

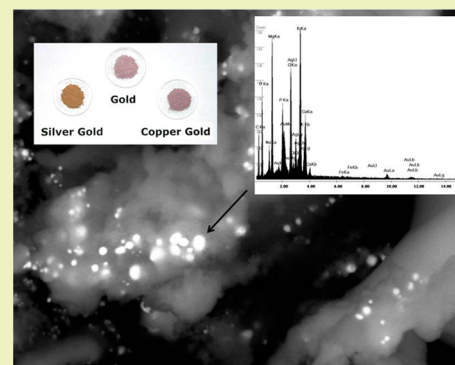
# In Vivo Effect of Copper and Silver on Synthesis of Gold Nanoparticles inside Living Plants

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## S Supporting Information

**ABSTRACT:** Living plants growing on mine waste were assisted to accumulate gold to a concentration greater than 3500 mg kg<sup>-1</sup> (0.35%) dry weight. Where Au was the only high-density metal present in the soil at high concentration, discrete biogenic nanoparticles of the metal were observed in preparations of shoot and leaf biomass. Where an equal concentration of silver was additionally present, a nanoalloy of Au and Ag was observed. An equal concentration of copper in the soil had no chemical effect on the Au and/or Ag structures synthesized and did not alloy with Au and/or Ag. However, both Cu and Ag reduced the size of Au nanoparticles, and the presence of Cu and/or Ag also limited the extent to which Au was reduced in plant tissues to Au(0). Ash prepared from Au-only plant material had a visible reflectance spectrum indicative of a Au surface plasmon resonance that was not apparent for the Au/Ag biomass. The Au concentration of the plant ash (3.2–7.2%) was of the same order of magnitude as Au reference catalysts. Therefore, biogenic synthesis is proposed as a valid technique to create high concentration Au structures in a carbon matrix. Our data suggest that such biogenic nanoparticles should be tested in a range of environmental and industrial reactions.

**KEYWORDS:** Gold, Biogenic nanoparticles, Copper and silver interactive effect, Nanoalloy, Catalyst synthesis



## INTRODUCTION

Gold (Au) accumulation in plants is well established in scientific literature. Studies to investigate Au accumulation have been directed by research into Au phytomining<sup>1,2</sup> or by research into environmentally friendly techniques for the laboratory synthesis of metallic nanoparticles. These Au nanoparticles may be important in a range of industrial, environmental, and medical applications,<sup>3,4</sup> and the potential synthetic advantages of using plants over conventional chemical techniques have been reviewed.<sup>5</sup> Nanoparticles in this context are defined as objects or structures that have at least one dimension in the range of 0.5–50 nm.<sup>6</sup> Bacteria, yeast, fungi, algae, and plants have all been reported at some time to absorb Au ions.<sup>7</sup> However, the potential of vascular plants to reduce absorbed Au ions to form crystalline nanostructures has intrigued many researchers since 2000. Nanoparticles accumulated and constructed by plants in vivo (within living tissues) have been described as biosynthetic or biogenic.<sup>4</sup>

The background concentration of Au in plants is very low, generally less than 0.1 mg kg<sup>-1</sup> dry weight.<sup>8</sup> This is because Au is not readily soluble in soil environments that allow plant growth. To yield Au concentrations in plants that are useful for phytomining or nanoparticle synthesis, the solubility of Au in the plant root zone must be increased. This can be achieved through growing the plants in a synthetic laboratory medium where soluble Au salts are present (hydroponic solution or

agar) or by applying chemical lixiviants to Au-containing soil to induce greater solubility and uptake. The concept of applying lixiviants such as sodium cyanide, ammonium thiocyanate, or sodium thiosulfate to Au-bearing mine waste on which plants are growing underpins the technology of Au phytomining.<sup>1,2,9</sup> To date, studies into biogenic nanoparticle synthesis have been generally limited to systems where hydroponic solution or agar is used as a growth media.<sup>3,4</sup>

Crystalline Au structures have also been recorded in plants growing in a natural environment. Through the analysis of field samples collected during biogeochemical surveys, Dunn showed, using scanning electron microscopy (SEM), the nucleation of 0.25  $\mu\text{m}$  diameter Au particles in black spruce (*Picea mariana*).<sup>8</sup> Dunn also identified crystalline elemental Au attached to a cell wall within the bark of mountain hemlock (dimensions approximately 1  $\mu\text{m}$   $\times$  0.5  $\mu\text{m}$ ).<sup>10</sup> In both studies, the atomic composition of the observed particles was confirmed using X-ray analytical techniques. However, in controlled laboratory studies, the observed form and distribution of Au is different. Gardea-Torresdey et al. observed Au particles in agar-grown alfalfa ranging from 2 to 40 nm in diameter.<sup>3</sup> Bali and Harris observed Au particles ranging from 5 to 20 nm in

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diameter in hydroponic *Brassica juncea* and *Medicago sativa* (alfalfa).<sup>4</sup> Literature evidence for Au nanoparticle biosynthesis indicates nanoparticle accretion within living tissues over time. Our interpretation of the literature is that rapid but controlled laboratory experiments, conducted using a high concentration of Au, appear to lead to a dominance of small nanoparticles, whereas longer-term accumulation of Au from soil appears to lead to the formation of less regular but large particles that have dimensions in the micrometer scale.

The described laboratory studies have been conducted in controlled media where the element composition of the plant root zone can be strictly managed. But these conditions are not representative of a soil environment where mine waste or tailings is the source of Au for plants, as is the case for phytomining. Such growth media will have a high concentration of other elements that may affect both the level of uptake and the form of nanoparticles synthesized in a plant. Published work has speculated that the high concentration of copper (Cu) that is induced into plants along with Au may affect the viability of plants during Au phytomining.<sup>2,11</sup> As Cu and silver (Ag) commonly occur in Au-rich mine waste and soil, the interactive effect of these metals on Au uptake and form inside a plant could be significant, both in terms of the potential usefulness of the resulting nanoparticles and in terms of an engineering solution to recover these metals from the plant material.

