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Pd-nanoparticles cause increased toxicity to kiwifruit pollen compared to soluble Pd(II)

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Nanoparticulate Pd – which resembles emissions from automobile catalysts – affects pollen to a higher extent than soluble Pd.

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ABSTRACT

In the present study, endpoints including *in vitro* pollen performance (i.e., germination and tube growth) and lethality were used as assessments of nanotoxicity. Pollen was treated with 5–10 nm-sized Pd particles, similar to those released into the environment by catalytic car exhaust converters. Results showed Pd-nanoparticles altered kiwifruit pollen morphology and entered the grains more rapidly and to a greater extent than soluble Pd(II). At particulate Pd concentrations well below those of soluble Pd(II), pollen grains experienced rapid losses in endogenous calcium and pollen plasma membrane damage was induced. This resulted in severe inhibition and subsequent cessation of pollen tube emergence and elongation at particulate Pd concentrations as low as 0.4 mg L^{-1}. Particulate Pd emissions related to automobile traffic have been increasing and are accumulating in the environment. This could seriously jeopardize *in vivo* pollen function, with impacts at an ecosystem level.

1. Introduction

Platinum group elements (PGEs) are rare and emitted to the environment only through anthropogenic activities. Their widespread use in many fields of technology, medicine and particularly in catalytic converters, and the inability of the environment to eliminate these elements has led to environmental accumulation of PGEs (Ek et al., 2004). PGE contamination initially occurs in airborne particulate matter, roadside dust, soil, sludge and water; which finally results in bioaccumulation in living organisms (Ravindra et al., 2004). Elevated PGE concentrations have been found in abiotic and biotic components of urban environments and even in Greenland snows (Barbante et al., 2001), indicating the possibility for long-range transport of PGE-containing particles. PGEs are largely released in particulate form (Pt > 95%, Pd > 85% and Rh > 90%) varying in their size from >10.2 μm to <5 nm. The coarse particles consist of alumina or silica that carry dispersed PGE particles with diameters of a few nanometers (Abthoff et al., 1994; Artelt et al., 1999; Rauch et al., 2000, 2002; Dongarrá et al., 2003). The fine particles of a few nm in diameter are mainly elemental PGEs and can make up to a third of the total PGE emission (Gómez et al., 2002; Kanitsar et al., 2003; Rauch and Morrison, 2008). Some of these evolved particles can be solubilized and transformed, preparing a proportion of the PGE compounds to enter the food chain (Jarvis et al., 2001).

Palladium (Pd) and platinum (Pt) are the main catalytic element in automotive catalysts nowadays. Their ratio varied within the last decades according to the metal's market prices. In the years 1998–2004, Pd amounts clearly exceeded those of Pt. Since 2004, Pd:Pt ratio is nearly equal and the Pd demand of car industry is almost constant since then (Johnson Matthey, 2009). However, a clear increase in Pd levels has been observed, especially in road dusts and roadside soils (Whiteley and Murray, 2003; Zereini et al., 2007; Leopold et al., 2008a). In Sweden, compared with Pt and Rh, higher Pd concentrations have been detected in feathers of different raptors suggesting larger environmental Pd mobility (Jensen et al., 2002). Greenhouse experiments, where different plant species were cultivated on soils collected from roadsides, demonstrated Pd was the most biologically available PGE (Schäfer et al., 1998). Similar results were obtained using the aquatic organism *Dreissena polymorpha* (Sures et al., 2002; Zimmermann et al., 2005).

The present work was aimed to evaluate the effects on *in vitro* pollen performance of Pd supplied both in a non contaminated,

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well-defined particulate form and as water-soluble Pd(II). Indeed, in this preliminary screening, the likelihood of interference from other metals, such as those occurring in road dust, as well as the variety of compounds derived by the transformation of soluble Pd forms was avoided. Pollen represents the haploid male generation of higher plants. Its unique and crucial task is to germinate and deliver the male gametes to the female partner for fertilization to occur. Interestingly, pollen represents a highly suitable experimental model to study the impact of a wide range of chemicals on plant cell metabolism (Kristen, 1997) and is considered a very sensitive indicator of air pollution (Mišik et al., 2007). Furthermore, several reports have demonstrated an increased sensitivity to environmental pollutants of the male gametophyte compared with vegetative plant structures (Varshney and Varshney, 1980; Bergweiler and Manning, 1999; Calzoni et al., 2007). Due to abundant pollen production in kiwifruit and ease of *in vitro* germination, pollen cultures of kiwifruit were a suitable choice for this study. Furthermore, we took advantage of our experience in managing kiwifruit pollen, already used to assess cytotoxicity of natural compounds or Cr (Antognoni et al., 2004; Speranza et al., 2007a,b; Scoccianti et al., 2008). Indeed, the present work should better elucidate the processes and effects of Pd-NP toxicity.

