

IN PLANTA “GREEN ENGINEERING” OF VARIABLE SIZES AND EXOTIC
SHAPES OF GOLD NANOPARTICLES: AN INTEGRATIVE ECO-FRIENDLY
APPROACH

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Daniel Lee Starnes

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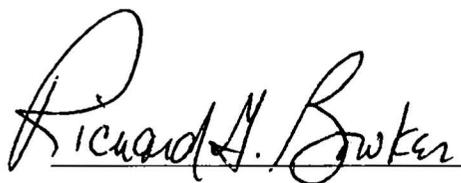
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Daniel Lee Starnes

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Directed by: Shivendra V. Sahi, Darwin Dahl, Linda Gonzales

Department of Biology

Western Kentucky University

Manipulating matter at the nanoscale creates materials endowed with unique optoelectronic and physicochemical attributes. Among the noble metals, the properties of gold in "nano" can be manipulated by varying, their shapes and sizes. Gold nanoparticles find several applications in electronics, medicine and environmental reclamation. Emphasis has been on the “green synthesis” of nanogold to mitigate the hazardous implications stemmed from conventional nanogold synthesis. However, it is not known if the *in planta* synthesis of nanogold particles could be “green engineered” as well for generating desirable sizes and exotic shapes. In the present study, we used inductively coupled plasma (ICP) analysis to determine the species-specific variability, if any, in uptake of gold across taxonomically diverse plant species (alfalfa, cucumber, red clover, rye grass, sunflower, and oregano). Seedlings of these species were grown in half strength Hoagland’s solution supplemented with 100 ppm potassium tetrachloroaurate (KAuCl₄) for 15d under controlled growth room conditions. Significant variations were detected in the ability of different plant species in accumulating gold in the root tissues ranging from 500 ppm (ryegrass) to 2500 ppm (alfalfa). Sunflower and oregano translocated significantly higher levels of gold into their aerial tissues compared to other species. This study thus suggested differential abilities of diverse plant species in uptake

of gold by roots and its mobilization to aerial parts. For further elucidation of the effects of different growth variables on *in planta* synthesis of different shapes and sizes of nanogold particles, alfalfa was selected due to its ability to accumulate large quantities of gold in the root tissues. Further, alfalfa was subjected to KAuCl_4 (50 ppm) treatment under variable growth conditions (duration of treatment, pH, temperature and light). Temporal analysis revealed that most of the nanogold particles formed within 6 h of treatment and majority fall within the size range of 10-30 nm. Spherical nanogold particles in the size range of 1-50 nm were detected ubiquitously across different treatments. Interestingly though, a noticeable shift was apparent towards the formation of nanogold particles of exotic shapes in response to specific treatments i.e., pH 3.8 (triangular), pH 7.8 (hexagonal), 15°C (rectangular). This study thus provides empirical evidence towards *in planta* “green engineering” of nanogold particles of exotic shapes and variable sizes. Efforts are now underway to decipher the mechanistic details governing the acquisition, synthesis and mobilization of nanogold particles in a model plant system. Furthermore, testing the efficacy of alternative non-lignified systems (callus and *in vitro* germinated pollen tubes) for nanogold particle production is of great interest in that it may be conducive for the extraction of nanogold particles.

Introduction

The term nanotechnology was coined by Norio Taniguchi, a Professor at Tokyo Science University, in 1974 to describe the processing of separation, consolidation, and deformation of materials by one atom or one molecule. Although the science of nanotechnology is only about four decades old, it has grown fast and has diversified, encompassing new approaches based upon molecular self-assembly to developing new materials within the size range of 100 nanometers or smaller in at least one dimension. To get the feel of the smallness one deals with while working on nanotechnology: the bacteria belonging to the genus *Mycoplasma*, the smallest cellular life form, are around 200 nm, a DNA double helix has a diameter around 2 nm, and the spacing between carbon-carbon atoms in a molecule is approximately 0.12-0.15 nm. The project on “Emerging Nanotechnologies” estimated that by 2008 over 800 manufacturer-identified nanotech products were publicly available including TiO₂ in sunscreen, cosmetics and some food products; ZnO in sunscreen and cosmetics, surface coatings and CeO₂ as a fuel catalyst(<http://www.nanotechproject.org>). Scientists in Japan discovered 10 years ago that gold displays fantastic catalytic abilities when reduced to 3 to 5 nm in size. If the gold particles are any bigger or smaller than this, the element resumes its inertness. At room temperature, nanogold can catalyze the conversion of carbon-monoxide (CO) to carbon-dioxide (CO₂) with 100-percent efficiency. Nanogold has now become a subject of intense research globally due to their unique optical, electronic, and molecular-recognition properties with applications in medicine, computer technology , cosmetics and the list is growing (Mukherjee et al., 2001; Shankar et al., 2004).

Traditional wet synthesis of nanomaterials requires the input of considerable amounts of energy and/or in the process of creating these materials there can be generation of large amounts of hazardous and/or toxic chemical substances. This has triggered the world-wide concern about the toxicity and environmental impacts of nanogold materials on the future applications of nanogold technology. These concerns have led to a debate among advocacy groups and governments on whether special regulation of nanogold technology is warranted. Therefore, to circumvent impending deleterious effects of technologies that are being employed for the generation of nanogold particles, focus has now being targeted towards “green synthesis” of nanogold particles. This could be made possible due to the unique ability of microorganisms and plants to convert metal salt (M^{x+}) into elemental metal (M^0). Several studies have advocated the use of bacterial systems for generation of nanogold particles (Dickson, 1999; Joerger et al., 2000; Nair and Pradeep, 2002; Shankar et al., 2003). Others have looked at the efficacy of plant systems for “green biosynthesis” of nanoparticles and results have been encouraging (Gardea-Torresdey et al., 2002, 2003; Sharma et al., 2007). However it is not known if the process of “green biosynthesis” could be further tuned for “green engineering” nanogold particles of desirable shapes and sizes. To explore this possibility, this study investigates the uptake of gold in taxonomically diverse species with the goal of identifying the most efficient system. The identified species was then subjected to different growth variables to see the effects, if any, on the formation of nanogold particles of exotic shapes and desirable sizes. Although the study showed the potentiality of tailoring the nanogold particles by fine tuning the growth conditions, the underlying mechanisms governing this process are still elusive. Attempts are now underway to use

plant model systems to decipher some of the enigmas attached to *in planta* synthesis of nanogold particles.

Materials and Methods

Seed Germination

Six taxonomically diverse plant species were selected for gold uptake analysis: alfalfa (*Medicago sativa*), cucumber (*Cucumis sativus*), oregano (*Organum vulgare*), red clover (*Trifolium pratense*), ryegrass (*Lolium multiflorum*), and sunflower (*Helianthus annuus*). The seeds were planted in autoclaved Premier PRO-MIX® 'BX' (Premier Horticulture) soilless medium. Flats of seeds were germinated in a growth chamber (25-28 °C; dark for 3 d followed by 16-h/8-h day/night cycle at 25°C with average PAR of 100 to 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by fluorescent tubes). Seedlings were allowed to germinate and grown for 15 d prior to the gold accumulation study.

Gold Treatment

Fifteen-day-old seedlings of cucumber, sunflower, alfalfa, red clover, ryegrass and oregano were removed from the soilless medium, washed thoroughly under running water followed by three rinses in sterile deionized (DI) H₂O. Single (cucumber and sunflower) or a group of 10-15 (alfalfa, ryegrass, oregano, red clover) seedlings were then transferred to plastic growth vessels (200 mL capacity) containing 75 mL sterile deionized water supplemented with 100 ppm of potassium tetrachloroaurate (KAuCl₄) acquired from Sigma-Aldrich and pH adjusted to 5.8 with MES buffer. Vessels were capped with aluminum foil allowing the aerial parts of the seedlings to pass through and suspending only the roots in the gold solution. Seedlings were then grown under controlled growth conditions for 15 d (16h-h/8-h day/night cycle at 25°C with average photosynthetically active radiation (PAR) of 100 to 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by

fluorescent tubes). For each treatment, roots were harvested from two independent biological replicates with three technical replicates from each.