A range of plant species have been used during Au accumulation studies. All tested plant species show the ability to accumulate Au if the metals can be made soluble in the root zone, and one research group has reported that *Brassica juncea* will accumulate Au, Cu, and Ag as a nanoalloy when grown on Au-rich soil.<sup>12</sup> Despite an early suggestion that plants may accumulate discrete Au nanoparticles from the soil environment,<sup>3</sup> plant physiology would suggest that plants accumulate a soluble Au complex. This would infer that plants reduce accumulated Au ions to the elemental state inside plant tissues to form nanoparticles. A mechanism for the formation of Ag nanoparticles in plants has been proposed,<sup>13</sup> but no such mechanistic determination has been made for Au.

To study the formation of potentially useful metal nanoparticles in plants, a high metal concentration in the growth media is necessary, and hydroponic experiments have used soluble Au concentrations of up to 1000 mg L<sup>-1</sup>.<sup>4</sup> Total Au concentrations of this order of magnitude do exist in mineral ores or concentrates, but another route to preparation is to spike an ore material that contains mineral surfaces with a solution of the metals under study. Mineral surfaces will retain cations as a function of cation exchange and specific adsorption,<sup>14</sup> and spiking these surfaces will generate a synthetic ore on which plants can grow. At the point of optimal biomass, a lixiviant solution is applied to the soil to dissolve the metals and thereby increase the concentration of soluble metal that is available for uptake. This was the approach followed in the current work.

The purpose of our research was to investigate the effect of Cu and/or Ag present in soil on the concentration of Au that is accumulated by *Brassica juncea* L. and to examine the subsequent form and distribution of Au that is stored within the leaf and shoot biomass. This was achieved by preparing soil with a target concentration of 100 mg kg<sup>-1</sup> of all combinations of Au and/or Cu and/or Ag, growing plants on this soil, and then treating the soil with a solution of potassium cyanide after a defined growth period to induce metal uptake. The interactive effect of Cu and/or Ag on Au nanoparticle biosynthesis may

have an important bearing on the value of these nanoparticles to industrial and catalytic applications. Furthermore, the nature of the nanoparticles could influence the choice of technology used to recover gold from the biomass product of any phytomining operation. The motivation for our research was to generate data that could allow for useful commentary on the interactive effect of Cu and Ag on Au nano and microstructures that might be applicable to the research areas of catalysis and biomass processing.

## ■ EXPERIMENTAL SECTION

**Plant Growth Experiment.** Plant growth experiments were conducted at Massey University in Palmerston North, New Zealand, under greenhouse conditions. The soil used for this study was waste non-acid generating rock from the Waihi Gold Mine in the Coromandel mining district of the North Island of New Zealand. The rock was the reject split from reverse circulation (RC) grade control drilling at the mine and comprised a heterogeneous collection of fragments less than 4 mm in diameter (50% less than 2 mm). The cation exchange capacity (determined as the summation of 1 M neutral ammonium acetate extractable cations K, Ca, Mg, Na, and extractable acidity) of the less than 2 mm fraction was 10 me/100g, with a pH (in water) of 5.70. Gold chloride solution prepared through the digestion of Au metal in concentrated nitro-hydrochloric acid (*aqua regia*) and copper nitrate and silver nitrate solutions prepared through the dissolution of analytical grade reagents were used to amend the waste rock with the seven metal combinations of Au-only, Cu-only, Ag-only, Au/Cu, Au/Ag, Cu/Ag, and Au/Cu/Ag, plus a control.

The level of nitrate and chloride in all waste rock preparations was kept the same through the balancing of these anions with either a blank nitro-hydrochloric acid digest solution (chloride with no Au) or calcium nitrate solution (nitrate with no Cu or Ag). Agricultural lime (80% CaCO<sub>3</sub>) at a rate of 5 g kg<sup>-1</sup> was added to every preparation to balance the added acidity of the acid chloride. The adopted methodology provided for the same level of chloride and nitrate amendment and salinity in all substrate preparations, including the control. The variable parameter was the relative presence of Au and/or Ag and/or Cu. Soil pH was determined in water at a soil:water ratio of 10 g:25 mL using a Radiometer PHM83 Autocal pH meter. Soil conductivity (EC) was determined in water at a soil:water ratio of 10 g:50 mL using a Hanna Instruments HI8633 conductivity meter.

The appropriate solutions were dripped into 1 kg amounts of waste rock, with one portion allocated to each treatment. The concentrated "ore" was dried in a 100 °C oven overnight according to the method of Msuya et al.,<sup>15</sup> then diluted with a further 5 kg of non-amended waste rock to produce a final growth substrate containing a target concentration of 100 mg kg<sup>-1</sup> of each metal. The eight preparations were then distributed into 1 L greenhouse pots (seven replicates; 800 g in each pot) and watered every second day for two weeks to allow the amendments to equilibrate with the waste rock. This sequence of metal spiking and equilibration is used to study heavy-metal uptake by plants and has been reported to be a viable system to synthesize soils with a high concentration of metal.<sup>16,17</sup> The aim of the two-step heating then equilibration cycle was therefore to ensure that all metal remaining in the system was present as free metal or metal complexed with the mineral constituents of the waste rock. The experiment consisted of 56 pots in total.

After the equilibration period, vertical core samples (6 g) were taken through the center of each pot. The core space was filled with control waste rock. Seeds of *Brassica juncea* were then added to the pots. Approximately 20 seeds were seeded per pot. Watering was continued every second day to maintain a water content at 80% of field capacity to mitigate leaching from the pots. Germination was fully apparent after six days, with seeds growing on the Ag treatments showing the slowest germination rate. After three weeks, the seedlings were thinned to leave five healthy plants per pot. A general purpose

N–P–K fertilizer was applied to each pot at the time of thinning at the rate recommended by the manufacturer.

After nine weeks of growth, potassium cyanide solution (100 mL of 4 g L<sup>-1</sup>) was irrigated onto four replicates of each treatment to promote metal solubility and uptake (0.5 g of KCN/kg soil). Cyanide has previously been shown to be the most effective chemical to promote gold uptake in plants.<sup>2,9</sup> Three pots for each treatment did not receive cyanide treatment and were designated non-treated controls. Regular watering was discontinued at this point, and 14 days after treatment, the roots and shoots from each pot were harvested, rinsed with deionized water, placed in paper bags, and then dried in an oven (70 °C) until constant weight. Soil was resampled from each pot at harvest using a vertical corer through the root zone (6 g core). Plant biomass was subsequently weighed and ground using a coffee grinder in preparation for Cu, Ag, and Au analysis. All soil samples were dried and then homogenized using a mortar and pestle.