2. Materials and methods

2.1. Preparation of Pd nanoparticle (Pd-NP) colloidal solution

A 20 mg Pd L^{-1} colloidal solution was prepared by aqueous chemical reduction of Pd^{II} to Pd^0 according to Leopold et al. (2008b). For this purpose, 1 mL of a standard Pd^{II} solution ($Pd(NO_3)_2$ in 0.5 M HNO_3 and 1000 mg Pd L^{-1}; Merck, Darmstadt, Germany) was added to 50 mL of ultra pure water. Subsequently, 0.5 mL of freshly prepared 0.03 M sodium borohydride solution ($NaBH_4$) from 22 mg of $NaBH_4$ (p.a., Merck) dissolved in 20 mL of ultra pure water was added slowly (1 µl s^{-1}) under gentle stirring at 0–5 °C. The solution color changed immediately to grey, which was evidence of the formation of black Pd-NPs. In order to separate the nanoparticles from the reagent solution, fast agglomeration was achieved by warming the solutions to 35 °C. The agglomerated Pd-particles (1–5 µm) were given 24 h to settle at room temperature; and the supernatant was poured off. To remove the adherent chemicals, the Pd agglomerates were re-suspended in ultra pure water and again provided 24 h to settle at room temperature before decanting the cleaning solution. This procedure was repeated twice to ensure complete reagent removal. Pd agglomerates were subsequently re-suspended in ultra pure water to a volume of 50 mL. The suspension was treated ultrasonically for 45 min to assure re-dissociation to Pd-NPs resulting in a 5–10 nm narrow particle size distribution (Leopold et al., 2008b).

Pd concentration of the nanoparticle colloidal solution was verified by graphite furnace atomic absorption spectrometry (GFAAS) confirming a quantitative reaction and loss-free cleaning procedure. Details on the GFAAS instrument and measurement procedure are given in Section 2.4.

2.2. Plant material

Kiwifruit pollen was obtained from plants of the male genotype (cv. Tomuri) of *Actinidia deliciosa* var. *deliciosa* (A. Chev) C. F. Liang et A. R. Ferguson growing in experimental plots of the Azienda Tarozzi, Faenza (Italy). Pollen was stored at −20 °C until use (Speranza et al., 2007a).

2.3. Toxicity tests

2.3.1. In vitro pollen performance

Pollen was rehydrated for 30 min at 30 °C under 100% relative humidity. Germination was performed in Petri dishes by suspending pollen in liquid (ultra pure water) basal medium (1 mg mL^{-1}) containing 0.29 M sucrose and 0.4 mM H_3BO_3 (Speranza et al., 2007a). Apart from control groups receiving no Pd-treatment, pollen was treated separately with either nanoparticulate or soluble Pd(II). Equal concentrations of the two treatments carried the same amount of Pd. Treated pollen cultures and controls received the same molarity of sucrose and boron.

The Pd-NP suspension was treated ultrasonically for 45 min; appropriate aliquots were immediately administered to cultures either at the beginning of incubation to obtain a final concentration of 0.1–7 mg L^{-1}; or to pollen incubated for 45 min to obtain a final concentration of 1 and 3 mg L^{-1}

$PdCl_2$ (99% purity grade, Sigma–Aldrich Chemical Co., St. Louis, MO, USA) was administered at the beginning of incubation as appropriate aliquots from a freshly made stock solution in ultra pure water, to obtain a final concentration of 0.1–15 mg L^{-1}.

Cultures were incubated for various time periods, as each time specified, up to 2 h, at 30 °C in the dark. Growth of NP-treated samples and controls was evaluated microscopically. When necessary, 0.05% Triton X-100 was added to cultures to disaggregate grains and tubes prior to observation. Percent germination and tube length were determined by scoring at least 1500 pollen grains and 500 tubes per sample, respectively, randomly chosen from digital images of non-overlapping fields of the cultures. An SSC-M370CE video camera (Sony, Tokyo, Japan) connected to an Axioplan microscope (Zeiss, Oberkochen, Germany), managed by AxioVision 3.1 software (Zeiss), was applied. Growth of $PdCl_2$-treated samples and corresponding controls was quantified photometrically at A_{500} according to the Pollen Tube Growth (PTG)-test (Kristen and Kappler, 1995; Scoccianti et al., 2008). The test evaluated pollen tube mass production. A V-530 Jasco spectrophotometer (JASCO Corporation, Tokyo, Japan) was used.

