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STANDARDIZATION OF SIZE, SHAPE AND CONCENTRATION OF NANOPARTICLE FOR PLANT APPLICATION

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ABSTRACT

A number of experiments were conducted to know the optimum size, shape and concentration of nanoparticles to be sprayed to plants for better penetration and translocation. Nanoparticles were capable of penetrating living plant tissues when sprayed on plant leaves, and migrated from leaves to different parts of plant. The results demonstrated 14.7% nanoparticles loss when sprayed by aerosol technique (using nebulizer) as compared to 32.5% loss by normal sprayer. Low concentration (< 5 ppm) of nanoparticles were better absorbed and translocated through plant leaves. Higher the particle size lower was nanoparticle penetration. Particle size <20 nm may be preferred to spray. Nanocube proved better shape to be sprayed to plants.

Keywords: Concentration, nanoparticle, shape, size, transportation

INTRODUCTION

Nano structured materials have received more attention because of their unusual and novel properties (Nel *et al.*, 2006). The development of nano-devices and nano-materials (Scott and Chen, 2003) has potential application in agriculture and biotechnology. Nanoparticles may be made from bulk materials and their action depends on chemical composition, size and shape of the particles (Brunner *et al.*, 2006). To understand the possible benefits of applying nanotechnology to agriculture, the first step should be to analyze the penetration and transport of nanoparticles in plants.

Phytotoxicity with five types of multiwalled nanoparticles at the level of seed germination and root growth in six higher plant species showed that seed germination, in general, was unaffected but root growth inhibition greatly varied among nanoparticles and plants (Lin and Xing, 2007). Further, root growth inhibition partially correlated to nanoparticles concentration. Zhang *et al.* (2005) analyzed the effects of nano-TiO_2 and non nano-TiO_2 on the germination and growth of naturally aged seeds of *Spinacia oleracea* by measuring germination rate and germination indexes, and found 0.25-4.0% increase in these indexes with nano-TiO_2 treatments. Moreover, the physiological effects were related to particle size, however non-nano-TiO_2 particles showed insignificant effect. Mahajan *et al.* (2011) demonstrated the optimum response of nano-ZnO on mung bean at 20 ppm and on chickpea at 1 ppm, beyond these concentrations the seedlings showed growth retardation. Gonzalez-Melendi *et al.* (2008) while studying the transport and deposition of nanoparticles inside the plants noticed the presence of nanoparticles both in extracellular space and within some cells. Battke *et al.* (2008) showed that palladium (Pd) uptake via roots depended on its particle diameter. The limited reports indicate that before applying nanoparticles to the plants we must standardize the concentration, size and shape of nano-particles to be used. Therefore, the aim of this work was to demonstrate the optimum size, shape and concentration of nano- particles to be selected to spray the plants for better penetration and transportation.

MATERIALS AND METHODS

Plant material and growth conditions

Watermelon (*Citrullus vulgaris*) cv. "Charlesteon gray" and corn (*Zea mays*) cv. "Corn Detectable Hybrid" plants were selected for the experiments due to their contrasting vessel size. Plants were grown in plastic pots (105 cm long and 122 cm dia.) filled with 250 g of 100% Calcined Fullers Earth (calcined clays) of TURFACE (mnp) with the nutrient solution 15:16:17 (Peat Lite 77220). Watermelon and corn seeds were germinated in petri-dishes with moistened filter paper. Two seeds were put in each pot. The plants were grown in a controlled environment growth house with a day/night temperature 28°/22°C, 16 h photoperiod and a photosynthetic photon flux density of 800 $\mu m\ m^{-2}\ S^{-1}$. Particles were sprayed in various experiments on the leaves of 15 to 21 days old plants and kept for 2 and 4 days to allow the penetration of particles to the plants. Then the plants were harvested.