**Elemental Analysis.** Analysis of all plant and soil samples for Cu, Ag, and Au concentration was conducted using flame atomic absorption spectroscopy (FAAS) at facilities of the Institute of Natural Resources, Palmerston North. Briefly, subsamples of plant material (0.2 g) were ashed and then digested in nitro-hydrochloric acid before analysis. Subsamples of soil (1 g) were digested in nitro-hydrochloric acid before analysis. A parallel quality assurance procedure was adopted using an internal reference sample for Cu<sup>18</sup> and a certified Standard Reference Material for Ag (PTM 1a).<sup>19</sup> The analytical results for the QA program differed by 3.3% of the published result for Cu and less than 1% of the certified value for Ag. X-ray absorption spectroscopy (XAS) data were collected on Beamline 2–3 at the Stanford Synchrotron Radiation Laboratory (SSRL). Detailed methodology for the atomic absorption and XAS analytical procedures are described in the Supporting Information.

**Electron Microscopy.** An objective for this research was to assess the possible implication of metal particle form and distribution on biomass processing at the end of a phytomining operation. As the first stage of processing is likely to be incineration of the biomass,<sup>9</sup> representative samples of each plant–metal combination were first ashed at 550 °C in porcelain crucibles for 4 h. This pretreatment had the added effect of concentrating the metal in the residual ash. Metal particles within the plant ash were imaged using scanning electron microscopy (SEM) (FEI Quanta 200 environmental scanning electron microscope). Ashed samples were placed upon a piece of tape mounted on stainless steel stubs and sputter coated with carbon prior to visualization under SEM. Electron microscopy microanalysis was conducted using back scatter imaging (BSE), and images were collected using a Dual segment BSE detector. The following parameters were used: accelerating voltage 20 KeV and spot size 3 for the BSE images to give good signal. The SEM was operating in the high vacuum mode. The chemical identity of the observed structures was confirmed using energy dispersive X-ray spectroscopy (EDS). The EDS spectra were collected at 20 KeV, with a larger spot size to adjust to a suitable count rate per second for spectrum collection. Each spectrum was collected using an EDAX detector in high vacuum mode.

**Reflectance Spectra.** Discrete Au nanoparticles impart a purple color to materials when present at a sufficiently high concentration.<sup>6</sup> In order to investigate the effect of plant metal combination on the absorbance of light by plant ash, the reflectance spectra of each ash sample prepared for the SEM study was obtained using a Field Spec Pro instrument (Analytical Spectral Devices, Inc., Boulder, CO) fitted with a plant probe (model A122317). Ash samples were transferred into a glass container and placed on top of the probe, and the reflectance spectra was obtained through illumination. The reflectance spectra were collected across the visible and infrared portions of the electromagnetic spectrum (350–2,500 nm).

**Statistical Analysis.** Metal concentration and plant biomass experimental data were analyzed using a one-way ANOVA (Tukey's studentized range test) with SAS/STAT statistical software (SAS Institute). Differences between means are reported at a probability of 0.05 ( $p < 0.05$ ).

## RESULTS AND DISCUSSION

**Metal Concentration in the Soil.** The Au, Cu, and Ag concentration in the soil at the time of sowing *Brassica juncea* is presented in Table 1. The final Cu concentration is elevated

**Table 1. Metal concentration, pH, and EC of Prepared Soils at Time of Seeding<sup>a</sup>**

soil treatment	soil pH and EC at time of planting		soil concentration for each element (mg/kg)		
	pH	EC (μS/cm)	soil Cu	soil Ag	soil Au
copper	8.04	148	139 (6.43) <sup>a</sup>	2.73 (0.30) <sup>b</sup>	<10 (–) <sup>c</sup>
silver	8.17	151	39.2 (2.94) <sup>c</sup>	27.37 (5.6) <sup>a</sup>	<10 (–) <sup>c</sup>
gold	8.20	127	46.6 (2.77) <sup>c</sup>	4.34 (0.42) <sup>b</sup>	115 (9.86) <sup>a</sup>
copper/silver	7.89	126	115 (0.51) <sup>b</sup>	33.8 (5.3) <sup>a</sup>	<10 (–) <sup>c</sup>
copper/gold	8.06	123	109 (5.92) <sup>b</sup>	5.01 (0.53) <sup>b</sup>	79.3 (3.58) <sup>b</sup>
silver/gold	8.06	152	43.4 (3.37) <sup>c</sup>	42.5 (6.2) <sup>a</sup>	95.5 (8.63) <sup>ab</sup>
copper/silver/gold	8.11	120	112 (2.98) <sup>b</sup>	41.9 (4.1) <sup>a</sup>	91.4 (4.24) <sup>ab</sup>
control	7.86	174	33.9 (2.1) <sup>c</sup>	3.29 (0.51) <sup>b</sup>	<10 (–) <sup>c</sup>

<sup>a</sup>Concentrations are mean and standard error,  $n = 7$ ; means with the same letter for each element are not significantly different;  $p < 0.05$ . pH and EC values were measured on a bulk soil sample generated as a composite of all same-treatment cores.

above the target of 100 mg kg<sup>-1</sup>, primarily due to the concentration of Cu already present in the soil (control concentration). The agreement of the analyzed Au and Cu concentration in the soil with the target concentration indicates good and rapid retention of these metals to the soil through adsorption onto mineral particles. The final Ag concentration is significantly below the target concentration of 100 mg kg<sup>-1</sup>. This may be due to leaching of a significant portion of the added Ag during the two-week equilibration period and prior to seeding (less adsorption of Ag to mineral surfaces relative to Cu and Au). Soil pH and conductivity (EC) at the time of seeding were similar for all treatments.

