects of common metal pollutants on terrestrial plants. We selected *Pisum sativum* (pea) as a model object because of its abundance and usability in hydroponic studies. A scheme of the experiment is shown in Fig. 2a. Seedlings were transferred into tubes filled with solutions of metal salts in two concentrations (200 μM and 1000 μM) or water (control). On day 10, the biomass gain and root elongation of the plants were assessed as indicators of growth inhibition. Representative pictures of the seedlings and plants grown in the presence of various metal salts are shown in Fig. 2b–f.

Unexpectedly, $RhCl_3$ demonstrated a strong toxic effect in *P. sativum*. At 1000 μM, it killed the plant on the first day of exposure (Fig. 2c and Fig. 3). The same effect was observed at other $RhCl_3$ concentrations higher than 600 μM (see Table S2). Soluble salts of other metals (iron, copper, nickel, and palladium; see Table S1) and rhodium (II) acetate dimer did not produce the comparable toxicity in *P. sativum* at 1000 μM, whereas rhodium (III) acetylacetonate induced no observable inhibitory effect at the maximum concentration achievable (400 μM) (see Fig. 3 and Tables S3-S6).

Nevertheless, the metal salts inhibited the plant growth to some degree. Fig. 3 shows Δ, which reflects the normalized difference between the biomass gain or root elongation in the control and test plants (0 corresponds to zero effect, and higher values correspond to higher inhibition; see Materials and methods for details). In the case of biomass gain, Δ varied significantly, depending on the salt ($\Delta_{200} = 0.39–4.52$ and $\Delta_{1000} = 0.86–4.73$). Thus, $Ni(OAc)_2$ was a