Synthesis of nanoparticles

Different size and shapes of Ag nanoparticles were synthesized in solution phase approach after using poly vinyl pyrrolidone (PVP) (Sun *et al.*, 2002; Xiong *et al.*, 2006) by adjusting the molar ratio of PVP to the salt precursor and by altering the molecular weight of PVP. PVP (mol. wt., 10,000 to 55,000) was dissolved in 8 mL water in a 20 mL vial, and heated at 60°C in air under magnetic stirring. Meanwhile, 3 mL aqueous $AgNO_3$ solution (188 mM) was rapidly added into the vial (molar ratio PVP: metal precursor, 5: 30). After capping the vial, the reaction mixture was heated in air at 60°C for 12 h. The samples were taken from the solution with a glass pipette at different stages of reaction, centrifuged and washed 3 times with water to remove excess PVP. Gold (Au) nanocages synthesized from Ag nanocubes via galvanic replacement reaction (Skrabalak *et al.*, 2008; Zhang *et al.*, 2009) were also made.

The samples for TEM and SEM studies were prepared by using a drop of aqueous suspension of particles on a piece of carbon-coated copper grid (Ted Pella, Redding, CA) or silicon wafer which were then transferred to a gravity-fed flow cell and washed for 1 h with deionized water to remove excessive PVP. The samples were then dried and stored in a vacuum for TEM and SEM characterization.

Experimental set up

In the first experiment, four concentrations (5, 10, 20 and 40 ppm) of Ag nanoparticles (cube shape) were sprayed, either by aerosol (nebulizer) or syringe spray, on the leaves of 21 days old watermelon plants and allowed to stay for 2 days. There were 4 replicates in each concentration under each spray condition. The effect on type of sprayer and concentration of nanoparticles were studied.

In second experiment, 15 days old corn plants were used to test the size effect. Silver nanoparticles of 20, 40 and 60 nm size were selected for spray. There were four replications in each treatment. Plants were sprayed by nebulizer and harvested after 4 days to see the penetration of nanoparticles in various plant parts.

In third experiment four different shapes viz., nanocube, nanoplate and nanowire (of Ag nanoparticles) and nanocage (of gold nanoparticles) were used. There were four replications of each treatment. The particles were spread on the leaves of 18 days old corn plants by nebulizer and plants harvested after 4 days for analyze with respect to the penetration of different shapes of nanoparticles.

Application of nanoparticles

Nanoparticles were sprayed, in a biosafety cabinet, on the leaves by a 1-jet collision nebulizers with a liquid flow rate of 3.3 mL h^{-1}. For comparison normal sprayer or droppers were also used. Extreme care was taken so that particles fall only on leaf surfaces.

Collection and processing of samples for analysis

At the end of experiment, the seedlings were washed with flowing tap water for 1 minute followed by 3 time rinsing with deionized water. The root, stem and leaves were separated and kept under different bags for drying. Plant biomass (leaf, shoot and root, separately) was measured after drying at 70°C for

48 h. The plants were digested at 180°C with HNO_3 : H_2O_2 (5:1) for 10 h till complete clear solution was obtained and made up constant volume with deionized water followed by centrifugation at 11,000 rpm for 15 min. by Eppendorf centrifuge 5804. The Ag and Au concentration in leaf, root and shoot were determined by inductively coupled plasma mass spectrometer (ICPMS), Perkin Elmer ELAN DRC II. The weight of each nanoparticle was calculated using the formula volume x density.

Microscopy analysis

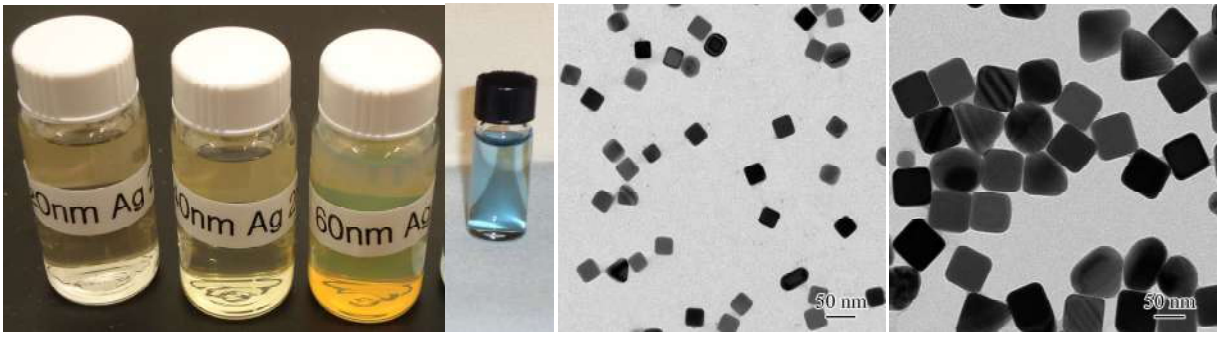
For correlative microscopy, 1-2 μm sections were cut from the polymerized blocks, observed on a light photomicroscope (Lietz, Germany) under phase contrast, bright field and dark field to identify the presence of nanoparticle aggregates (González-Melendi *et al.*, 2008) and photographed using an Olympus Dc 10 digital camera. The regions of interest were trimmed and 70-100 nm ultra-thin sections obtained. The sections were counterstained with 5% uranyl acetate for 30 min, rinsed in bi-distilled water, dried and observed on a transmission electron microscope.