**Metal Concentration in the Plant.** The metal concentration in *B. juncea* biomass harvested from each of the cyanide-treated soil preparations is presented in Table 2. *Brassica juncea* accumulated Cu, Ag, and Au from all soil preparations, with the final concentration of each metal a function of the metal combination in the soil. At harvest, all cyanide-treated biomass was severely affected by chemical or metal toxicity and showed signs of chlorosis or necrosis. However, this was an expected result. During soil-based gold phytoextraction, the chemical treatment is only applied at a point of maximum biomass. Over the following several days, any treated plant will begin to die as it accumulates the pulse of soluble metal. Biomass is harvested once evapotranspiration is inhibited by necrosis. A physiological mechanism for metal uptake after EDTA application to soil was described by Nowack et al.<sup>20</sup> Presumably, a similar mechanism can be expected for cyanide, where the increased salt concentration in soil solution associated with KCN treatment disrupts the natural barrier at the endodermis, allowing the metal complex to flood in via the xylem. Prior to treatment, there was no visual sign of plant stress that could be attributed to the growth media, and there was no evidence for increased metal uptake in the non-cyanide treated plants relative to the

**Table 2. Biomass Yield and Metal Concentration in the Cyanide-Treated *Brassica juncea* Shoot (Cu, Ag, and Au) and Root (Au only) Biomass and Total Average Harvested Au Yield as a Function of Soil and Cyanide Treatment<sup>a</sup>**

soil treatment	shoot harvested biomass yield (g) and concentration (mg/kg)				root mass and concentration		harvested gold yield (mg)
	mass	Cu	Ag	Au	mass	Au	
copper	3.03 (1.26) <sup>b</sup>	800 (74.9) <sup>a</sup>	14.5 (2.03) <sup>c</sup>	10.48 (1.89) <sup>d</sup>	1.54 (0.10) <sup>ab</sup>	17.6 (1.58) <sup>c</sup>	0.06
silver	4.15 (0.82) <sup>b</sup>	35.7 (16.3) <sup>c</sup>	355 (37.5) <sup>ab</sup>	<10 (–) <sup>d</sup>	1.19 (0.10) <sup>b</sup>	10.2 (1.58) <sup>c</sup>	0.06
gold	4.48 (0.19) <sup>ab</sup>	36.14 (3.59) <sup>c</sup>	13.4 (1.62) <sup>c</sup>	3612 (404) <sup>a</sup>	1.90 (0.16) <sup>ab</sup>	1824 (241) <sup>a</sup>	27.68
copper/silver	6.78 (0.59) <sup>a</sup>	386 (21.7) <sup>b</sup>	282 (30) <sup>b</sup>	16.4 (2.32) <sup>d</sup>	1.75 (0.11) <sup>ab</sup>	15.8 (3.83) <sup>c</sup>	0.09
copper/gold	4.44 (0.37) <sup>ab</sup>	452 (39.4) <sup>b</sup>	14.3 (1.50) <sup>c</sup>	1803 (103) <sup>b</sup>	1.60 (0.21) <sup>ab</sup>	2224 (62.9) <sup>a</sup>	12.54
silver/gold	5.35 (0.22) <sup>ab</sup>	77.9 (53.1) <sup>c</sup>	313 (34.9) <sup>b</sup>	882 (77.7) <sup>c</sup>	1.30 (0.03) <sup>b</sup>	1101 (87.3) <sup>b</sup>	5.58
copper/silver/gold	3.76 (0.15) <sup>b</sup>	334 (26.9) <sup>b</sup>	461 (45.8) <sup>a</sup>	1592 (213) <sup>b</sup>	2.06 (0.21) <sup>a</sup>	1342 (101) <sup>b</sup>	7.09
control	3.42 (0.48) <sup>b</sup>	30.8 (1.48) <sup>c</sup>	21.3 (1.86) <sup>c</sup>	28.9 (2.10) <sup>d</sup>	1.23 (0.23) <sup>b</sup>	24.1 (2.35) <sup>c</sup>	0.12

<sup>a</sup>Values are mean and standard error,  $n = 4$ ; means with the same letter for each element are not significantly different,  $p < 0.05$ . Metal concentration in the harvested control biomass (non-cyanide treated,  $n = 3$ ) is presented in Table S1 of the Supporting Information.

respective metal-treatment control (Table S1, Supporting Information). Gold, Cu, and Ag was not available for plant uptake until the cyanide treatment was applied to the soil.

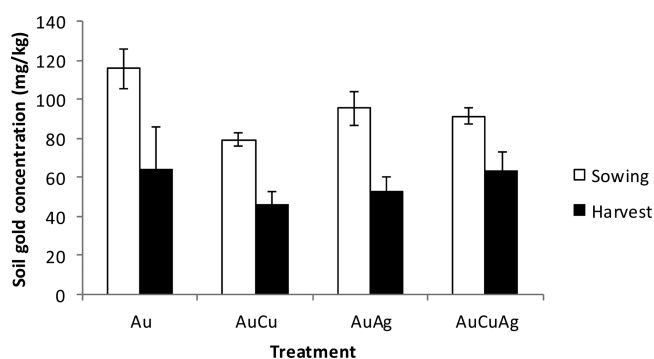
**Uptake of Cu and Ag.** The concentration of Cu in the plant was decreased where Ag and/or Au was also present in the soil. The highest concentration of Cu in *Brassica juncea* (800 mg kg<sup>-1</sup>) was recorded for the plants growing on the Cu-only amended soil. There was no differential effect of Au and/or Ag on the Cu concentration in the plants. With respect to Ag, there was no clear effect of Cu and/or Au on the Ag concentration that was accumulated after cyanide treatment of the soil.

**Uptake of Au.** The maximum Au concentration was recorded for plants grown on the Au-only substrate (3612 mg kg<sup>-1</sup> or 0.36% dry weight). The Au uptake concentration was significantly reduced where an elevated concentration of Cu and/or Ag was also present in the soil and, therefore, in the plant after cyanide treatment. The order of this effect from least to greatest was Cu < Cu/Ag < Ag, and the Au concentration of the Au/Ag plants was significantly lower than that of the Au/Cu plants. The Au concentration in plants growing in the Au/Ag soil was reduced to only 25% of that recorded for plants growing in the Au-only soil.

