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In vitro toxicity of silver nanoparticles to kiwifruit pollen exhibits peculiar traits beyond the cause of silver ion release

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ABSTRACT

The vast use of silver nanoparticles (AgNPs) mandates thorough investigation of their impact on biosystems at various levels. The cytotoxicity of PVP coated-AgNPs to pollen, the aploid male gametophyte of higher plants, has been assessed here for the first time. The negative effects of AgNPs include substantial decreases in pollen viability and performance, specific ultrastructural alterations, early changes in calcium content, and unbalance of redox status. Ag^+ released from AgNPs damaged pollen membranes and inhibited germination to a greater extent than the AgNPs themselves. By contrast, the AgNPs were more potent at disrupting the tube elongation process. ROS deficiency and overproduction were registered in the Ag^+- and AgNP-treatment, respectively. The peculiar features of AgNP toxicity reflected their specific modes of interaction with pollen surface and membranes, and the dynamic exchange between coating (PVP) and culture medium. In contrast, the effects of Ag^+ were most likely induced through chemical/physicochemical interactions.

1. Introduction

The rapid development of nanotechnology over the past two decades has impacted the fields of electronics, materials, catalysis, nanoscale assembly, biosensing, environmental remediation, and nanomedicine. Gold and silver nanoparticles (AgNPs) have long been associated with biotechnology, exploiting their surface plasmon resonance upon light excitation for high-resolution sensing of DNA hybridization and Raman spectroscopy. As one of the most produced nanomaterials, AgNPs have also been employed as an antimicrobial and antifungal agent in clothing, food containers, pharmaceuticals, and electronics (Nair et al., 2010). In addition to AgNPs, other classes of nanomaterials such as metal oxides and carbon-based nanostructures have been rapidly developed in pursuit of their unique physical and physicochemical properties (Lee et al., 2010; Mauter and Elimelech, 2008).

Along with the technological developments of nanomaterials, it has been realized that their mass production could also compromise human health and environmental sustainability through host erosion, release during use, accumulation, and point source emission (Jin et al., 2010). An increasing body of literature delineates the complex behaviour of nanomaterials interacting with aquatic species, plants, and soil (Chen et al., 2010; Lin and Xing, 2007; Nair et al., 2010; Navarro et al., 2008a). Upon administration, nanomaterials can be translocated within plant organisms and also transmitted to their progeny through seeds (Lin et al., 2009). Pristine and surface functionalized AgNPs, specifically, have been known for their ready aggregation and dissolution in the aqueous phase, and their released silver ions have often been attributed – albeit controversially – as a major cause for AgNP-induced toxicities in eukaryotic and prokaryotic species (Fabrega et al., 2009; Sotiriou and Pratsinis, 2010; Croteau et al., 2011; Lin and Lenhart, 2012). Furthermore, AgNPs naturally produced through biotic and abiotic syntheses can be found in the environment (El-Shanshoury et al., 2011; Lin and Lenhart, 2012).

We have recently shown that nanoparticulate Pd strictly similar to that emitted from automobile catalytic converters produced invariably more harmful effects on kiwifruit pollen than soluble Pd(II) (Speranza et al., 2010). This pointed out to a potentially

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serious risk for *in vivo* pollen function when the pollen is exposed to road dusts in heavily trafficked areas (Ek et al., 2004; Leopold et al., 2008).

Pollen culture is known to work as an excellent model system for toxicity assessment (Kristen, 1997). In consideration of the vast use of AgNPs and their various possible routes of entry into the environment (Som et al., 2011), the potential toxic activities of both particulate and soluble silver (AgNPs and Ag^+ ions) to kiwifruit pollen are examined here for the first time. For the present study, we assumed pollen mortality and inhibition on germination as main endpoints, and we also studied the effects of AgNP exposure on morphology, redox status, and protein turnover of the cells. Despite the abundance of reports on the toxicity of AgNP elicited in a vast variety of organisms (see for instance Navarro et al., 2008b; Ahamed et al., 2010; Park et al., 2010; Oukarroum et al., 2012), data on AgNP toxicity in plants are scarce (Miralles et al., 2012; Ma et al., 2010; Nair et al., 2010) and entirely absent in the case of pollen. However, male function of plants and its changes due to pollution stress are strictly relevant to environmental concerns *per se*. Pollen function in fact is deemed crucial for the survival of many native species and also for crop plant yield, which largely consists of seeds and fruits.