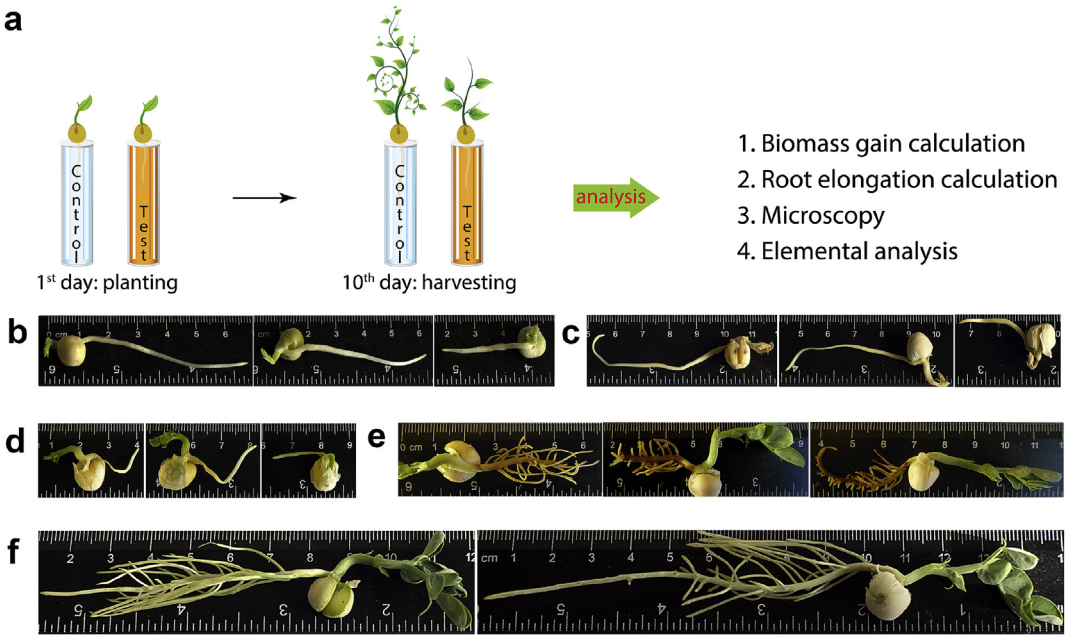
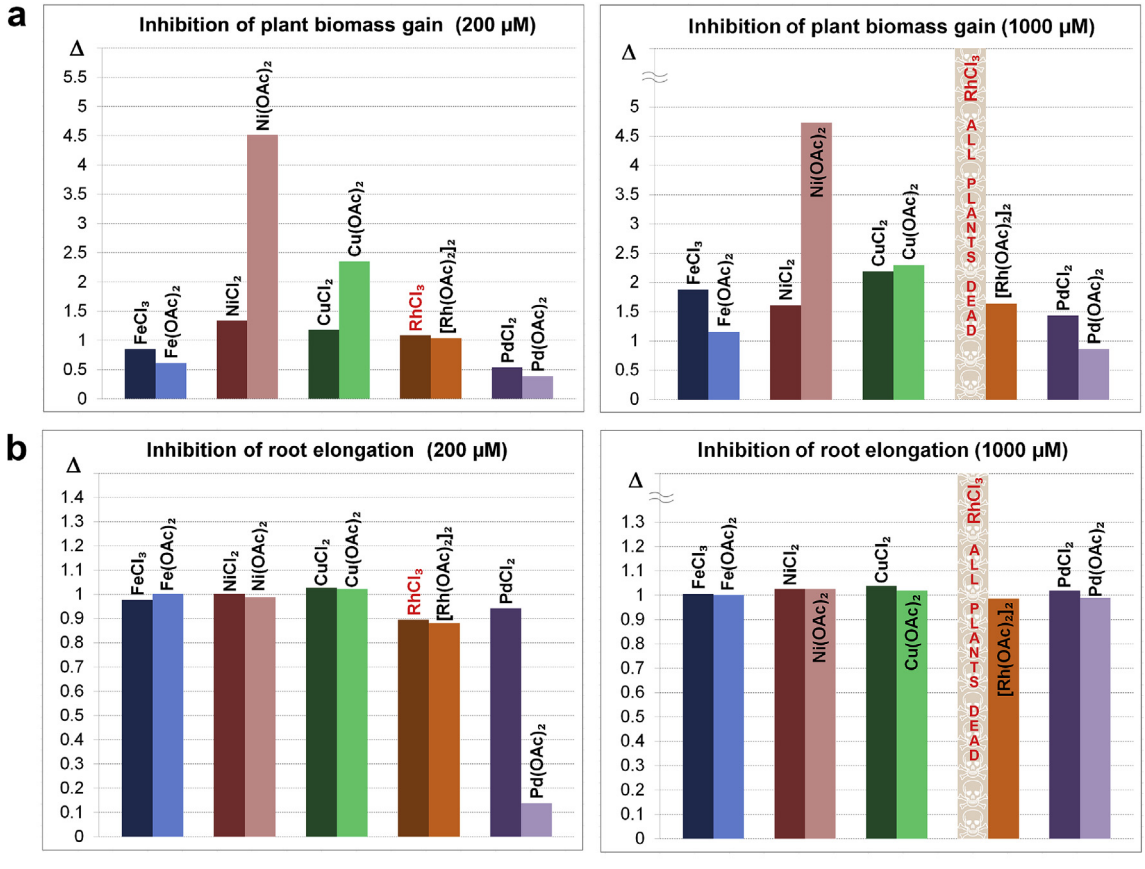


Fig. 2. Scheme of plant hydroponic experiment. (a) Seedlings were transferred into tubes filled with salt solutions (test) or water (control). During the experiment, water was added as necessary to compensate absorption and evaporation. On day 10, the biomass gain and root elongation of the plants were calculated. Other analytical tools were also employed. *P. sativum* seedlings before planting (b) and 10-day plants exposed to 1000 μM aqueous solutions of $RhCl_3$ (c), $Ni(OAc)_2$ (d), or $Pd(OAc)_2$ (e), or water (f). (Images from www.all-free-download.com are used in the figure.)



