Reducing Strength Prevailing at Root Surface of Plants Promotes Reduction of Ag\(^+\) and Generation of Ag\(^0\)/Ag\(_2\)O Nanoparticles Exogenously in Aqueous Phase

Peddisetty Pardha-Saradhi\(^1\)*, Gupta Yamal\(^{1,2}\), Tanuj Peddisetty\(^3\), Peddisetty Sharmila\(^4\), Shilpi Nagar\(^1\), Jyoti Singh\(^5\), Rajamani Nagarajan\(^5\), Kottapalli S. Rao\(^2\)

1 Department of Environmental Studies, University of Delhi, Delhi, India, 2 Department of Botany, University of Delhi, Delhi, India, 3 Department of Petroleum Engineering & Earth Sciences, University of Petroleum and Energy Studies, Dehradun, Uttarakhand, India, 4 Department of Chemistry, Indian Institute of Technology Delhi, Hauz Khas, New Delhi, India, 5 Department of Chemistry, University of Delhi, Delhi, India

Abstract

Potential of root system of plants from wide range of families to effectively reduce membrane impermeable ferricyanide to ferrocyanide and blue coloured 2,6-dichlorophenol indophenol (DCPIP) to colourless DCPIPH\(_2\) both under non-sterile and sterile conditions, revealed prevalence of immense reducing strength at root surface. As generation of silver nanoparticles (NPs) from Ag\(^+\) involves reduction, present investigations were carried to evaluate if reducing strength prevailing at surface of root system can be exploited for reduction of Ag\(^+\) and exogenous generation of silver-NPs. Root system of intact plants of 16 species from 11 diverse families of angiosperms turned clear colorless AgNO\(_3\) solutions, turbid brown. Absorption spectra of these turbid brown solutions showed silver-NPs specific surface plasmon resonance peak. Transmission electron microscope coupled with energy dispersive X-ray confirmed the presence of distinct NPs in the range of 5–50 nm containing Ag. Selected area electron diffraction and powder X-ray diffraction patterns of the silver NPs showed Bragg reflections, characteristic of crystalline face-centered cubic structure of Ag\(^0\) and cubic structure of Ag\(_2\)O. Root system of intact plants raised under sterile conditions also generated Ag\(^0\)/Ag\(_2\)O-NPs under strict sterile conditions in a manner similar to that recorded under non-sterile conditions. This revealed the inbuilt potential of root system to generate Ag\(^0\)/Ag\(_2\)O-NPs independent of any microorganism. Roots of intact plants reduced triphenyltetrazolium to triphenylformazon and impermeable ferricyanide to ferrocyanide, suggesting involvement of plasma membrane bound dehydrogenases in reduction of Ag\(^+\) and formation of Ag\(^0\)/Ag\(_2\)O-NPs. Root enzyme extract reduced triphenyltetrazolium to triphenylformazon and Ag\(^+\) to Ag\(^0\) in presence of NADH, clearly establishing potential of dehydrogenases to reduce Ag\(^+\) to Ag\(^0\), which generate Ag\(^0\)/Ag\(_2\)O-NPs. Findings presented in this manuscript put forth a novel, simple, economically viable and green protocol for synthesis of silver-NPs under ambient conditions in aqueous phase, using root system of intact plants.

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* Email: ppsaradhi@gmail.com