Analysis of Gold in Plant Tissue

Root and shoot samples were weighed and placed in a 15 mL screw capped Teflon beaker. Concentrated HNO₃ (3 ml) was added to the sample and beaker was placed on a hot plate (100 °C) overnight, and then evaporated to dryness. Samples were allowed to cool and made up to a volume of 20 mL with sterile DIH₂O. The ICP-analysis was carried out using an external calibration procedure.

Energy Dispersive X-ray spectroscopy (EDS)

Fifteen-day-old seedlings of alfalfa were grown as described above, then treated with KAuCl₄ (100 ppm) for 3 d under controlled growth conditions (pH 5.8, 25°C, and average PAR between 100-120 μmol m⁻² s⁻¹). Following treatment, roots were isolated from aerial tissues, frozen, fractured in liquid nitrogen, freeze-dried for 24 h, and then mounted on aluminum stubs. The roots were viewed in a JEOL JSM-5400LV scanning electron microscope with an IXRF Systems EDS system with a Moxtek AP3.3 light element entrance window. Samples were viewed under high vacuum mode with the backscatter detector in place with an accelerating potential of 20 kV.

Gold Treatment under Variable Growth Conditions

Alfalfa was selected for temporal analysis of nanoparticle formation and characterization experiments based on the ability to sequester gold into its tissues.

Alfalfa seeds were germinated as described above and transferred to hydroponic set up. Plants were treated with 50 ppm KAuCl_4 in DIH_2O (pH 5.8, 25°C , and average PAR between $120 \mu\text{mol m}^{-2} \text{s}^{-1}$) for varying time intervals (30 min, 1h, 6 h, 12 h, 1 d, 3 d, 5 d, and 7 d) to determine a minimum time required for an initiation of *in planta* formation of nanogold particles and the effects on its shapes and sizes. The seedlings were also subjected to 50 ppm KAuCl_4 treatments for 3 d under different light conditions ($0 \mu\text{mol m}^{-2} \text{s}^{-1}$, $120 \mu\text{mol m}^{-2} \text{s}^{-1}$), temperatures (15°C , 25°C , 35°C), and pH (3.8, 5.8, and 7.8). After exposure to the gold solution under variable growth conditions, the plants were washed thoroughly under running water and then rinsed thrice in DIH_2O . For each treatment, roots were harvested from two independent biological replicates with three technical replicates from each.

Transmission Electron Microscopy of gold containing tissues

Roots (~50 mg) were cryo-ground in liquid nitrogen and suspended in 2 ml of dH_2O . A 500 μL aliquot of the root extract was transferred to a 1.5 ml micro-centrifuge tube and pulse-sonicated (Sonics and Materials Inc. VCX 130 Ultrasonic processor using the 3 mm standard probe) on ice (10 seconds at 95% power followed by a 15 second rest with 5 pulses per cycle). After each sonication cycle, the root extract was centrifuged at 4°C for 30 seconds at 1000 rpm in a Sorvall[®] Legend[®] Micro 21 table top centrifuge. The sonication-centrifugation cycles were repeated thrice to maximize the breakdown of the root cells and tissues. A final pulse centrifugation was carried out for 3 sec to pellet the root material. Subsequently, sonicated root extract (10 μL) was then transferred to a 400 mesh copper Formvar[®] coated grids, and then viewed at 100 kV in a JEOL JEM 100CX

transmission electron microscope and micrographs were taken at a magnification of 66,000x or as indicated.

Statistical Analysis

Statistical significance of differences between mean values was determined using Student's t-test. Different letters on the histograms are used to indicate means that were statistically different at $P < 0.05$.

Results

Taxonomically Diverse Species Show Differential Spatial Accumulation of Gold

Several studies have shown the phytomining ability of plants in extracting desirable noble metals with commercial value such as gold (Girling 1978; Anderson *et al.*, 1998). Although X-ray absorption near edge spectroscopy (XANES) provided further evidence towards the conversion of metal salt (M^{x+}) into elemental metal (M^0) by plants, studies so far have been confined to species belonging to Fabaceae (*Sesbania drummondii* and *Medicago sativa*), Brassicaceae (*Brassica juncea*) and Bignoniaceae (*Chiopsis linearis*) (Gardea-Torresdey *et al.*, 2000, 2002, 2007; Sharma *et al.*, 2007). However, there are no comparative studies available on the spatial distribution of gold in taxonomically diverse species. The present study species belonging to Fabaceae (alfalfa, red clover), Cucurbitaceae (cucumber), Asteraceae (sunflower), Lamiaceae (oregano), and Poaceae (ryegrass) were investigated to determine variations, if any, in the spatial distribution of gold (Fig. 1). All the species investigated accumulated gold in roots in the range of 500 ppm (rye grass) to 2500 ppm (alfalfa) (Fig. 1 A). However, no significant ($P < 0.05$) variations were observed in accumulation of gold between the roots of taxonomically diverse red clover and sunflower and also between cucumber and oregano. The study did not reveal any species- and/or family-specific trend of gold accumulation in the roots. A lack of influence of species diversity was all the more evident with respect to accumulation of gold in the shoots (Fig. 1 B). Interestingly though, oregano and sunflower accumulated significantly ($P < 0.05$) higher levels of gold in the shoots compared to the other species. Differential efficacies of these species in translocating gold from the roots to the shoots were apparent with values ranging from about 2.5%

(alfalfa) to 18.0% (oregano) (Fig. 1 C). At present, it is just a matter of conjecture whether other members of Lamiaceae would also exhibit high per cent translocation of gold to the shoots and this question may warrant further studies. Nonetheless, the study does provide empirical evidence towards the differential potentialities of taxonomically diverse species in accumulating gold in their aerial tissues. Since among the species investigated alfalfa exhibited maximum accumulation of gold in its root, it was subsequently used for documenting the formation of nanogold particles of different shapes and sizes under variable growth conditions.

Energy Dispersive X-ray spectroscopy (EDS) Validates the Fidelity of the Nanogold Particles

Energy dispersive X-ray spectroscopy (EDS) is an analytical technique used for the elemental analysis or chemical characterization of a sample. EDS analysis has been used earlier to visualize the distribution of different elements (Hirsh et al., 2006). We used this technique for validating the formation of nanogold particles and also for determining whether they are merely adsorbed on the root surface or actually formed inside the root tissues. Alfalfa seedlings were grown as described earlier and treated with 50 ppm K-AuCl₄ for 3d. As a control, one set of seedlings were grown without KAuCl₄ treatment. The cross section of the control plants (Fig. 2A left panel) did not show any detectable gold peak (Fig. 2A right panel). Whereas, the cross section of the seedlings (Fig. 2B left panel) treated with gold revealed detectable gold peaks (Fig. 2B right panel), thereby confirming the presence of gold being deposited inside the root tissues.

Temporal Effects on the Sizes of Nanogold Particles

Although *in planta* synthesis of nanogold particles in different species has been demonstrated in several studies (Gardea-Torresday et al., 2002; Haverkamp et al., 2007; Sharma et al., 2007; Marshall et al., 2009), the temporal effects on the formation and size distribution of these particles have not been elucidated. To decipher this, alfalfa seedlings were grown as described earlier in the presence of 50 ppm KAuCl_4 and root tissues were harvested sequentially at different time intervals (30 min, 1h, 6 h, 12h, 1d, 3d, 5d, and 7d) and subjected to TEM analysis to document the temporal effects on the size distribution of nanogold particles (Fig. 3A). *In planta* synthesis of nanogold particles could be detected as early as 6h following KAuCl_4 treatment. The sizes of approximately 65% of the nanoparticles formed were found to be in the range of 11-20 nm. The remaining gold nanogold particles were found in the size range of 1-10 nm and 21-30 nm constituted about 12-15% each, while larger nanogold particles (31-50 nm) represented only a small percentage. A similar trend of size distribution was observed during longer periods of KAuCl_4 treatment with nanogold particles in the size range of 11-20 nm constituted the predominant form. Since the distribution of nanoparticles shifted to a more uniform bell curve upon 3 d treatment, this time point was used for subsequent analysis of the effects of variable growth treatments on nanogold particle formation.