$$\Delta = \frac{GAIN(control) - GAIN(test)}{GAIN(control)}$$

Fig. 3. Phytotoxic effects of metal salts. Effect of 10-day exposure to 200 μM and 1000 μM solutions of metal salts on (a) biomass gain and (b) root elongation in *P. sativum*. Δ between test and control samples is shown; medians from representative experiments are used for calculation. 0 corresponds to zero effect; higher numbers correspond to higher inhibition. The figure is based on the data provided in Tables S3-S6.

powerful inhibitor of the biomass gain ($\Delta_{1000} = 4.73$), whereas $Fe(OAc)_2$ and $Pd(OAc)_2$ produced the least pronounced effect ($\Delta_{1000} = 1.16$ and 0.86, correspondingly); in most cases (with the exception of $NiCl_2$, $Ni(OAc)_2$ and $Cu(OAc)_2$), these effects were concentration-dependent (Fig. 3a). At the same time, all the studied salts had similar inhibitory effects on the root growth which mostly did not depend on the compound concentration ($\Delta_{200} = 0.88–1.03$ and $\Delta_{1000} = 0.99–1.04$), the only exception being $Pd(OAc)_2$, which did not impose significant inhibition at 200 μM ($\Delta_{200} = 0.14$) (Fig. 3b). Of note, even $Ni(OAc)_2$ did not kill the plant during the exposure, in spite of the strong inhibitory effect (compare Fig. 2c and d).

The metals predominantly accumulated in the roots of the plants, which was in accordance with possible absorption of metal ions on the root surface (Table 1). The highest relative metal contents in the roots were observed for Fe(III), Ni(II), Cu(II) and Rh(III) (see entries 1, 3–7 in Table 1). It should be noted that, in spite of their rapid death, the plants accumulated considerable relative amounts of Rh(III). In contrast to the other metals, only traces of Fe(III) and Pd(II) were found in the shoots (see entries 1, 9 and 10 in Table 1).

The changes of the metal content in the media during the exposure are provided in Table S7. The Pd content in the solutions was not measured due to technical difficulties. According to the data, Fe(II) was totally depleted from the solution on day 10, indicating its high bioavailability and importance to the plant. Similarly, the content of Fe(III) decreased significantly. The levels of Ni(II) and Cu(II) decreased to some degree (about 20–30%), whereas the contents of Rh(III) and Rh(II) remained essentially unchanged.

Sensitivity of plants to metals sometimes demonstrates broad inter- and intraspecific variability (Kochian, 1995). In order to establish whether the rhodium effect was species-specific, we studied it in other plants: *Lupinus angustifolius* (lupine) and *Cucumis sativus* (cucumber). Lupine, as well as pea, belongs to the Fabaceae family, whereas cucumber belongs to the Cucurbitaceae family. 1000 μM $RhCl_3$ killed *L. angustifolius* and *C. sativus* during the first days of exposure, similarly to *P. sativum* (see Tables S8-S9 and Fig. S4-S5).