**Plant Metal Mass Balance.** The biomass recorded from each of the soil treatments shows an effect of metal combination in the soil on plant dry-matter yield (Table 2). Shoot biomass was significantly increased for the Au-only soil relative to the control soil. This was an unexpected result, and no reason for this observation can be proposed. Root biomass production was not as dramatically affected by the metal combination in the soil. The Au concentration in roots was greatest for the Cu/Au treatment (2223 mg kg<sup>-1</sup>). The root Au concentration was not significantly different to the shoot Au concentration for the treatment where Cu and/or Ag was also present in the soil but was lower than the shoot Au concentration for the Au-only soil. The ratio of shoot:root Au was 2 for the Au-only soil but 1 for the mixed Au treatments. These ratios indicate no preferential retention of Au in roots and more active translocation in the absence of Cu and/or Ag.

Table 2 presents a calculation of the total amount of Au (mg per pot) extracted by *Brassica juncea* for each treatment. The combination of high Au concentration and large biomass results in an increased Au yield for the plants growing on the Au-only soil. The yield was greater than twice that of the plants growing on the Cu/Au soil, four times that of the plants on the Cu/Ag/Au soil, and five times that of the plants growing on the Ag/Au

soil. There was a reduction in the Au concentration in soil at harvest relative to the time of sowing (Figure 1), and detailed



**Figure 1.** Mean Au concentration in the Au-only treatment soils at the time of sowing ( $n = 7$ ) and at harvest ( $n = 4$ ). Error bars represent the standard error of the mean. For all treatments, the soil Au concentration at harvest was significantly lower than that at sowing ( $p < 0.05$ ). There were no differences in the soil Au concentration between the treatments at harvest.

mass balance calculations show that much of this Au can be accounted for through plant uptake (Table 3). Cyanide irrigation and subsequent watering was designed to mitigate the risk of leaching out of each pot. However, net Au loss from the experimental system indicates that some mobilization of Au may have occurred to areas outside the plant root zone. In contrast, mass balance calculations for Cu and Ag show a net increase of these metals in the cored region of each pot (Table S2, Supporting Information). This may indicate mobilization and movement of these metals into the root zone but restricted uptake. Net loss of Au from the root zone but net gain of Cu and Ag may indicate preferential uptake of Au over Cu and Ag; the concentration of Au in treated plants was higher than Cu or Ag.

**Interactive Effect of Cu and Ag on Concentration and Form of Au in *Brassica juncea* Aerial Biomass.** Copper and Ag in the soil have an effect on the aerial plant biomass and on the Au concentration in the plants. With respect to biomass, plant growth appears to be increased when Au is present in the soil (relative to the control). This effect is more apparent for shoot biomass than root biomass. Both Cu and Ag in the plant depress the final Au concentration that is accumulated in the aerial biomass. This effect was more apparent for the Ag/Au treatment and could be due to the presence of Ag in the plant. However, this effect was ameliorated to some extent where Cu

**Table 3. Mass Balance for Au in Experimental System Expressed as Average for Each Pot from each Soil Treatment. The total amount of Au in soil is calculated using the Au concentration at sowing and harvest, assuming 0.8 kg of soil per pot<sup>a</sup>**

soil treatment	gold in system at sowing		gold in system at harvest				mass balance
	soil Au (mg/kg)	Au in soil = total Au in system (mg)	soil Au (mg/kg)	Au in soil (mg)	Au in plant (mg) <sup>b</sup>	total Au in system (mg)	
gold	115	92.0	64.4	51.5	27.7	79.3	lost 12.7 mg
copper/gold	79.3	63.4	46.4	37.1	12.5	49.6	lost 13.8 mg
silver/gold	95.5	76.4	53.3	42.6	5.58	48.2	lost 28.2 mg
copper/silver/gold	91.4	73.1	63.4	50.7	7.09	57.8	lost 15.3 mg

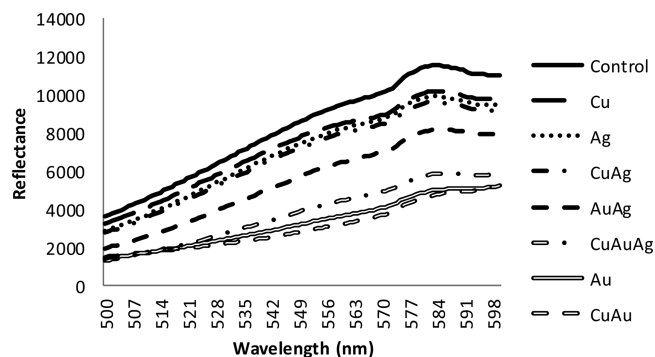
<sup>a</sup>Statistical parameters for Au in soil and biomass are presented in Tables 1 and 2. Similar mass balance calculations for Cu and Ag are presented in Table S2 of the Supporting Information. <sup>b</sup>Data from Table 2.

was also present. The concentration of Au in the aerial biomass was the same for both the Cu/Au and Cu/Ag/Au treatments and was significantly greater for these two treatments than the Ag/Au treatment.

To prepare the metal-containing biomass for further characterization, a bulk sample of each treatment was ashed at 550 °C for 4 h. This allowed for visual record of the interactive effect of Cu and Ag on Au uptake through observation of the color of the plant ash. The color was affected by the structure of metals in the ash. The concept that plants store Au in the form of nanoparticles was first described in 2002,<sup>3</sup> and this finding has been corroborated by a number of research groups. Red or purple is the color typically associated with nanoparticulate Au,<sup>6</sup> and it is a function of absorption of light in the green part of the electromagnetic spectrum (about 520 nm). This wavelength corresponds to the frequency at which a plasmon resonance occurs with the Au.<sup>6</sup> Bulk (non-nanoparticulate) Au is yellow due to reflection of light with a wavelength corresponding to the red–yellow part of the spectrum.<sup>21</sup>

In ashed samples of the current study, a purple color indicative of nanoparticles was observed for the Au-only and Au/Cu treatments but not for the Au/Ag treatment. Plant ash with a high concentration of Au/Ag was brown. Similarly, the ash of plants with a high concentration of Cu/Ag/Au was brown with a slight indication of purple. These observations can be quantified through consideration of the reflectivity spectra of the samples in the range of 500–600 nm (Figure 2). The reflectivity spectrum for the Au and Au/Cu samples across this range is similar and reduced relative to the samples that do not show a dominant purple color. Low reflectivity at 520 nm indicates relatively strong absorption of light at this wavelength. The spectrum for the Au/Ag sample is closer to that of the Ag-only than to the Au-only sample. The spectrum of the Au/Ag/Cu biomass is similar to that of the Au-only and Au/Cu biomass indicating some formation of discrete Au nanoparticles in this sample. All metal-enriched ash samples show a reflectivity spectrum that is reduced relative to the control biomass, indicating increased absorption of energy across this portion of the electromagnetic spectrum. This is likely a function of the increased concentration of all metals in the ash.