Effects of Variable Growth Conditions on the Sizes of Nanogold Particles

Previous studies have demonstrated the feasibility of using variable pH conditions (2-6) for the bioreduction of KAuCl_4 by ground dry wheat biomass (Armedariz et al., 2009). However, the effects of pH and other growth variables on the *in planta* formation of nanogold particles have not been elucidated. Plant species respond differentially to

growth variables such as pH, light and temperature conditions. In addition to the pH of the medium, temperature also has substantial effects on the growth responses of plants. To acclimatize to variable temperature regimes, plants have evolved a complex process comprising an array of physicochemical responses, including changes in membrane structure and function, water content of the tissue, compositions of protein and lipids, and global gene expression (Scott et al., 2004). Availability of photosynthetically active radiation (PAR) ($\mu\text{m}^{-2} \text{s}^{-1}$) is also pivotal for optimal photosynthetic activity and the production of photosynthates (sugars). Sugars not only act as a metabolite but also function as signaling molecules influencing many vital processes in the plant life cycle from germination, root and leaf development, flowering, embryogenesis, to senescence (Rolland et al., 2006). Although the mechanisms governing the shapes and sizes of *in planta* synthesized nanogold particles are far from being elucidated, this research hypothesized the likely influence of these growth variables on their synthesis in plant biomatrix. Therefore for deciphering the effects of growth variables, one set of alfalfa seedlings were grown under controlled conditions (pH 5.8, 25°C, and average PAR between $120 \mu\text{mol m}^{-2} \text{s}^{-1}$), and others were grown under variable pH (3.8 and 7.8), temperatures (15°C and 35°C) and in dark. Figure 3B shows the effect of variable pH of the growth medium on the per cent distribution of nanogold particles across five different size ranges. The higher pH (7.8) tends to push the sizes of nanogold particles formed into the 11-20 nm range. Whereas, at pH 3.8 and pH 5.8 the particles revealed more of a bell curved distribution across size classes ranging from 1-10 nm to 41-50 nm. Figure 3C illustrates the effects of different temperature regimes on per cent size distribution of nanogold particles. At 35°C, high percentages of nanogold particles formed were in the

range of 11-20 nm range. Interestingly, lower temperature (15°C) resulted in a significant shift towards higher size classes (21-30 nm and 31-40 nm). Relatively, the per cent size distribution in the control (25°C) displayed a uniform bell shaped curve. Figure 3D shows the effect of light (PAR 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$, pH 5.8 and at 25°C) and dark (0 PAR, pH 5.8 and at 25°C) conditions on the per cent size distribution of the nanogold particles being formed. Under light condition, per cent size distribution revealed bell shaped curve. Whereas, under dark condition there was a perceptible shift more towards lower size range of 11-20 nm. The data thus revealed noticeable effects of different growth variables on per cent distribution of *in planta* synthesized nanogold particles. The study provides empirical evidence towards the potential engineering of the sizes of nanogold particles by manipulating growth conditions.

Effects of Variable Growth Conditions on the Shapes of Nanogold particles

Among gold nanoparticles of varied shapes (rods, flat sheets, spherical, hexagonal, icosahedral and irregular shaped), nanotriangles have found extensive applications in therapeutic medicine. Gold nanotriangles were found to have spectral tunability and have can efficiently absorb in the near-infrared wavelengths and transmit that energy into desired tissues (Loo et al., 2004). Therefore, the effects of different growth variables (temperature, pH and light conditions) were also investigated on the shapes of nanogold particles. Interestingly, low pH (3.8) triggered the formation of nanogold triangles and represented about 8% of the total nanogold particles (Fig. 4A). Whereas, about 15% of the particles formed were hexagonal at higher pH (7.8) (Fig. 4B). A shift was apparent towards the formation of nanogold rectangles (20%) at lower temperature (15°C) (Fig.

4C). Relatively, nanogold sphericals were very ubiquitous across different growth conditions and constituted more than 65% - 80% of the total nanogold particles (Fig. 4D). Furthermore, exotic shapes (triangular, rectangular, and spherical) could be found across different size classes ranging from 1-10 nm to 41-50 nm (Fig. 5). Figure 6A shows ubiquitous nanogold spheres produced under standard growth conditions (PAR $120 \mu\text{mol m}^{-2} \text{s}^{-1}$, pH 5.8 and at 25°C) and Figure 6B shows the variable sizes of nanogold triangles that are generated under low pH conditions (PAR $120 \mu\text{mol m}^{-2} \text{s}^{-1}$, pH 3.8 and at 25°C). The study thus provide ample evidences toward the feasibility of manipulating plant system for “green engineering” nanogold particles of desirable configurations.

Discussion

Taxonomically Diverse Species Show Differential Spatial Accumulation of Gold

Earlier studies have shown the *in planta* synthesis of nanogold particles in the members of Fabaceae (*Sesbania drummondii* and *Medicago sativa*), Brassicaceae (*Brassica juncea*) and Bignoniaceae (*Chiopsis linearis*) (Gardea-Torresdey *et al.*, 2000, 2002, 2007; Sharma *et al.*, 2007). However, from these studies it was not apparent if the efficacy of gold uptake and consequent *in planta* synthesis of nanogold particles in roots and aerial parts of the plants was in any way related to a specific species or a member of a particular family. It was difficult to draw any correlation from these studies because the focus was largely on a specific species and conditions used for gold treatment were variable across different studies. Therefore, to decipher whether there are any species and/or family-specific effects on the uptake of gold by roots and its mobilization to shoots, we selected species representing taxonomically diverse families i.e., Fabaceae alfalfa, red clover (Fabaceae), cucumber (Cucurbitaceae), sunflower (Asteraceae), oregano (Lamiaceae), and ryegrass (Poaceae). The purpose of selecting two species from Fabaceae was to see if there is any similarity in the gold uptake potentialities of the members of the same family.

Although ICP analysis of gold content in the roots and the shoots of these species did reveal significant variability, no species-and/or family-specific correlation could be established (Fig. 1). For instance, alfalfa and red clover are both leguminous species but exhibited significant variability in accumulating gold in their roots. The mechanistic details governing the uptake and mobilization of gold in different plant species remains elusive. Since gold is not an essential element required by plants for its growth and

development, it is quite likely that it is taken up by the plant by some default mechanism or uses a transport system evolved for essential nutrients. The detection of nanogold particles in the cytoplasm of the root cells of *Sesbania drummondii* is a testimony of an active symplastic transport system that is being used for the uptake of gold by the roots and its subsequent mobilization to aerial parts (Sharma et al., 2007). A previous study has shown the inability of plant species to differentiate between essential nutrients and ones which closely related but could be unessential and potentially toxic to plants. For instance, in soils the most abundant arsenic species is arsenate [As (V)] and its toxicity is derived from its close chemical similarity to phosphate (Pi). The similarity between these anions makes plants highly sensitive to As (V) due to its inability to differentiate between the two and thus easily is incorporated into cells through high-affinity Pi transport system (Catarcha et al., 2007). Due to the potential similarity of Au³⁺ with some of the metal cations, the likely influence of cation transporters on the uptake and mobilization of gold could not be ruled out. Furthermore, there is prevalence of crosstalk across several essential and nonessential elements. For instance, excess aluminium (Al) induces iron (Fe) deficiency symptoms in rice (Clarke et al., 1981) and the uptake, transport and use of several essential nutrients (Ca, Mg, K, P and Fe) (Foy, 1992). However, at present our understanding of the effects of gold on nutrient homeostasis, morphophysiological, and molecular responses are far from being elucidated. Perhaps this is important for a better tailoring of plant system not only to increase its efficiency of gold uptake but also converting them to nanoparticles. In this regard, identification of precise molecular mechanisms that regulate gold uptake and mobilization could provide the opportunity of manipulating them further either by mutation or by overexpressing the genes under

constitutive promoters. Use of model plant species like *Arabidopsis thaliana*, and *Medicago truncatula* with their whole genome having been sequenced would facilitate molecular analysis and would thus expedite the process of solving some of the enigmas attached to *in planta* synthesis of nanogold particles.