3.2. Phytotoxicity of $RhCl_3$ does not relate to pH of the media

The results indicated that the observed extreme toxicity of $RhCl_3$ at high concentrations in plants was metal- and ligand-specific. Moreover, the salt of Rh(II) did not cause such dramatic inhibition. In order to gain insight into the cause of the effect, we verified whether the change of pH could be the main reason of the toxicity. Chlorides of some metals, such as iron, are prone to hydrolysis in aqueous solutions (Egorova and Ananikov, 2016); in particular, Rh(III) was shown to form various species depending on the media pH and the presence of available inorganic ligands (Sánchez et al., 2002; Colombo et al., 2008). First, we compared pH of aqueous solutions of $RhCl_3$ and the other metal salts and found that 1000 μM $FeCl_3$ was more acidic than 1000 μM $RhCl_3$ (pH 2.68 vs. 3.14 immediately after preparation; see Table S10). $FeCl_3$ is the product of the interaction between a strong acid and a weak base which explains the acidity of its solutions. Only its cation undergoes hydrolysis; pH of the solution did not changed significantly with time (2.68 and 2.65 on days 1 and 10, respectively; see Table S10). Similarly, pH of the $RhCl_3$ solution did not change significantly during the experiment (3.14 and 3.40 on days 1 and 10, respectively; see Table S10). 1000 μM solutions of all the other salts and distilled water were more alkaline than 1000 μM $RhCl_3$. In most cases, pH of the media increased during the experiment, the only exceptions being 1000 μM $Cu(OAc)_2$ and 1000 μM $[Rh(OAc)_2]_2$. Thus, $Fe(OAc)_2$ and $Pd(OAc)_2$ are hydrolyzed by both the cation and the anion; during the experiment, the pH of these solutions increased significantly towards the neutral medium due to hydrolysis and changes in the salt concentration through absorption by the plant (3.72 vs. 5.60 and 3.93 vs. 5.05 for $Fe(OAc)_2$ and $Pd(OAc)_2$ on days 1 and 10, respectively; see Table S10). It also should be noted that pH of the control (distilled water) decreased significantly (from 6.67 to 4.80), possibly due to the secretion of the metabolism products from the pea roots.

We also studied the influence of HCl solutions on the growth of *P. sativum*. Even at 3000 μM (pH 2.13 at 21.0 °C), HCl did not kill the plants on the first day of exposure; at 1000 μM and 200 μM, its inhibitory effects were moderate and low, respectively, in comparison with the studied metal salts (see Tables S11 and S12). Thus, according to our results, the observed toxic effects of $RhCl_3$ could not be attributed to acidification of the growth media.

Table 1
Metal content in 10-day plants grown in 1000 μM solutions of test metal salts.[a]

Entry	Salt	Roots, mg g^{-1}	Shoots, mg g^{-1}
1	$FeCl_3 \cdot 6H_2O$	32.0	0.093
2	$Fe(OAc)_2$	4.4	0.12
3	$NiCl_2 \cdot 6H_2O$	15.0	3.7
4	$Ni(OAc)_2 \cdot 4H_2O$	18.0	3.2
5	$CuCl_2$	30.0	2.2
6	$Cu(OAc)_2$	21.0	2.5
7	$RhCl_3 \cdot xH_2O$	20.0	3.4
8	$[Rh(OAc)_2]_2$	3.8	0.35
9	$PdCl_2$	3.5	0.0067
10	$Pd(OAc)_2$	3.4	0.0042

[a] As determined by ICP-MS. The content of the corresponding metal is shown in each case. The following metal contents were found in the control plants: Fe, 0.26 (shoots) and 0.12 (roots); Ni, 0.02 (shoots) and 0.0029 (roots); Cu, 0.10 (shoots) and 0.01 (roots); Rh, 0.0094 (shoots) and 0.0047 (roots); Pd, 0.0097 (shoots) and 0.0027 (roots).

3.3. Morphological manifestation of $RhCl_3$ toxicity in *P. sativum*

The exposure of *P. sativum* to the studied metal salts in all cases resulted in the development of some morphological defects, which were not observed in the control plants (Table S13; representative pictures of the plants are shown in Fig. S6). At 1000 μM, all the compounds inhibited the root elongation and shoot proliferation, and all the salts except for $Fe(OAc)_2$ and $Pd(OAc)_2$ arrested the formation of lateral roots. $CuCl_2$, $NiCl_2$, $RhCl_3$ and $[Rh(OAc)_2]_2$ also induced the leaf necrosis. However, only $RhCl_3$ caused the plant death on the first day of exposure.

Morphology of the root was investigated by scanning electron microscopy. On the 5th day of exposure, in the plants, which had been exposed to 1000 μM $RhCl_3$ and had perished by then, the root stele consisted solely of three protoxylem bundles. No metaxylem and sclerenchyma were discernible, while these tissues had fully differentiated by this stage in the control plants (Fig. 4a and b; see also Fig. S7).