The shape and distribution of the high atomic number (high density, specifically Au and Ag) metals within the ashed plant biomass was further investigated using the back scatter imaging function of scanning electron microscopy (SEM) (Figure 3). Although metals were unevenly distributed throughout the plant ash, the chemical composition of high-density particles within each ash preparation was homogeneous (analytical data in Figure S1, Supporting Information). The electron micros-

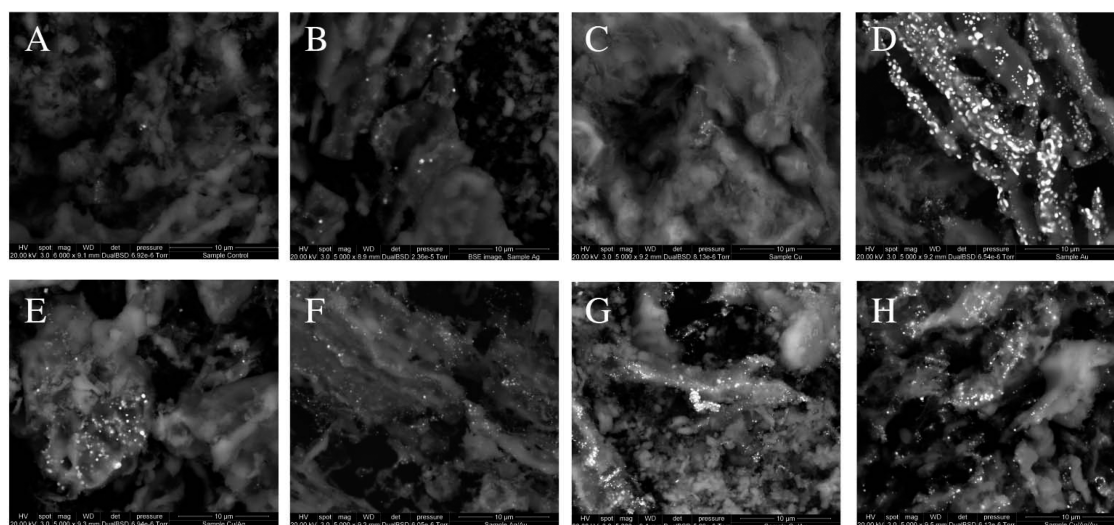


**Figure 2.** Spectral reflectance of plant ash prepared for each treatment across the green/yellow visible portion of the electromagnetic spectrum (500–600 nm). A low reflectance value for one treatment preparation relative to another indicates increased absorption of visible light at any given wavelength. Reflectance is a unitless parameter and is calculated as the ratio of the amount of light reflected by a material under study to that reflected by a pure white standard. Each spectrum is an average of three scans. The standard deviation for each mean was less than 1% of the mean value.

copy images reported here are, therefore, representative of each treatment preparation.

The control biomass showed a low density of high-contrast particles that were identified using energy dispersive X-ray spectroscopy (EDS) as Au and Ag (Figure 3A). These particles represent the native Au and Ag present in the soil that was taken up by plants once made soluble by potassium cyanide. The Cu biomass showed a similar low density of high-contrast particles (Figure 3C). However, no Au and Ag signal could be detected in these particles. There was no apparent particulate distribution of Cu in the plant material, and no Cu was detected using EDS. The plants grown on the Ag-only soil show a moderate density of Ag particles (Figure 3B). The plants grown on the Au-only soil show a high density of large particles that do not fit the definition of nanoparticles (longest dimension from 1 to 2  $\mu\text{m}$  with no dimension less than 50 nm), although there was a background of smaller particles within the nanoparticle size range (Figure 3D). The identity of both the Ag and Au particles was confirmed using EDS as being Au or Ag in combination with Cl, P, S, and base metals.

Imaging of the Cu/Ag plant material showed high-contrast particles of Ag, with a shape and distribution similar to that of the Ag-only biomass (Figure 3E). The Cu/Au plant material showed a high density of Au particles, but these were smaller than the particles observed for the Au-only biomass (Figure 3G). The largest particles in the Cu/Au preparation had dimensions less than 300 nm with an abundance of particles



**Figure 3.** Scanning electron microphotographs (SEM) of high-density metal structures in the plant ash prepared for each treatment. All images were obtained using a dual segment BSE (back scatter) collector that shows high-density particles as bright objects. The chemical identity of the particles in these images was confirmed using X-ray microanalysis (EDS). (A) control, (B) silver-only, (C) copper-only, (D) gold-only, (E) copper and silver, (F) silver and gold, (G) copper and gold, and (H) copper, silver and gold.

with dimensions less than 50 nm. For both the Cu/Ag and Cu/Au samples, EDS showed Ag or Au only, in combination with Cl, O, and base metals, but no Cu. The Au/Ag biomass showed a regular distribution of nanoparticles (Figure 3F), and these particles were again smaller than the Au particles recorded for the Au-only biomass (all dimensions less than 330 nm with an abundance of particles less than 50 nm). Microanalysis with EDS indicated no distinction between Au and Ag in these particles and no presence of Cu. The Cu/Ag/Au biomass showed a high density of particles that were smaller than the Ag or Au particles recorded for the Ag-only or Au-only biomass (all particles less than 335 nm with an abundance of particles less than 50 nm; Figure 3H). These particles were analyzed as a mixture of Au and Ag associated with Cl, P, base metals, and Fe.