Manipulating Growth Variables Facilitate *In planta* Synthesis of Exotic Nanogold Particles

To investigate growth variables, it might seem most beneficial to choose the species with the highest amounts of gold being deposited into the aerial tissues. However in practice it is desirable to select the species which has the highest concentration of gold nanoparticles. Therefore selection was based upon the accumulation of gold in the root tissues. For this reason alfalfa was chosen for nanogold particle characterization studies. The bulk of nanogold particle formation occurred in the first 6h (Fig. 3 A). This time frame is not that different from the approximate 4 hour time established for formation when using *Aloe Vera* plant extract (Chandran et al., 2006). The difference in times between those of this study and those of Chandran could be attributed to the fact that the plants we used were not boiled extracts, thereby decreasing the speed at which KAuCl_4 could be reduced to form nanogold particles. To ensure that the nanogold particles formed were not merely on the root surface but actually mobilized into the root, we employed Scanning Electron Microscopy (SEM) attached with an EDS detection system (Fig. 2). The analysis conclusively demonstrated the presence of gold in the interior of the roots. In the literature search, we did not find any relevant information on the distribution of size and shapes of nanogold particles as a function of growth variants.

Length of time the plants were exposed to the gold treatment did not play a critical role in the per cent size distribution of nanogold particles with most being formed in the range of 11-20 nm. Interestingly though, 3 day exposure did yield a bell shaped distribution across 5 different size categories (1-10nm, 11-20nm, 21-30nm, 31-40 nm, and 41-50nm). However, the study showed the formation of larger nanoparticles at lower temperatures and pH values (Fig. 3 B and C). This could be due to the reduction in catalytic activities of various enzymes that could be involved and or genes that are being inactivated or depressed under those conditions. The absence of light did appear to favor the production of smaller nanogold particles in the size range of 11-20 nm (Fig. 3D).

The catalytic activity of nanogold particles is dependent on both their size and shape. The vertices and edges of the nanoparticles are sites of high catalytic interactions, therefore it would be of great benefit to try and direct the shapes of the particles being produced. Interestingly, we observed that the pH of the nutrient solution had the biggest effect on the shapes of the nanoparticles produced with respect to the formation of nanotriangles and nanohexagons (Fig. 4 A, B). Whereas, the formation of nanorectangles appears to be most prevalent at low temperatures (Figure 4C). Furthermore, these exotic nanogold triangles were represented across different sizes ranging from 1-10 nm to 41-50 nm (Fig. 5). Finally, we show that *in planta* synthesis of nanogold particles could be manipulated by using specific growth conditions resulting in the formation of well defined nanotriangles (Fig. 6) and other exotic types with clear and distinct vertices.

Extraction of Nanogold Particles from Plant Biomatrix is a Bottleneck

The extraction of these nanoparticles from the plant biomatrix is essential to determine their functions for different applications. Attempts were made to extract nanogold particles from the plant biomatrix using acid digestion (See Appendix). But so far we have not been met with much success. This could be due to the lignified tissues that may hinder the isolation process. An earlier study had also reported encountering a similar problem (Marshall et al., 2009). This aspect warranted further detailed investigation for using *in planta* synthesis of nanogold particles at a commercial scale. Efforts are underway to identify alternative non-lignified plant system that could be potentially used for this purpose. Cell wall materials in pollen tubes are made of pectin and the contents could then be easily released into the medium by simple osmotic shock. Furthermore, pollen grains could be easily germinated under *in vitro* condition and the germination medium could be supplemented with KAuCl_4 to facilitate the uptake of gold by growing pollen tubes. Will gold treatment have any detrimental effect on the germination or growth of the pollen tubes, can pollen tube synthesize the nanogold particles, if formed could they be released from the pollen tubes by digestion/osmotic shock? Many of these questions could be answered by testing a few species like petunias, maize, and crotalaria which produces sufficient amounts of pollen grain and would be conducive to *in vitro* germination. Another aspect that needs to be addressed, is the effect of tissue specificity on the configuration of nanogold formation. Perhaps use of calli generated from different explants could provide some insight into this aspect. The use of calli allows for the isolation of specific plant tissues thus allowing the investigations into tissue specific nanoparticle production.

Conclusions

With the information gathered from this research, it is deemed possible to control the size and shape of gold nanoparticles being produced *In planta*. Hence bio-engineering of tailor-made nanoproducts to meet specific requirements of commercial applications appears feasible. Future studies are required to further refine the manipulation of growth conditions as they relate to nanoparticle production. Other conditions outside of the obvious temperature, pH and Light variables, will also need to be explored. Additional studies will need to address the still unresolved issues of extracting the nanomaterials from the biomatrices of the plant material in a rapid, cost effective, and controlled manner. If these enigmas associated with *In planta* synthesis were to be resolved the large scale application of nanomaterials generated *In planta* could be efficiently and sustainably implemented.

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Figures

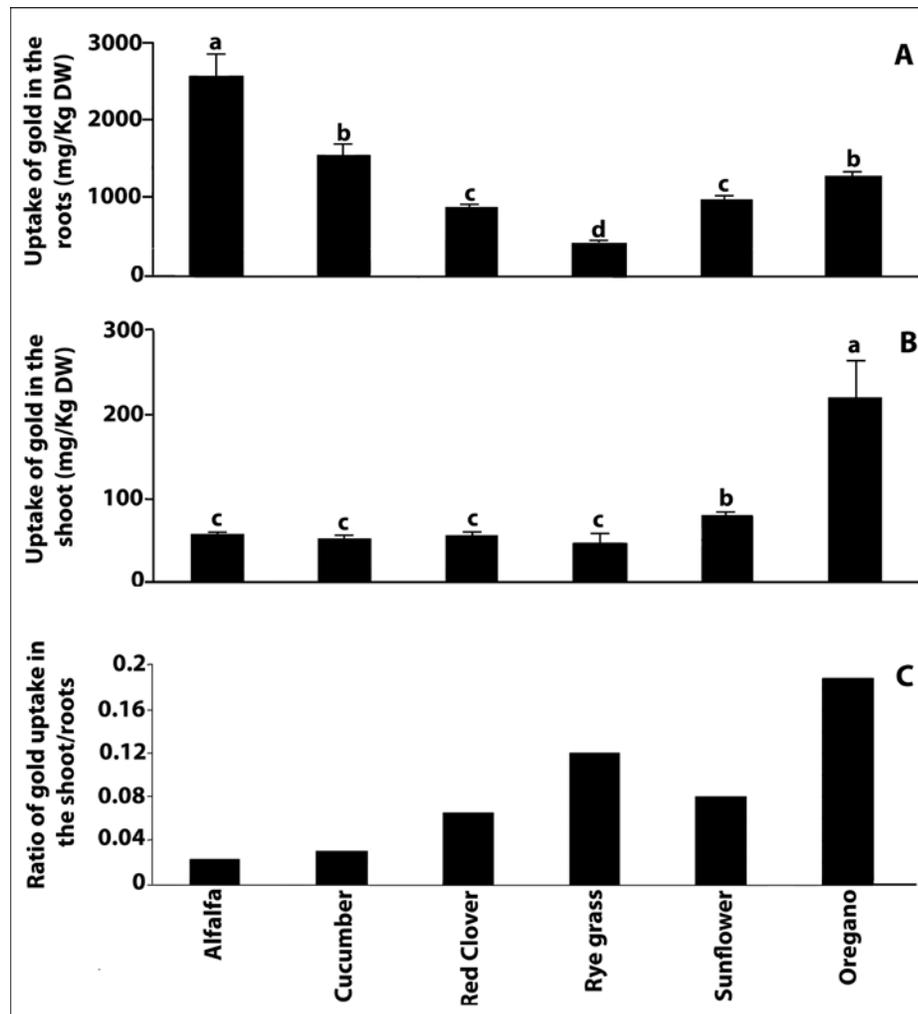


Figure 1. Differential uptake of gold by taxonomically diverse plant species. Different plant species (alfalfa, cucumber, red clover, rye grass, sunflower, and oregano) were germinated on soilless medium for 15d under greenhouse conditions and subsequently transferred to a hydroponic set up containing modified 0.5x Hoagland's solution supplemented with 100 ppm KAuCl_4 for 15d under controlled growth room conditions. The data are presented for the ICP analysis of uptake of gold in the (A) roots, (B) shoot, and (C) shoot/root. Values are means \pm SE and different letters on the histogram represent means that differ significantly ($P < 0.05$).