To study the dependence of the manifestation of $RhCl_3$ toxicity in *P. sativum* on the growth stage, we conducted the following experiment: plants were transferred into the salt solution on days 1, 3, 5 and 7 of their growth in the aqueous media, and the inhibitory effects were assessed on day 10. The plants transferred into 1000 μM $RhCl_3$ on days 1 and 3 died shortly after the transfer, whereas those transferred on days 5 and 7 survived, albeit with apparent damage (see Tables S14-S15 and Fig. S8). Root and shoot elongation seemed arrested due to the transfer to the $RhCl_3$ solution; necrotic zones emerged on the leaves. The living plants that were transferred into the salt on day 5 were used for the analysis of Rh distribution in the tissues.

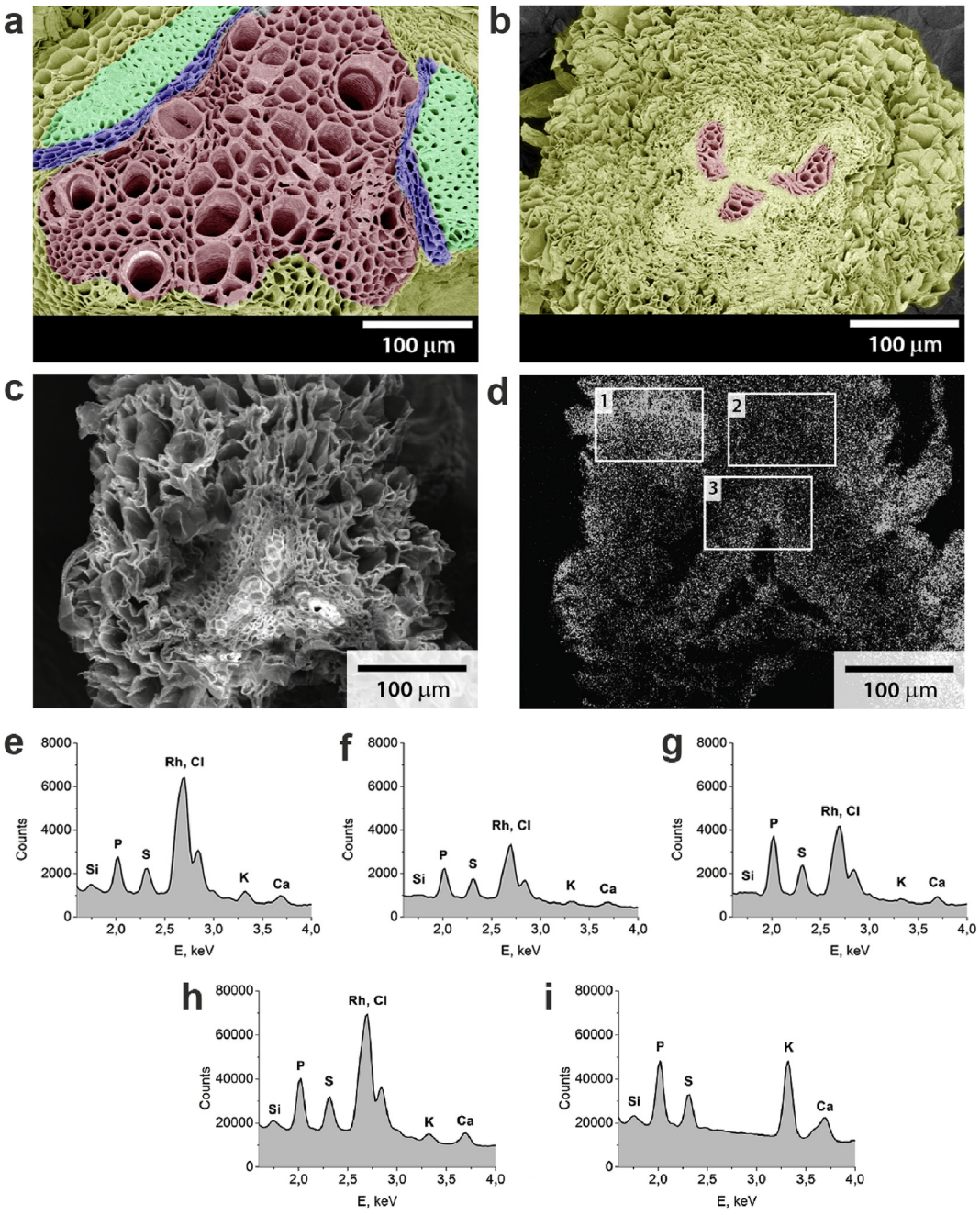


Fig. 4. SEM-EDX study of rhodium and chlorine distribution in pea root (elongation zone). Pseudo-colorized FE-SEM images of cross-section of root of 5-day plant grown in (a) water or (b) 1000 μM $RhCl_3$; (c) FE-SEM image of cross-section of root of 10-day plant transferred to 1000 μM $RhCl_3$ on day 5 after planting; (d) distribution of Rh and Cl (sum) within the sample (location of sites 1–3 is shown); fragments of EDX spectra collected from (e) site 1, (f) site 2, (g) site 3 or (h) total cross-section area; (i) fragment of EDX spectrum of control sample without $RhCl_3$. In (a) and (b), tentative tissue designation is shown by color: green, sclerenchyma; pink, protoxylem and metaxylem; blue, phloem; yellow, parenchyma. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

During the exposure, Rh was predominately accumulated in the roots, though some amount of it was also transported to the shoots, as established by ICP-ES (see Table S16). In the roots, Rh was abundant in the meristem, elongation, maturation and branching zones, whereas lower quantities of the metal were found in the lateral roots (see Table S17 and Fig. S9). The distribution of Rh and Cl in a slice of the elongation zone of the pea root was studied by scanning electron microscopy combined with X-ray microanalysis (SEM-EDX) (Fig. 4c–h). An electron microscopy image of the sample area, which was chosen for elemental mapping, is shown in Fig. 4c. Due to overlapping of the rhodium and chlorine X-ray emission lines, an EDX map was constructed with the use of a wide spectral window; thus, the map reflected the $RhCl_x$ content in different parts of