Dry plant material containing gold with or without Cu and/or Ag was also analyzed using X-ray absorption spectroscopy (XAS-XANES) to allow quantification of the speciation of Au inside the biomass (Figure 4 and Figure S2, Supporting Information). The XANES data show that for the Au-only biomass 88.5% of Au was present as Au(0) with 14.1% present as AuOH (Au<sup>+</sup>). For the Ag/Au, Cu/Au, and Cu/Ag/Au biomass, the split was approximately 50:50 between the reduced and non-reduced forms Au(0) and Au<sup>+</sup> (Table 4).

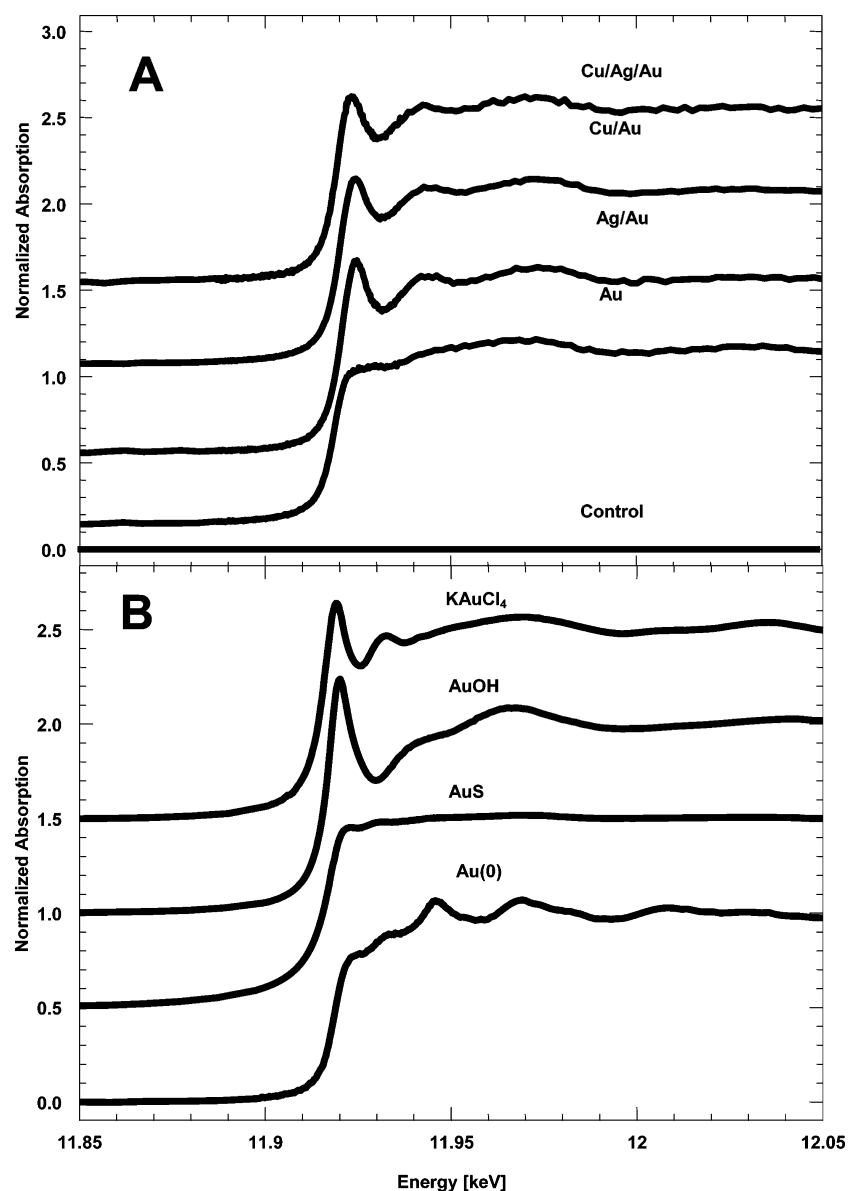
**General Discussion.** Results from the XAS study confirm the presence of reduced gold inside the shoot biomass of *Brassica juncea* (a higher plant) as has been previously reported.<sup>12</sup> The XAS data furthermore show that the amount of Au reduced from Au(I) to Au(0) is affected by the presence of Cu and/or Ag in plants and that there is an interactive effect of Cu and/or Ag on the speciation of Au stored within plant tissues. Assuming the reduction of Au occurs by way of physiological function as the plant is viable, XANES data could indicate that the uptake of Cu and/or Ag in addition to Au causes physiological stress within the plant. The effect of Cu and/or Ag could explain the reduced size of Au particles in the biomass that also contains Cu and/or Ag. We proposed that living plants “build” nanoparticles as they accumulate the metal. This means that Au nanoparticles will increase in size with time. The same Cu- and/or Ag-effected stress that inhibits the

reduction of Au(I) to Au(0) could also inhibit the aggregation of Au atoms inside plant tissues to form large nano and microstructures.

Electron microscopy imaging of the ash preparations supports the formation of metallic particles of Ag and/or Au inside *B. juncea* biomass. The metallic structures were a mixture of nanoparticles and microparticles and were analyzed to be Au or Ag or an alloy mixture of Au and Ag. In contrast to reported literature, Cu was not detected in the identified particles. Copper did not alloy with Au and/or Ag in the ash. The color of the ash prepared for each treatment supports the SEM observations. The purple color of discrete Au nanoparticles was observed for the Au and Au/Cu preparations but not where Au was alloyed with Ag.

The findings from the current research have implications for the synthesis of nanoparticles for industrial use. Despite the suggested application of plant biomass enriched in Au nanoparticles to industrial catalysis, few studies have investigated such a proposition. The first proposals were for the use of Au-rich biomass or ash as catalysts for heterogeneous reactions such as the oxidation of carbon monoxide or the water–gas shift reaction. The catalytic reduction of 4-nitrophenol to 4-aminophenol in an aqueous phase has been reported,<sup>22</sup> and this represents the best scientific evidence that Au nanoparticles inside plant biomass can act as catalysts for industrial or environmental reactions. In the current study, nanoparticles in plant ash were large, but discrete, and thus had an identifiable surface area on which reactions may occur. However, the importance of the size of a nanoparticle is a recurring theme with respect to catalytic activity.<sup>23</sup>

Previous attempts at Massey University to use Au-rich biomass prepared through ashing or enzymatic degradation of cell tissues have failed to show activity toward the oxidation of carbon monoxide, and this lack of activity has been attributed to a low Au concentration (for enzymatically degraded tissues)<sup>24</sup> or size (ashed preparation). But the range of reactions investigated in these studies has been limited. Catalytic activity for the reduction of 4-nitrophenol occurred at a dry weight concentration as low as 80 and 100 mg kg<sup>-1</sup> for the shoots and roots of *Sesbania drummondii*, respectively.<sup>22</sup> In



**Figure 4.** XANES spectra of samples analyzed by X-ray absorption spectroscopy from 11.85 to 12.05 keV to determine the speciation of Au inside the plant biomass. (A) Gold-containing plant samples (dry weight biomass). (B) Gold model compounds.