2. Materials and methods

2.1. AgNP source; Ag^+ release

PVP BioPure™ AgNPs of 10 nm in diameter (1.00 mg mL^{-1}) and PVP BioPure supernatant were purchased from nanoComposix (San Diego, CA, USA). The size distributions of the AgNPs in water and in pollen culture medium were characterised using transmission electron microscopy (TEM) and dynamic light scattering, as described in the Supplementary Information (Figs. S1 and S2). A 1.0 mL aliquot of AgNP solution in pollen culture medium (10 mg L^{-1} or 0.003 μM of silver) was taken at different times (0.5–24 h) for measuring the amount of released Ag^+. These aliquots were centrifuged at 7500 RCF (relative centrifugal force), for 30 min, and filtered using Microcon centrifugal filters (Amicon Bioseparations, 3000 MWCO) made of regenerated cellulose. An inductively coupled plasma mass spectrometer (ICP-MS) (X Series 2, Thermo Scientific) was used to monitor the content of Ag^+ in the filtrates. Approximately 100 μL of each filtrate was mixed with 2% HNO_3 solution to make a total volume of 10 mL prior to the ICP-MS experiment.

2.2. Plant material

Kiwifruit pollen of the male genotype (cv. Tomuri) of *Actinidia deliciosa* var. *deliciosa* [(A. Chev) C. F. Liang et A. R. Ferguson] was collected and managed as described in Speranza et al. (2010).

2.3. In vitro pollen performance

Germination was performed in Petri dishes by suspending pollen (1 mg mL^{-1}) in basal medium including 0.29 M sucrose and 0.4 mM H_3BO_3 in Milli-Q water (Speranza et al., 2010). Apart from control groups receiving no treatment, pollen was treated separately with various amounts of either nanoparticulate (AgNPs) or soluble Ag ($AgNO_3$). A further group received the same aliquot of PVP supernatant carried by the highest NP concentration tested (20 mg L^{-1}). Cultures were incubated for various times up to 2 h, at 30 °C in the dark. The growth of the cultures was evaluated microscopically by scoring at least 1500 pollen grains and 500 tubes per sample, randomly chosen from digital images recorded (Speranza et al., 2010).

2.4. Pollen viability

Pollen viability was evaluated in terms of plasma membrane integrity with the fluorochromatic reaction (FCR) (Heslop-Harrison et al., 1984). Pollen samples, from either the particulate or soluble silver treatments at 30 or 90 min of incubation were managed as described in Speranza et al. (2010). The fluorescence of a minimum of 1000 pollen grains per sample was determined with UV epifluorescence (490–520 nm) (Speranza et al., 2010).

2.5. Electron microscopy of pollen

Controls and pollen treated with AgNPs (2 and 20 mg L^{-1}) or $AgNO_3$ (200 and 500 μg L^{-1}) were analysed by electron microscopy (SEM and/or TEM), following the protocols as described in Speranza et al. (2010).