the sample. The elements of interest distributed relatively uniformly through all the tissues, with a local excess on the cortex periphery, supposedly due to the sorption on the root surface, whereas protoxylem bundles were the least accumulating zones (Fig. 4d). Variations in the elemental composition can be clearly seen in the EDX spectra collected from several sites on the sample surface (Fig. 4e–g), as well as from the total cross-section area (Fig. 4h). It should be mentioned that the shapes of the lines in all the spectra are almost identical, so one can conclude that the

Rh/Cl ratio within the sample is constant. Analysis of the control sample showed no presence of Rh and Cl (Fig. 4i); therefore, the only source of these elements was the hydroponic solution.

3.4. $RhCl_3$ demonstrates low cytotoxicity, in comparison with salts of other metals

Since the inhibitory effect of $RhCl_3$ on *P. sativum* was outstanding among the various metal salts tested, we also studied its cytotoxicity, being one of the first and most popular indicators of biological activity of a chemical (Egorova and Ananikov, 2017), in a human fibroblast cell line (3215 LS). 24-h IC_{50} are provided in Table 2. To our surprise, $RhCl_3$ was among the least cytotoxic salts and was inferior only to $FeCl_3$ and $Fe(OAc)_2$ in terms of cytosafety (compare entries 1, 2 and 7 in Table 2). Of note, $RhCl_3$, $PdCl_2$ and $Pd(OAc)_2$ demonstrated significantly lower cytotoxicity than the corresponding salts of Ni(II) and Cu(II) (compare entries 3–7, 10 and 11 in Table 2). The most pronounced difference in the cytotoxic effects was observed between the salts of Rh(III) and Rh(II): 24-h IC_{50} of $[Rh(OAc)_2]_2$ was by an order of magnitude lower than that of $RhCl_3$ (compare entries 7 and 8 in Table 2). At the same time, $Rh(acac)_3$ was not toxic in the concentration range tested (entry 9 in Table 2). These results are in agreement with the data obtained earlier in other cell lines (Schmid et al., 2007). Coordination complexes of rhodium also demonstrated significantly lower cytotoxicity in murine fibroblasts and embryonic human lung cells than these of palladium and platinum (Bünger et al., 1996). High cytotoxicity of $[Rh(OAc)_2]_2$, in comparison with $RhCl_3$, can be explained by the ability of Rh(II) compounds to cross-link DNA strands and to inhibit DNA and RNA polymerases in cells (Erck et al., 1974; Howard et al., 1979).