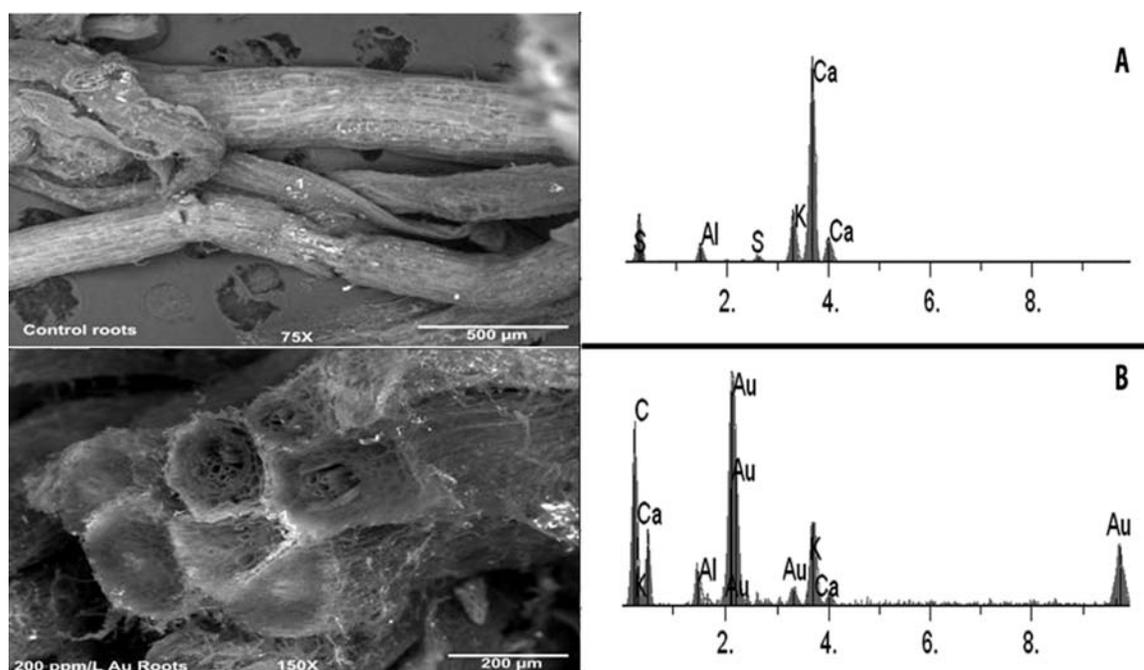


Figure 2. Scanning Electron Microscopy and Elemental Energy Dispersive X-Ray Spectroscopy (EDS) microanalyses for gold presence in plant tissues. Alfalfa was grown on soilless medium and transferred to hydroponics set up as described in the legend to Figure 1 and treated with 100 ppm KAuCl_4 for 3 d. (A) left panel SEM micrograph of roots 0 ppm (control) and right panel EDS spectra, and (B) left panel SEM micrograph of roots treated 100 ppm KAuCl_4 and right panel EDS spectra.

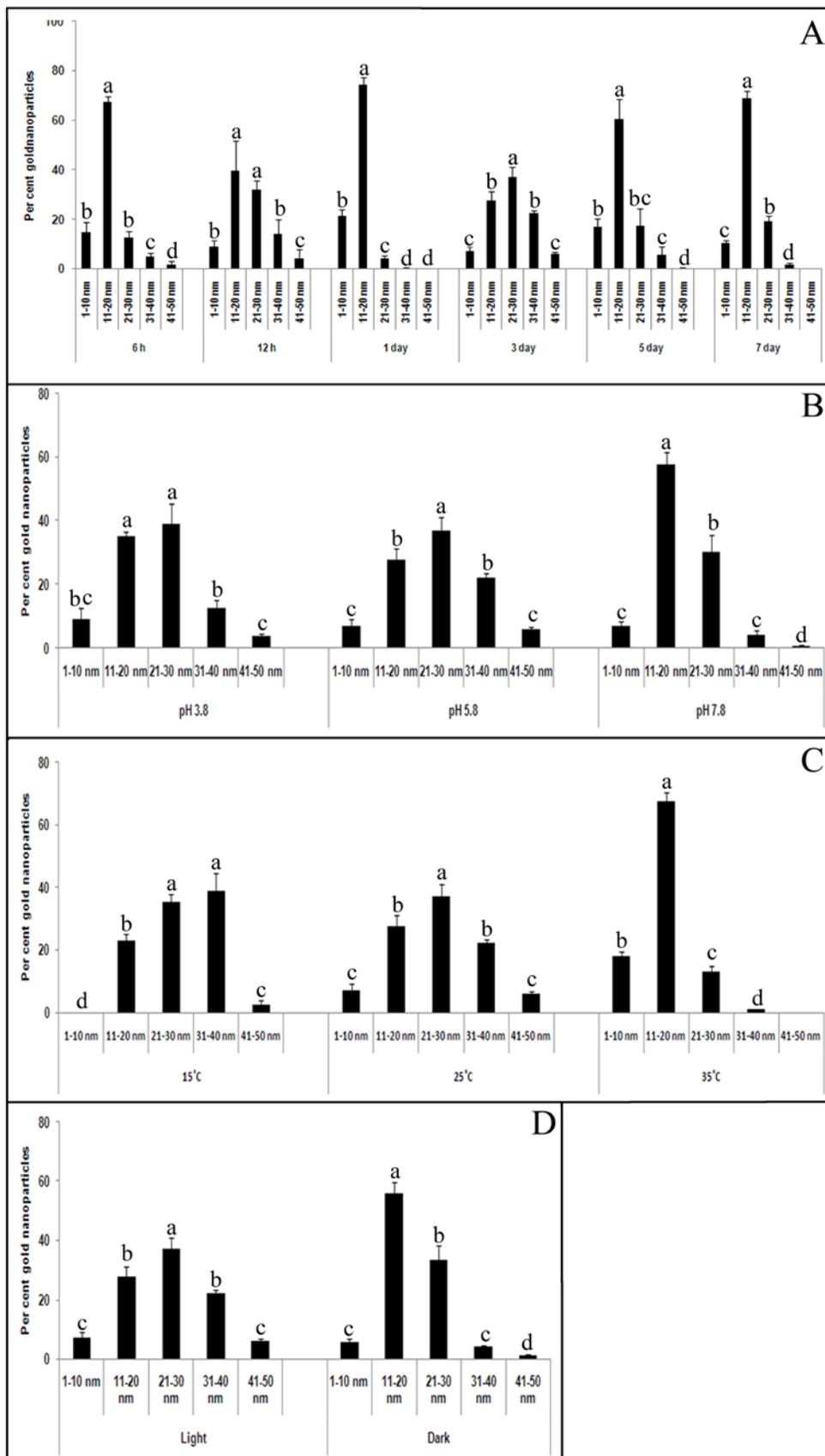


Figure 3. Effects of different growth conditions on *in planta* synthesis of variable sizes of gold nanoparticles. Alfalfa (*Medicago sativa*) was grown on soilless medium as described in the legend to Figure 1 and subsequently transferred to a hydroponic set up containing 50 ppm KAuCl_4 in deionized water under variable growth conditions. Roots were harvested, washed thrice with deionized water, ground to fine powder in liquid nitrogen and then transferred to a 400 mesh copper grid coated with FormVar and observed under transmission electron microscope. The data on the effects of (A) temporal, (B) pH, (C) temperature, and (D) light conditions on the per cent distribution of different sizes of gold nanoparticles are presented. Values are means \pm SE and different letters on the histogram represent means that differ significantly ($P < 0.05$).