For preparation of plant tissues for TEM analysis, the samples were immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 24 h at 4°C and washed 3 times (each 5 min. duration) in 0.1 M cacodylate buffer, pH 7.4. The samples were placed in 1% osmium tetraoxide in 0.1M cacodylate buffer, pH 7.4 for 1 h. The samples were again washed thrice (each 5 min. duration) in 0.1 M cacodylate buffer, pH 7.4. The samples were stained with 2% aqueous uranyl acetate for 1 h and dehydrated for 2 × 10 min in 50% ethanol (EM grade). The samples were placed in 50/50 acetonitrile/ spurr's resin for overnight. The mold containing samples was placed in oven at 60°C for 24 h. TEM images were taken using JEOL-1200Ex11 microscope operated at 80 kV. The data was statistically analyzed using Microsoft Excel 2000 for standard errors of mean and presented where necessary.

RESULTS

Size and shapes of nanoparticles

Silver nanoparticles of 20, 40 and 60 nm sizes and 40 nm size of gold nanoparticles were prepared (Fig. 1). Four different shapes of nanoparticles (nanocube, nanoplate, nanowire and nanocage) were prepared and used for the study (Fig. 2). A comparison was made between aersol spray (nebulizer) and normal syringe spray of nanoparticles under different concentrations. In general, 14.7% nanoparticles were lost during aersol spray while 32.5% nanoparticles were lost during normal syringe spray (Table 1). Higher the particle concentration, more were the losses, except during spray.

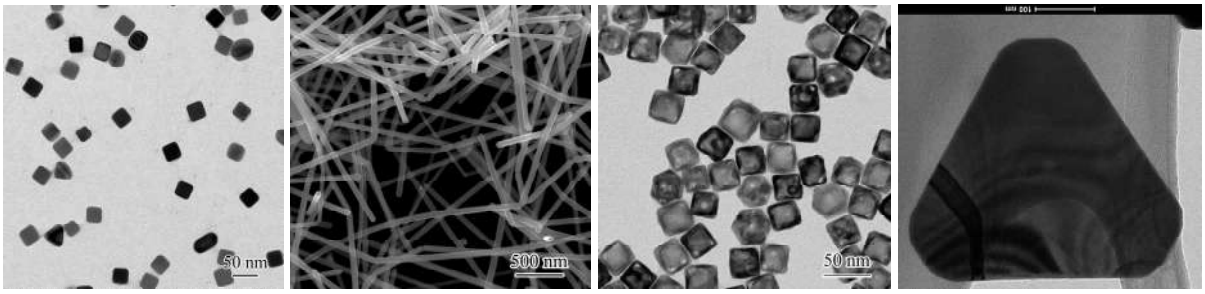


(a) (b) (c) (d) (e) (f)

Fig. 1: Size of different nanoparticles used in the study (a) silver nanoparticle of 20 nm size (b) silver nanoparticle of 40 nm size (c) silver nanoparticle of 60 nm size (d) gold nanoparticle of 40 nm size (e) TEM images of silver nanoparticles (f) TEM images of gold nanoparticles.