3.5. Possible ecological consequences

Recently, a case of genotype-dependent acute toxicity of aluminum in *P. sativum* has been described (Kichigina et al., 2017). 10-day exposure to 110 μM $AlCl_3$ led to complete growth inhibition, whereas exposure to 80 μM $AlCl_3$ significantly inhibited root and shoot elongation in aluminum-sensitive plants. The authors suggested that Al^{3+} disturbed the uptake and distribution of nutrients in the plant (Kichigina et al., 2017). Aluminum is a known toxicant for *P. sativum*; it affects the root apex elongation and causes oxidative stress (Matsumoto and Motoda, 2012). However, no such pronounced effect has been described for rhodium in terrestrial plants. There are separate reports on rhodium toxicity in algae, moss and aquatic plants (Gagnon et al., 2006; Bednarova et al., 2014). The available studies in terrestrial plants mostly concern the accumulation of PGMs upon the exposure to low metal concentrations (Lesniewska et al., 2004; Odjegba et al., 2007; Orecchio and Amorello, 2010), although the effect of the Pd uptake on barley leaves was assessed (Battke et al., 2008). Pd as nanoparticles and as a salt was shown to accumulate in *Sinapis alba* and to have moderate effects on the biomass gain, but not on the growth and morphology (Kińska et al., 2018). Gawrońska and coworkers demonstrated phytotoxic effects of Pt (II) in *Arabidopsis thaliana* at concentrations above 2.5 μM (Gawrońska et al., 2018). In 1934, Brenchley described severe inhibitory effects of $PdCl_2$ on pea growth and morphology (Brenchley, 1934), but the data on interactions of *P. sativum* with PGMs are scarce.

On the other hand, high bioavailability of rhodium has been demonstrated in plants (Odjegba et al., 2007; Nischkauer et al., 2013; Shams et al., 2014). In *Brassica napus* (rapeseed), exposure to a 0.5 mg L^{-1} mixture of Pt, Pd and Rh for 4 weeks resulted in the accumulation of ~600 mg kg^{-1} Rh in roots, which was higher than the corresponding values for Pd and Pt (Nischkauer et al., 2013). However, no pronounced toxicity or lethality accompanying this accumulation was described.

Heavy metals can influence the overall growth and biomass gain of a plant by inhibiting the growth and functioning of the root. Possible mechanisms of heavy metal toxicity in plants include (1) competition with and/or displacement of essential cations; (2) interaction with sulfhydryl groups of proteins leading to structural and functional disturbances; and (3) generation of reactive oxygen species (ROS). Roots, in their turn, can counteract the heavy metal exposure via several mechanisms, such as impeding the metal uptake and transporting the metal ions to the aboveground parts of the plant, where they can be stored in vacuoles, thus being rendered harmless (Ovečka and Takáč, 2014; Singh et al., 2015). In our experiments, Rh, as well as the other metals, accumulated predominantly in the roots of *P. sativum*, though some part of Rh was transported into the shoots. Partially, the plant death could be attributed to the termination of root elongation and cell differentiation; however, it could not explain the swiftness of the effect. The ligand environment is known to affect the oxidation potential of a metal ion. Higher constants of complex stability correspond to weaker oxidative properties of a cation (Rizvi, 2015). Rhodium has a rather high oxidative potential (+0.758 V), which manifests as high ability of Rh^{3+} to reduce to Rh^0 by oxidizing available organic substances. Bioorganic molecules present in the plants can possibly serve as such reducing agents, and their corresponding oxidation can lead to their deactivation and failure to participate in various biochemical processes. In contrast, acetate ions can coordinate around cations of a metal thus decreasing its oxidative properties which can presumably explain the differences in phytotoxicities of $RhCl_3$ and $[Rh(OAc)_2]_2$. The exact mechanism of $RhCl_3$-induced acute toxicity in the plants remains to be revealed.