**Table 4.** LC-XANES Fittings for Au in *Brassica juncea* Shoot Biomass Harvested from Au-only, Ag/Au, Cu/Au, and Cu/Ag/Au Soil Showing Speciation of Au within Each Sample

soil treatment	% Au(0)	% KAuCl <sub>4</sub>	% AuOH	% AuS
gold	88.5	0.0	14.1	0.0
silver/gold	45.0	0.0	55.0	0.0
copper/gold	50.8	0.0	49.1	0.09
copper/silver/gold	51.3	0.0	49.7	0.0

the current work, a DW Au concentration of between 1600 and 3600 mg kg<sup>-1</sup> was recorded for the shoots of *Brassica juncea* (range dependent on the combination of Au/Cu/Ag in the plant). The ash weight Au concentration will have been up to 20 times higher (3.2–7.2%) in a carbon matrix. Hydroponic studies have shown Au concentrations of a similar order of magnitude, and these concentrations are all of the same order of magnitude as reference Au catalysts for industrial and

environmental chemistry (1 % wt/wt).<sup>25</sup> Therefore, concentration should not be a factor restricting catalytic activity.

On the basis of our findings, we propose that soil-grown biogenic nanoparticles should be further investigated as industrial and environmental catalysts or reactive surfaces in both gaseous and aqueous mediums. There may be good application for biogenic structures in aqueous reactions. For example, Au nanoparticles (less than 100 nm diameter) have been shown to remove elemental mercury from wastewater.<sup>26</sup> The nanoparticle size range and alloy chemistry of plant-synthesized structures may impart certain interesting and useful properties on these metallic moieties that have application to specific reactions, although the support matrix of the nanoparticles must be better quantified. In the current work, there is evidence that the addition of Cu and/or Ag to the growth medium may inhibit the accretion of larger particles with time, potentially restricting the formed nanostructures to less than a maximum size range. In the presence of other catalytically important metals, plants may synthesize a range of

interesting and potentially useful structures. Co-metallic influence on the synthesis of in vivo gold nanoparticles may overcome size and chemistry limitations on the potential usefulness of biogenic gold nanoparticles. In 2012, a research consortium of York University, University of British Columbia, and Yale University received funding from the G8 Research Councils Initiative on Multilateral Research Funding: Material Efficiency to investigate the catalytic activity of palladium (Pd) in plants to industrially important reactions ([www.phytocat.org](http://www.phytocat.org)). Biomass for this research could be produced according to the methodology we have described, where Pd is added to mine waste in place of, or in addition to, Cu, Ag, and Au.

The synthesis of a Au/Ag nanoparticle alloy would be difficult using a hydroponic or agar medium, as the soluble Au salt commonly used ( $\text{KAuCl}_4$ ) would cause the precipitation of soluble Ag. Therefore, for nanoalloy investigations, use of a soil media is appropriate, where the solubility of separately added or native Au and Ag can be induced using an appropriate chemical lixiviant (induced phytoextraction). Mass balance calculations from the current work suggest that the amendment of a soil or ore with soluble Au, Cu, and Ag salts will lead to retention of these metals on the mineral matrix after an appropriate incubation period (through adsorption to mineral structures). This could be a valid technique to generate media that would allow for the laboratory production of nanoalloys. If the biogenic synthesis of nanoparticles was proven to be a viable technique to produce nanoparticles/nanoalloys for specific industrial uses, then plants could be grown on high-grade Au ore. Ores or concentrates with a Au concentration in the order of  $100 \text{ mg kg}^{-1}$  can be readily found at many mining operations, and Au concentrations in excess of  $5000 \text{ mg kg}^{-1}$  have been reported for bonanza-style mineral deposits.<sup>27</sup> These ores and concentrates will also have a high concentration of Cu and Ag. Such an operation would not represent large-scale phytomining but rather a niche application of the technology, where any environmental risk of using cyanide to induce metal uptake could be managed through the use of a liner in an enclosed area or by growing plants in a designed growth facility. Any inherent toxicity of cyanide to the plants is unimportant as the plants are harvested once signs of toxic metal shock are visible.

The findings of this work are also applicable to commercial and large-scale phytomining operations that are becoming increasingly attractive (economically) at current world gold prices (in excess of US\$1500/ounce). Such operations seek to exploit the residual precious metal concentration in mine waste and would not necessarily yield metal concentrations in plants that might be suitable for catalysis. The current research shows that Cu and Ag will be made soluble by cyanide and accumulated by plants in the process of making Au soluble. The concentration of each of these metals accumulated by plants will subsequently limit the concentration of Au accumulated by the plant. This should be considered in locations where Au-rich soil has a high concentration of Ag and Cu, as is the case, for example, in the Au mining areas of Mexico, a location where phytomining is being actively developed.<sup>9</sup> An understanding of the form of nanoparticles and nanoalloys inside plant biomass and ash is important in the processing of biomass to recover the valuable product. A nanoalloy of Au and Ag represents a chemically refined product. Therefore, the use of a processing system that involves the chemical oxidation of all metals in the biomass, with the selective extraction and subsequent reduction of Au and Ag,

may be unnecessarily complicated. Instead, physical methods could be developed that separate the high-density valuable metals or alloys with secondary refinement of a concentrate.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Detailed methodology for the analysis of Au, Cu, and Ag in plants (FAAS and XAS), metal concentration in the non-cyanide-treated biomass, detailed mass balance of Cu and Ag in the experimental system, EDS scans for metallic particles in the SEM photomicrographs, and XANES spectra for gold-containing dry plant material. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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