2.3.2. Lethality

Pollen lethality due to either Pd-NPs or $PdCl_2$ was tested by fluorochromatic reaction (FCR), which assessed plasma membrane integrity (Heslop-Harrison et al., 1984). Aliquots from untreated and treated cultures were withdrawn at 30 or 90 min of incubation and allowed to react with fluorescein diacetate (Calzoni et al., 2007). The fluorescence of a minimum of 1500 pollen grains per sample was determined under an Eclipse E600 microscope (Nikon Co., Tokyo, Japan) connected to an Eclipse DXM 1200 video camera (Nikon) and managed by ACT1 (Nikon) software, after fitting the microscope with UV epifluorescence (490–520 nm).

2.4. Pd and calcium content

Ungerminated pollen, pollen incubated for 30 and 90 min without treatment (controls) and incubated for 30 and 90 min with Pd-NPs or $PdCl_2$ were analyzed for Pd and calcium content. Controls and $PdCl_2$-treated samples were collected by centrifugation at 1400 *g* for 2 min, washed with fresh basal medium and re-pelleted as above. Pd-NP-treated pollen was separated from the medium by Millipore vacuum filtration (5.0 µm ∅ pore size) and washed with fresh basal medium. Pollen samples were decomposed in a mixture of 4 mL of HNO_3 (65%, per analysis, Merck, Darmstadt, Germany) and 1 mL of H_2O_2 (30%, per analysis; Merck) by using microwave assisted, pressurized digestion in a Multiwave sample preparation system (Anton Paar, Graz, Austria) as described by Battke et al. (2008). Clear, colourless solutions were obtained after digestion and were filled to a final volume of 10 or 25 mL with ultra pure water, depending on the Pd content expected.

An aliquot of this solution was subsequently analyzed for Pd concentration by GFAAS with a SIMAA 6000 instrument (Perkin Elmer, Überlingen, Germany). Standard instrumentation parameters were applied according to the recommendations of Perkin Elmer for Pd analysis (wavelength 247.6 nm, slit width 0.7 nm). Instrument calibration (10–500 µg Pd L^{-1}) was performed by measuring aqueous solutions of Pd^{II}, freshly prepared by dilution of adequate aliquots of stock standard $Pd(NO_3)_2$ solution in 0.5 M HNO_3, and 1000 mg Pd L^{-1} (Merck) in 0.25 M HNO_3. The Pd blank value for the sample preparation procedure resulted in a GFAAS signal intensity of 0.0009 ± 0.0001 A-s, which is more than 5-times lower than the detection limit for the method (13.2 µg Pd L^{-1}). Three replicates of each standard and sample digestion solution were measured.

The digest calcium content was determined using total reflection x-ray resonance fluorescence (TXRF) analysis with an Atomika 8010 instrument (Oberschlessheim, Germany). For internal calibration, Vanadium (V) was chosen in a concentration range of 1–10 mg Ca L^{-1}. For this purpose, an adequate volume of a V-standard solution (NH_4VO_3 in 0.5 M HNO_3 and 1000 mgV L^{-1}; Merck) was added to a defined volume of the sample digestion solution. After thorough mixing, 1 µL of the sample solution was pipetted onto a silicon wafer, desiccated by water evaporation on a heated plate and measured by TXRF. The measured values were corrected by a reagent blank resulting from the sample preparation; the detection limit for this method was 10 µg Ca L^{-1}.

2.5. Electron microscopy

Controls and pollen treated with either Pd-NPs or $PdCl_2$ were analyzed by scanning (SEM) and transmission (TEM) electron microscopy. SEM samples were fixed overnight at 4 °C using 2.5% paraformaldehyde and 2.5% glutaraldehyde, in 0.1 M cacodylate buffer, with a pH 7.2. Following three 30 min washes with fresh buffer, samples were post-fixed for 1 h with 1% osmium tetroxide and 0.15% ruthenium red in cacodylate buffer. Samples were washed three times in distilled water (15 min each), block stained with 1% uranyl acetate in distilled water, washed once again for 15 min, and dehydrated in a graded acetone series. Samples were adsorbed onto pieces of poli-L-lysinate coverslips and then dried by the critical point method using CO_2 in a Balzers Union CPD 020. Samples were subsequently attached to aluminium stubs using a carbon tape and gold sputter-coated in a Balzers MED 010 unit. Observations were made by a JEOL JSM 5200 electron microscope.

TEM samples were fixed and dehydrated as described above. Infiltration and embedding were completed in an Epon-based resin. Thin sections were made with a Reichert Ultracut ultramicrotome using a diamond knife, collected on copper grids,