As we emphasize in our recent reviews, the available data on toxicity of heavy metals are scattered and controversial, and cannot be used for deriving general ecological profiles of metal influence on the environment (Egorova and Ananikov, 2016, 2017). The biological impact of a metal compound depends on: (1) the nature and oxidation state of the metal; (2) the nature of its ligands; (3) the nature of the biological object being affected; and (4) the environment.

Table 2
Cytotoxicity of metal salts towards human fibroblasts.[a]

Entry	Salt	24-h IC_{50}, mg L^{-1}	24-h IC_{50}, μM
1	$FeCl_3 \cdot 6H_2O$	785 (686–884)	2905 (2537–3272)
2	$Fe(OAc)_2$	752 (622–881)	4322 (3578–5067)
3	$NiCl_2 \cdot 6H_2O$	25 (22–28)	105 (92–118)
4	$Ni(OAc)_2 \cdot 4H_2O$	30 (27–33)	121 (109–133)
5	$CuCl_2$	16 (15–17)	118 (110–126)
6	$Cu(OAc)_2$	20 (16–24)	111 (90–132)
7	$RhCl_3 \cdot xH_2O$	395 (321–470)	1498 (1216–1779)
8	$[Rh(OAc)_2]_2$	28 (24–33)	64 (54–74)
9	$Rh(acac)_3$[b]	>140	>360
10	$PdCl_2$	145 (128–162)	817 (722–911)
11	$Pd(OAc)_2$	119 (98–140)	529 (435–623)

[a] 24-h IC_{50}, 24-h half maximal inhibitory concentration. 95% confidence intervals are shown in parentheses.
[b] The maximum concentration tested (360 μM) was limited by low water solubility of $Rh(acac)_3$; at this concentration, no cytotoxic effect was observed during 24 h.

4. Conclusions

To summarize, the extraordinarily fast development of lethal acute toxicity upon the exposure of *P. sativum* to $RhCl_3$ at concentrations above 600 μM is described in this work for the first time,

presenting an evidence of the deficiency in our knowledge on possible environmental dangers of newly widespread metal pollutants. According to our experimental data, the growth stage, at which the exposure occurred, had a crucial impact on the toxicity manifestation: at earlier stages, 1000 μM $RhCl_3$ killed the plants within 24 h. Similar effects were observed in *L. angustifolius* and *C. sativus*; however, $RhCl_3$ was relatively low-toxic in human fibroblasts. Therefore, the observed effect was plant-specific, but not species- or family-specific.

More generally, the following keypoints should be emphasized:

- During the whole history of the living world, living organisms have never been in a systematic contact with platinum group metal chemicals (salts or complexes of Rh, Pd, etc.), because these metals are very rare in nature (i.e. there are only traces of them in the Earth's crust) and are localized in small geological deposit areas, often as bulk metals (i.e. in a chemically inert form).
- Human activity in the last 20 years has led to global redistribution of platinum group metals and to their spreading in the environment in non-metallic forms (i.e. in chemically reactive forms).
- Living organisms have no evolutionary developed protective mechanisms against these compounds. A case of extraordinarily rapid lethal toxicity of a rhodium salt in terrestrial plants demonstrated in this study supports this notion.

A number of further studies will be required to reveal the topic in more details and to estimate possible environmental impacts as well as mechanisms of biological action of $RhCl_3$ in plants.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.02.043.

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