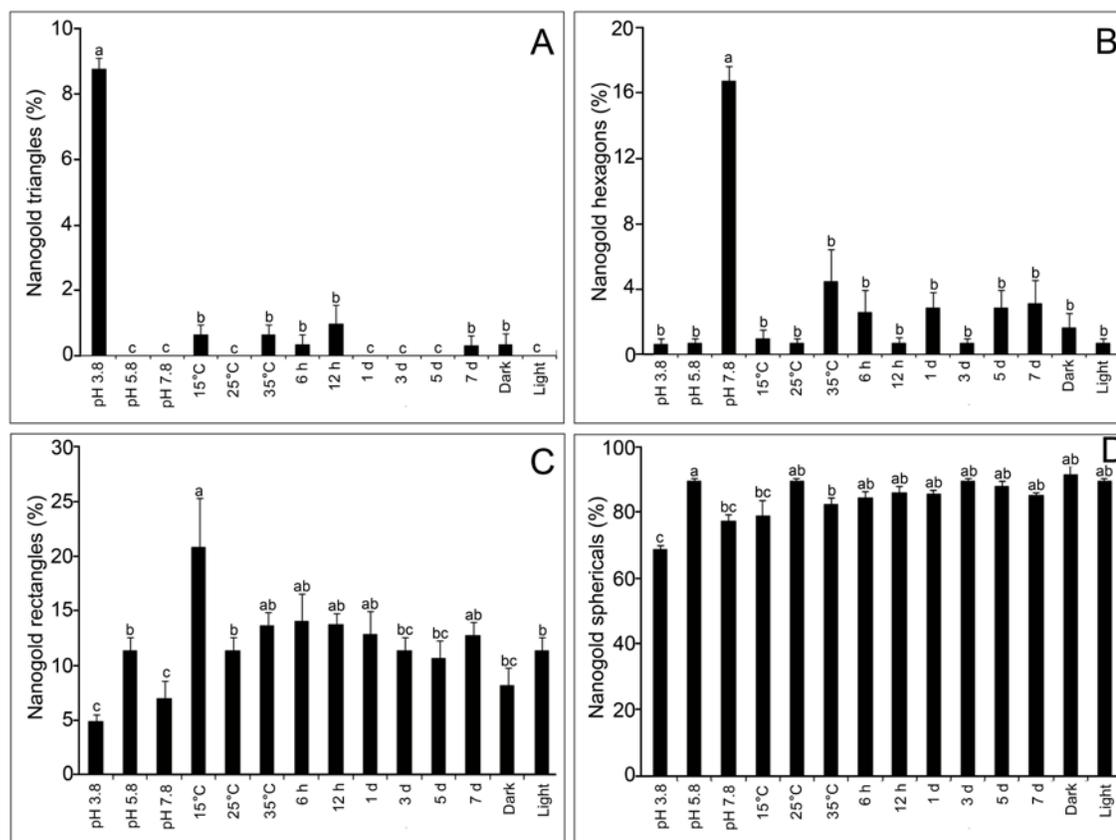


Figure 4. Effects of different growth conditions on *in planta* synthesis of variable shapes of gold nanoparticles. Alfalfa (*Medicago sativa*) was grown on soilless medium and treated with KAuCl_4 in a hydroponic set up and root tissues were processed for TEM analysis as described in the legend in Figure 3. The data on the effects of variable growth conditions on the per cent formation of nanogold particles depicting (A) triangular, (B) hexagonal, (C) rectangular, and (D) spherical shapes are presented. Values are means \pm SE and different letters on the histogram represent means that differ significantly ($P < 0.05$).

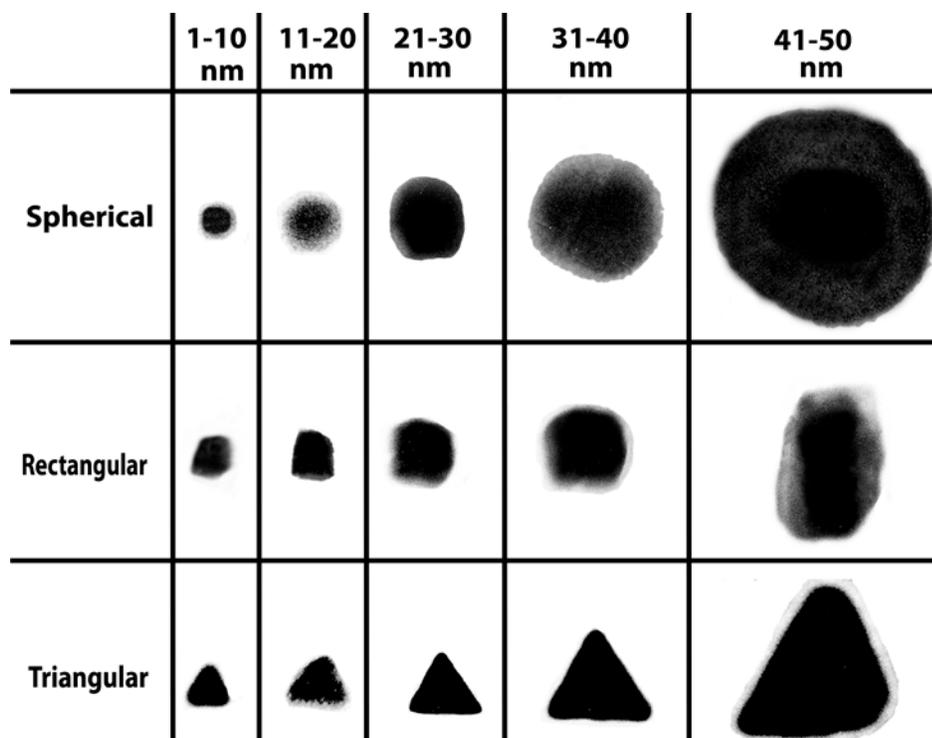


Figure 5. *In planta* synthesis of variable shapes and sizes of nanogold particles. TEM micrographs of gold nanoparticles formed by Alfalfa grown under variable growth conditions and prepared for TEM analysis as described in the legends to Figure 3 and to 4, respectively. The data presented are representative micrographs depicting variable (triangular, rectangular, spherical) shapes across 5 different size classes.