Table 1: Nanoparticles of Ag (40 nm cube shaped) sprayed and recovered under two different spray system on watermelon plants

Concentration of nanoparticles (ppm)	No. of nanoparticles sprayed (x 10^8)	Particle recovery in aerosol spray (x10^8)	% loss	Particle recovery in syringe spray (x10^8)	% loss
5	241.5	221.9	8.1	193.0	21.1
10	483.0	469.0	3.0	348.0	28.0
20	966.0	810.0	16.1	667.0	31.0
40	1932.0	1590.0	17.7	1236.0	36.0



(a) (b) (c) (d)

Fig. 2: Different shapes of nanoparticles prepared for the study (a) nanocube (b) nanowire (c) nanocage (d) nanoplate

Optimized the concentration of nanoparticles to be sprayed

An experiment was conducted with watermelon plants to find out the concentration of nanoparticles to be sprayed for better transport to plants. The distribution of nanoparticles after 48 h of spray to 21 days old watermelon plants showed (Table 2) more than 22% nanoparticles transported to shoot and root when the concentration was 5 ppm. However, with increase in concentration the transport of nanoparticles to shoot and root was much less and at 40 ppm concentration only 2% of nanoparticles could travel through shoots in both the spray condition. The results clearly suggest that lower concentration (5 ppm or less) of nanoparticles can absorb and penetrate better through plant leaves.

Table 2: Nanoparticles present in root, shoot and leaf of 21 days watermelon plant (after 48 h spray)

Treatments (ppm)	Nanoparticles in root (×10^8)	Recovery (%)	Nanoparticles in shoot (×10^8)	Recovery (%)	Nanoparticles in leaf (×10^8)	Recovery (%)
5	5.79	3.00	36.89	19.11	150.32	77.89
10	3.15	0.91	59.16	17.00	285.69	82.09
20	6.00	0.90	74.62	11.19	586.38	87.91
40	7.44	0.60	16.63	1.35	1211.93	98.05

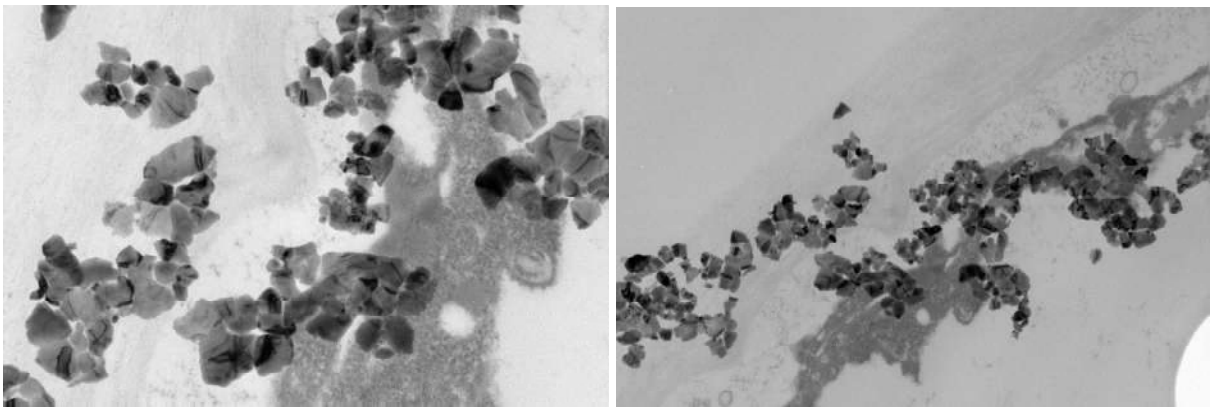
Optimized the size of nanoparticles

To see the effect of nanoparticle size on penetration and transport in corn plants, 15 days old plants were sprayed with 20 ppm concentration of 14 mL of 20, 40 and 60 nm size of Ag nanoparticles. Plants were harvested after 4 days of spraying. The results demonstrated that the penetration of nanoparticles increased significantly when the particle size was less than 40 nm size (Table 3). Only 36.2% of sprayed nanoparticles were present in leaves after 4 days of spray when particle size was 20 nm whereas when sprayed with 60 nm sizes of particles there were 78.3% nanoparticles in leaves. The results showed that with higher particle size the penetration of nanoparticles into the plants was less whereas nanoparticle size of 20 nm were better transported through plants.