2.6. Determination of silver and calcium content

Ungerminated pollen, and pollen incubated for 30 and 90 min without treatment or, separately, with AgNPs (2 and 20 mg L^{-1}) or $AgNO_3$ (200 and 500 μg L^{-1}) were analysed for Ag and Ca content. Pollen samples were digested in 10 mL of HNO_3 (65%, Suprapur, Merck, Darmstadt, Germany) using a microwave oven (MDS-2100-CEM-Microwave Technology Ltd., Buckingham, United Kingdom). Clear, colourless solutions were obtained after digestion and were filled to a final volume of 15 mL with ultrapure water. Metal concentrations were measured by using a Perkin Elmer (Norwalk, USA) AAnalyst-300 atomic absorption spectrophotometer with flame atomization (FAAS). For Ag, a 5% acetic acid (AnalaR, BDH, England) solution was added to standards and digested samples to increase silver sensitivity. For Ca, a 0.1% cesium chloride lanthanum chloride buffer solution (Sigma–Aldrich, Steinheim, Germany) was added to reduce interferences. Traces of calcium in Milli-Q water and culture medium were also determined; they were 0.054 and 0.105 μg L^{-1}, respectively. The limits of detection (LODs) were calculated on the basis of 10 determinations of the blanks as 3 times the standard deviation of the blank. The LOD for pollen was 0.6 mg L^{-1} for both metals. The Ca LOD for water and culture medium was 0.01 mg L^{-1}.

2.7. ROS production

Ungerminated or germinated pollen without any treatment, or exposed to AgNPs (2, 7 and 20 mg L^{-1}) or, separately, $AgNO_3$ (200 and 500 μg L^{-1}) for various time periods were used to determine total ROS production. The nitroblue tetrazolium (NBT) assay described by Wang et al. (2009) was used, with slight modifications concerning incubation time (30 min) and temperature (30 °C). The amount of formazan produced by NBT reduction was spectrophotometrically measured at 530 nm after dissolving the samples in methanol. For the controls, formazan production in the presence of the superoxide-dismutase and catalase mimic compound, Mn-5, 10, 15, 20-tetrakis(1-methyl-4-pyridyl) 21H, 23H-porphin (Mn-TMPP; Sigma–Aldrich, Milan, Italy) was also tested. Mn-TMPP at 100 μM, which completely inhibits pollen germination (Speranza et al., 2012), resulted in a 40% decrease in ROS generation over the controls (not shown). Overtime H_2O_2 release in culture medium by the controls and by AgNP- (2 and 20 mg L^{-1}) or $AgNO_3$-treated pollen (200 and 500 μg L^{-1}) was detected with the FOX1 method (Jiang et al., 1990; Wolff, 1994) as predescribed (Speranza et al., 2012).

2.8. Statistics

Samples were collected in triplicate. Percent data of germination and viability were analyzed after arcsin$\sqrt{p}$ transformation. Linear or non-linear regression analysis, and analysis of variance (ANOVA) with a Dunnett's Post-Hoc-Test ($\alpha = 0.05$) were performed with the GraphPad Prism package, version 5.0a for Macintosh (GraphPad Software, San Diego, CA, USA). Effective median concentration (EC_{50}) was calculated with the Log-probit method (Speranza et al., 2010).

3. Results

3.1. Ion-release from AgNPs

Ion release from metallic nanoparticles, including AgNPs, is an important environmental consideration to understand their fate, transport and biological impacts (Kittler et al., 2010; Liu and Hurt, 2010; Yang et al., 2011). Accordingly, we analysed the ion release kinetics of PVP-coated AgNPs in the pollen culture medium. The particles rapidly dissolved into ions over a span of one day, and the dissolution did not reach saturation during our observation times (Fig. 1). A maximum of 11.8 wt% ion release was observed. This implies that the AgNPs were not completely dissolved; hence, any effect on the pollen could be a combination of both the AgNPs and their released ions.

3.2. Pollen viability and performance

A 30 min-exposure to AgNPs lowered pollen viability, following a linear trend. At 2 h, the dose-dependent lethal effect was further strengthened, except for the 15- and 20 mg L^{-1}-treatments (Fig. 2a). Pollen viability tests after the PVP-treatment let us register values that were not distinguishable from those of controls without PVP (30 min: 1.32 ± 0.01 for PVP-controls, and 1.32 ± 0.054 for controls without PVP; 2 h: 1.30 ± 0.03 for PVP-controls, and 1.31 ± 0.028 for controls without PVP). For Ag^+ exposure, the pollen