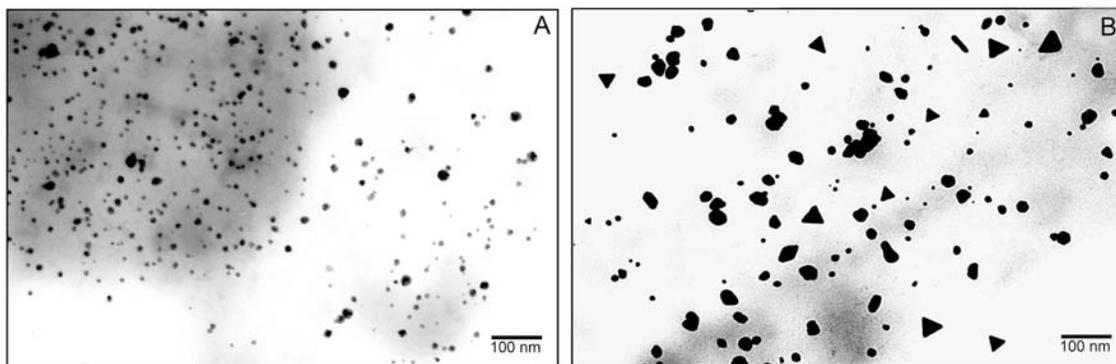


Figure 6. Low pH triggers *in planta* synthesis of nanogold triangles. Alfalfa (*Medicago sativa*) was grown on soilless medium as described in the legend to Figure 1 and subsequently transferred to a hydroponic set up containing 50 ppm KAuCl_4 in deionized water and adjusted to different pH. TEM micrographs of gold nanoparticles produced in the roots of alfalfa were prepared for viewing as described in the legend of Figure 3. The micrographs show (A) spherical nanoparticles generated under standard growth conditions (PAR $120 \mu\text{mol m}^{-2} \text{s}^{-1}$, pH 5.8 and at 25°C), and (B) formation of triangular nanoparticles triggered by low pH growth condition (PAR $120 \mu\text{mol m}^{-2} \text{s}^{-1}$, pH 3.8 and at 25°C).

Appendix I

General Introduction

Manipulating matter at the nanoscale (1-100 nanometers) generates a large surface to volume ratio thereby creating a myriad of new materials endowed with unique optoelectronic and physicochemical attributes. Among the noble metals, the properties of gold in "nano" form change dramatically from that of bulk gold and acts as an effective catalyst, and is no longer a metal - instead it turns into a semiconductor (Shankar et al., 2004; Mohanpuria et al., 2008). The properties of the nanoparticles could further be modified by manipulating their shapes, sizes and controlled dispersity.

Applications of nanogold particles: All that glitters is not gold, goes the old adage, however, nanogold does not glitter but its future looks bright (http://en.wikipedia.org/wiki/Colloidal_gold). In fact, if gold is created in small enough chunks, it turns red, blue, yellow and other colors. Nanoparticles have significant adsorption capacities due to their relatively large surface area, therefore they are able to bind or carry other molecules such as chemical compounds, drugs, probes and proteins attached to the surface by covalent bonds or by adsorption. Nanotechnology has experienced rapid growth in recent years and particularly the usage of nanogold particles with its broad applications in industry, superconductors, drug delivery systems, and in imaging and diagnostics (Fei and Perrett, 2009). Among the variety of gold nanoparticles shapes (rods, flat sheets, spherical, hexagonal, icosahedral and irregular shaped), nanotriangles have found extensive applications in therapeutic medicine. For instance, a recent study has proposed the introduction of gold nanotriangles into the body with antibodies or biomarkers for infra-red radiation mediated cauterization of cancerous cells

(Loo et al., 2004). Furthermore, nanogold particles have extremely dense electron clouds and this property has been exploited by microscopists in the field of optics as a contrasting and staining material (Murphy et al., 2005). The potential exists for the precise and tunable property of light scattering by gold nanoparticles making them an excellent candidate for molecule sensing (Schultz et al., 2003, McFarland and Van Duyne, 2003).

Hazardous implications of nanotechnology: Although there is an unprecedented development of novel techniques that are being employed for generating gold and other metal nanoparticles, there are concomitant growing apprehensions on the risks of generating hazardous by-products causing environmental concerns (<http://www.futureforall.org/nanotechnology/risks.htm>). Furthermore, there are growing concerns about the toxicity of nanoparticles especially in situations where these nanoparticles serve as drug carriers. So grave is the global concern that it has led to the emergence of the new field of “Nanotoxicology” focusing on unpredicted adverse biological effects of nanotechnology on living organisms (<http://www.aspbs.com/toxic.html>, Scheringer, 2008). Nowadays, with an emerging concern of reducing the carbon footprint caused by nanotechnology-related activities, it has become imperative to adopt stringent measures for mitigating the adverse effects of nanotechnologies. In this regard, several methods that are being employed for generation of nanoparticles will be described, along with a brief discussion of their pros and cons.

Wet Synthesis of Gold Nanoparticles: An intense light pulse for 4 to 10 minutes using ultraviolet or visible light is a relatively easy way of photo production of nanoparticles on a small scale and requires only a nominal amount of energy. Although this technique at

small scale does not pose any serious environmental concern, at commercial scale it is not only economically infeasible but also lacks environmental sustainability. Sodium citrate is another traditional wet synthesis technique that is being employed for the generation of nanoparticles (McFarland et al., 2004). The use of the sodium citrate is energy intensive, requiring the boiling of the tri-sodium salt of gold. Again on the bench top scale this method is nominally energy intensive and the byproducts do not pose any serious concern to environment or human health. However, the amount of energy required increases dramatically during industrial/commercial scale production. With the rise in energy costs this alone would be enough to deter the implementation of this technique for the mass production of gold nanoparticles. In addition, the cost factor and environmental concerns associated with the disposal of waste materials make this technique less attractive. The nanoparticles produced with these or other techniques such as chemical reductions by organic and inorganic reducing agents, laser ablation, gamma and electronic irradiations (Bönnemann and Richards, 2001; Lee et al., 2001; Mallick et al., 2004; Bogle et al., 2006; Long et al., 2007; Khaydarov et al., 2009). Nanoparticles produced by the above mentioned methods can be less stable over time leading to aggregation events ending with the nanoparticles falling out of solution. Therefore, there is a need to find a suitable matrix that not only allows the formation of gold nanoparticle of desirable shapes and sizes but also keeps them stable for long-term commercial usage.

Green chemistry for synthesis of nanogold particles: The potential efficacy of various biological organisms, both unicellular and multicellular as sources of “green chemistry” for fabricating nanoparticles in a sustainable eco-friendly environment has been an

attractive and economically viable alternative to “wet synthesis” (Mukherjee et al., 2001; Bhattacharya and Gupta, 2005; Kumar and Yadav, 2009).

Use of microorganisms for the synthesis of nanogold particles: One of the biggest advantages of using microorganisms as a system for the production of nanogold particles is the availability of well developed technologies for growing, maintaining, and harvesting them. In addition microorganisms are amenable to different physiological growth conditions, thereby providing a wider scope of engineering nanogold particles of desirable shapes and sizes. Interestingly, magnetotactic bacteria and other various species that use the earth’s magnetic field for orientation are known to produce iron nanomaterials naturally (Dickson, 1999). The accumulation of iron nanomaterials in these species could be an adaptive evolutionary response to high availability of iron in the environment in which they grow. Diatoms and magnetotactic and S-layer bacteria are also capable of producing nanoscale materials (Pum and Sleytr, 1999). Likewise, *Pseudomonas stutzeri*, a bacterial strain isolated from silver mines, has demonstrated an ability to synthesize a composite nanomaterial of carbon and silver (Joerger et al., 2000). These biologically produced carbon-silver composite materials could potentially provide a viable alternative to the currently used physical and chemical vapor deposition methods that are expensive and require a large commitment of capital and energy. A strain of the common *Lactobacillus* also has the potential for producing pure nanoparticles of gold and silver as well as nanoparticles of gold-silver alloy (Nair et al., 2002). Despite accumulating stable nanoparticles up to 35% of their dry weight biomass, these bacteria sustained normal growth. Production of nanoparticles is not limited to the realm of prokaryotes. For instance, actinomycete (*Rhodococcus* sp.) has the ability to concentrate

gold nanoparticles on the cytoplasmic membrane with some degree of control on size of the nanoparticles being generated and without having any perceptible detrimental effect on its growth but lacked sufficient information to determine the mechanisms controlling the sizes of particles being generated (Ahmad, et al., 2003). Furthermore *Colletotrichum* sp., an endophyte associated with geraniums (*Pelargonium graveolens*), has the ability to produce polydisperse spherical gold nanoparticles in an extra-cellular fashion (Shankar et al., 2003). However, isolation of nanoparticles from microorganisms poses a challenge and therefore a limitation in using this system for viable commercial purposes.