Table 3: Effect of size of nanoparticles on penetration and transport in 15 days old* corn plants

Nanoparticle size (nm)	Particle in leaves (%)	Particle in shoot (%)	Particle in root (%)
20	36.2±2.3	10.4±1.1	53.4±3.0
40	72.5±1.9	5.8±0.7	21.7±1.2
60	78.3±2.1	10.2±0.5	11.5±1.0

*Plant harvested for analysis 4 days after spraying



(a) (b)

Fig. 3: TEM images of accumulation of nanoparticles in plants 4 days after spray (a) shoot (b) root

Optimization of shape of nanoparticles

Four shapes of nanoparticle were tested on 18 days old corn plants to find best shape for optimum penetration. The plants were harvested four days after spray and analyzed for the particles present in leaves, shoots and roots. Better penetration (28%) was achieved when particles were cube shaped as compared to 9.1% in cage shape, 10% in plate shape and 16.2% in wire shape (Table 4). A considerable amount of particles (21.9%) traveled to roots when particles were cube shaped. The penetration and accumulation of particles under different plant parts when sprayed on leaves are presented as Fig. 3. The result demonstrated that particle may penetrate through the cuticle/epidermis and stomata.

Table 4: Effect of the shape of nanoparticles on penetration and transport in 18 day old* corn plants

Shape nanoparticle	Particle in leaves (%)	Particle in shoot (%)	Particle in root (%)
Nanocube	72.0±2.5	6.1±0.9	21.9±1.2
Nnaoplate	90.0±1.9	1.2±0.3	8.8±1.1
Nanowire	83.8±1.7	1.8±0.5	14.4±1.2
Nanocage	91.9±1.2	3.8±0.7	4.3±1.0

*Plant harvested for analysis 4 days after spraying

DISCUSSION

Penetration rates of foliar applied polar solutes are highly variable and the mechanism involved is not yet fully understood. Our results demonstrate that the size, shape and concentration of nanoparticle determine the penetration rate. Eichert *et al.* (2008) reported that in *Allium porrum* and *Vicia faba* size exclusion limits the lateral heterogeneity of stomatal foliar uptake pathway for water-suspended nanoparticles. The results suggested that the stomatal pathway differ fundamentally from the cuticolour foliar uptake pathway. Gonzátez-melendi *et al.* (2008) showed penetration and translocation of magnetic nanoparticles in whole living plants and into plant cells. The magnetic character allowed nanoparticles to be positioned in the desired plant tissues by applying a magnetic field gradient. Since

no particles with a diameter size bigger than 50 nm have been detected inside plants, a size based selection mechanism seems to be operating, they predicted that probably cell wall and waxes act as a barrier. Fabrega *et al.* (2009) indicated toxicity of silver nanoparticles on *Psondmonas fluorescens* over 24 h exposures at 2000 ppb concentration. Corredon *et al.* (2009) reported that nanoparticles were capable of penetrating living plant tissues and migrating to different regions of the plant.

Nanoparticles uptake into plant body will depend on the size and surface properties of nanoparticles. Permeation properties differ between cuticles on the epidermal cells and trichomes on stomata (Schreiber, 2011). Nanoparticles that enter intercellular gas space of leave by passage through the stomatas are deposited on cell wall of substomatal cavity or neighbouring cells (Dietz and Herth, 2011). Nanoparticles must traverse the cell wall before entering the intact plant cell protoplast. Solute exclusion techniques provide information on limiting pore size in cell walls. Based on such experimental data, the maximum pore size of plant cell walls is usually in the range of a few nanometers; for example, 3.5-3.8 nm in root hairs and 4.5-5.2 nm in palisade parenchyma cells (Carpita, 1979). Similar pore sizes are obtained with chromatographic methods using ethanol-extracted cell walls. Exclusion pore sizes are affected by pH, divalent ions and boric acid (Fleischner, 1999), which suggests that only nanoparticles less than 5 nm dia. will be able to traverse the cell wall of undamaged cells efficiently. A globular protein of 30 kDa is approximately 3 nm in size, suggesting that nanoparticles smaller than 3 nm might also traffic between cells and through the phloem. Overall, our results show the efficiency of nanoparticles translocation is low.

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