Use of plant biomass/ extract for the synthesis of nanogold particles: Interestingly several groups have shown that the production of nanomaterials is not limited to microorganisms or living organisms for that matter (Shankar et al., 2003, Chandran et al., 2006, Li et al., 2007). The use of biomass and extracts of plants have been shown to produce gold nanoparticles in an extracellular fashion and the appearance of a pinky to ruby red color is a good visual indicator of its synthesis. One of the benefits of using a biomass system is that waste materials from industrial or agricultural systems could be effectively used in the formation of the nanoparticles. Biomass of wheat (*Triticum aestivum*), oat (*Avena sativa*), alfalfa (*Medicago sativa*), and different plants parts i.e., leaf extracts of geranium (*Geranium sanguineum*), lemongrass (*Cymbopogon marginatus*), neem (*Azadirachta indica*), tamarind (*Tamarindus indica*), *Aloe vera* and fruit extract of *Emblica officinalis* have been successfully employed for an efficient and rapid extracellular reduction of Au (III) to Au (0) nanoparticles by reductases and other reducing equivalents (Armendariz et al., 2009; Kumar and Yadav, 2009; Shankar et al.,

2003; Shankar et al., 2005; Shankar et al., 2004a; Ankamwar et al., 2005a; Chandran et al., 2006; Ankamwar et al., 2005b). The use of this approach facilitated generation of an amalgam of rods and many other shapes (Shankar et al., 2003). Since optical properties often play an important role in the functionality of nanogold particles, several studies have also directed their efforts in conferring nanogold particles with desirable shapes and sizes by manipulating the volume of the extract, temperature and pH of the reaction conditions. A study suggested that by using different quantities of aloe extract that the size of nanotriangles being produced can be manipulated, by increasing the concentration of aloe extract from 100 μ L to 4 ml the average size of triangles being produced decreased (Chandran et al., 2006). In addition, soybean extracts successfully reduced gold salts to nanoparticles and were found to be non-toxic when tested by MTT assays which measure the effects of cytotoxic substances on reductases and dehydrogenases in living cells. The nanoparticles were coated with a variety of phytochemicals which imbued them with *in-vitro* stability in a range of chemical solutions (Shukla et al., 2008). The variety of nanogold particles produced using biomass or extract, could have a wide range of potential applications in medicine and medical imaging. However, plant extracts provides a cornucopia of enzymes and several biological constituents that makes it difficult to identify the reactive component actively involved in the reduction of ionic gold. Although there have been several studies demonstrating the feasibility of plant extracts in synthesizing nanogold particles of different shapes and sizes, none has specifically identified a particular correlation between the type of extract used and their commensurate effects on shapes and sizes of the gold nanoparticles generated (Armendariz et al., 2009; Kumar and Yadav, 2009; Shankar et al., 2003; Shankar et al.,

2005; Shankar et al., 2004a; Chandran et al., 2006; Ankamwar et al., 2005a,b). Therefore, with only a limited knowledge available on the actual mechanisms that are involved in the formation of the gold nanoparticles, a plethora of questions need to be answered prior to the use of biomass/extract for commercial production of nanogold particles.

Furthermore, plant extracts are not amenable for fine tuning of the biochemical processes that may be actively involved in the synthesis of nanogold. In addition, removing the nanogold particles from the biomatrices of extract tissues still remains very challenging.

Only partial success has been achieved in extracting nanogold particles from the reaction medium by using sodium citrate or cetyltrimethylammonium bromide (CTAB) (Armendariz et al., 2009).

In planta synthesis of nanogold particles: Among higher organisms, certain plant species incorporate remarkably high levels of heavy metals including Pb, Hg, and Au into their tissues (Anderson et al., 1998; Srivastava et al., 2007; Venkatachalam et al., 2009).

The potential of metal-tolerant and metal-hyperaccumulating plants have been extensively exploited for ecological restoration of contaminated and degraded soils, phytoremediation, phytomining and biogeochemical reconnaissance

(<http://phytoremediation.com.au/about-us-resume-alan-j-m-baker.html>). In addition,

hyperaccumulators could also act as a potent source for phytomining commercially important heavy metals such as gold, silver, and platinum for *in planta* synthesis of nano materials for multiple and diverse applications in an economically and ecologically friendly process. (Gardea-Torresdey et al., 2002; Anderson et al., 1999; Nedelkoska and Doran, 2000, Marshall et al., 2007; Rodriguez et al., 2007). To date, only a few studies have attempted to discern between elemental metal (M^0) deposition and metal salt (M^{x+})

accumulation. Using X-ray absorption near edge spectroscopy (XANES), the ability of *Sesbania* to reduce KAuCl_4 into more than 80% of elemental monodisperse gold nanoparticles ranging in size from 6-20 nm was demonstrated (Sharma et al., 2007). The study also revealed symplastic transport of nanoparticles from the root to shoot resulting in an accumulation of 120 ppm gold (dry weight basis) in aerial tissues. In addition, the authors demonstrated *in situ* catalytic function of gold nanoparticles imbedded in the biomatrix by its ability to reduce 4-Nitrophenol; a primary material for many fungicides and a potential source of environmental contamination. This study amply demonstrated the efficacy of the plant system for “green manufacturing” of gold nanoparticles. *In planta* synthesis has attracted the attention of many other researchers (Gardea-Torresday et al., 2002, Kumar and Yadav, 2009). For instance, using desert willow (*Chilopsis linearis*) researchers found that there was a correlation between the average sizes of the nanoparticles formed, the concentration of Au found in plant tissues, and where the gold is located within the plant tissues (Rodriguez et al., 2007). Additionally alfalfa (*Medicago sp.*) was shown to absorb ionic gold and its translocation to the shoots while maintaining valency. Subsequently the absorbed gold was reduced and coalesced into nanogold particles ranging from 2 to 20 nm in diameter (Gardea-Torresday et al., 2002). Additionally, *Brassica juncea* has been shown to accumulate nanogold particles during growth on gold enriched soils (Marshall, et al., 2007). The authors also suggested the use of enzymatic digestion as a potential solution for separating the gold nanoparticles from the biomatrix. However, presently it is not known whether the gold nanoparticles potentially could also be “green engineered” *in planta* to form the desirable exotic shapes such as nanotriangles and nanorods having commercial viability.

General Conclusions

Biological systems offer many potential advantages that overcome the shortcomings of wet synthesis such as the high inputs of energy and generation of undesirable byproducts. Since usage of biological pathways can be eco-friendly, it is a potential system for mass scale production. Studies to date have shown the utility of plants in “green synthesis” of nanogold particles. Whether nanogold particles could also be “green engineered” to generate their desirable shapes and sizes by altering various growth parameters like temperature, pH, light and nutrient composition is a matter of conjecture and need further detailed investigations. In addition, extraction of nanogold particles from biological matrix is the biggest obstacle in the implementation of this system for commercial production. It is perceived that usage of plant systems /tissues with relatively less lignification of their tissues could provide better chances of isolating nanogold particles. Further investigations are needed to either confirm or dispute this hypothesis.

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Appendix II

Nitric Acid Extraction of Nanoparticles from Plant Biomatrix

Alfalfa plants that were prepared as described above in Materials and Methods (pages 17-19). After dilution to the 20 ml as required for ICP analysis a 1 ml sample was taken from three replicates for TEM analysis. Each 1 ml sample was mixed thoroughly in a 50 ml disposable conical tube and then diluted to a volume of 20 ml. Subsequently 10 μ L was then transferred to a 400 mesh copper Formvar® coated grids, and then viewed at 100 kV in a JEOL JEM 100CX transmission electron microscope. After viewing 3 grids insufficient nanoparticles were found for observation and to make assessment of the distribution of size and or shapes being produced. With lack of success the use of strong acids were abandoned for the remainder of the project.