Introduction

Nanotechnology has witnessed spectacular advancement in fabrication and utilization of nanomaterials. Owing to small size and large surface to volume ratio, nanoparticles possess unique physicochemical and biological properties which differ entirely from the bulk material. Amongst different metal nanoparticles, the synthesis and application of silver NPs received wide attention, as silver nanoparticles find application in almost every sphere of life. Due to immense antimicrobial properties, silver nanomaterials have wide application in medicine especially artificial teeth, bone coating, medical catheters, wound dressings besides surgical instruments. Silver nanoparticles are also extensively used in daily commodities such as cosmetics (viz. lotions, creams etc.), toothpastes, detergents, soaps, surface cleaners, room sprays, shoe insoles and textiles. Utility of silver nanoparticles in home appliances (such as washing machines, air and water filters), automotive upholstery, paints and food storage containers is also known [1–4]. Silver NPs find usefulness in sensing applications such as biolabeling, optical imaging of cancer and detection of DNA sequences [5,6]. Electronic application of silver nanoparticles includes its usage for the preparation of optical devices, inks for circuit boards, high density recording devices, battery-based intercalation materials [6–9]. Large surface area of silver NPs provides high surface energy for catalysis [10]. Silver NPs of different sizes and shapes are routinely synthesized by various chemical and physical methods [11–13]. Various physical, chemical and physico-chemical approaches such as ion sputtering, laser ablation, inert gas condensation, mechanical milling, thermal or laser irradiation, chemical reduction, sol-gel technique, photochemical reduction and electrochemical techniques have so far been employed to generate metal NPs [12–14]. The Lee-Meisel method using sodium citrate along with heating for 1 h for generation of silver nanoparticles continues to be the standard by which other
generate silver nanoparticles in size range of 10–50 and 10–
20–600 nm [15,16]. Recently, we demonstrated potential of
synthetic methods are compared. Although, the method is simple,
ial and safety measures [32]; (ii) it is difficult to precisely identify the biomolecule(s) amongst the
cocktail of molecules in extracts of various biological materials/
plant biologists clearly established that root
it wise to exploit root system of intact plants for generating silver
in search for an alternate and ideal green method, we felt
bacteria, actinomycetes and fungi) [11,20–24]; (ii) cell organelles
(iii) extract from various components (viz. leaves, seeds, flowers, fruit or fruit peel, latex, tuber, bark etc.) of
numerous plant species [17,24–28] and (iii) live plants (which
restricted to cause least damage to the plants as well as to the
root system at the time of collection.

Raising plants under sterile conditions

Seeds of *Brassica juncea* cv. Varuna, *Vigna mungo* cv. PS-1, and *Lycopersicon esculentum* cv. Pusa Ruby were purchased from Seed Sales Counter, Indian Agricultural Research Institute (IARI), New Delhi, and that of *Triticum aestivum* cv. H1-1544 and *Cicer arietinum* cv. PG-114, were gifted by Dr. A.N. Mishra (Head, IARI Regional Station, Indore, India) and Dr. Jiendi Kumari (Principal Scientist, Pulse Division, IARI, New Delhi, India), respectively. Seeds were treated with 0.5% cetrimide for 5 min, washed with distilled water; surface sterilized with 0.1% (w/v) mercuric chloride for 3 min and washed thoroughly with sterile distilled water. Capped glass bottles of (2.5×5") dimensions containing glass beads (~75 g) with 25 ml of mineral growth

Materials and Methods

Plant material

Sixteen plant species from 11 distinct families of diverse
taxonomic groups of Angiosperms were collected from wild and
maintained garden beds of North/Main campus of University of Delhi, details of which are given in Table 1. In all the cases utmost care was taken to cause least damage to the plants as well as to the root system at the time of collection.

| Botanical Name          | Common Name          | Family          | Size of NPs (nm) |
|-------------------------|----------------------|-----------------|------------------|
| *Amaranthus gracilis*   | Slender Amaranth     | Amaranthaceae   | 10–30            |
| *Cannabis sativa*       | Hemp                 | Cannabinaceae   | 20–50            |
| *Catharanthus roseus*   | Madagascar Periwinkle| Apocynaceae     | 10–30            |
| *Cynodon dactylon*      | Couch Grass          | Poaceae         | 20–50            |
| *Euphorbia hirta*       | Asthma Plant         | Euphorbiaceae   | 20–40            |
| *Medicago sativa*       | Alfalfa              | Fabaceae        | 10–40            |
| *Ocimum sanctum*        | Holy Basil           | Lamiaceae       | 20–50            |
| *Phyllanthus fraternus* | Gulf Leaf Flower     | Euphorbiaceae   | 10–50            |
| *Portulaca grandiflora* | Moss Rose            | Portulacaceae   | 20–50            |
| *Tagetes erecta*        | Marigold             | Asteraceae      | 5–30             |
| *Vernonia cinerea*      | Little Ironweed      | Asteraceae      | 20–50            |
| *Brassica juncea*       | Indian Mustard       | Brassicaceae    | 5–40             |
| *Cicer arietinum*       | Chickpea             | Fabaceae        | 5–30             |
| *Lycopersicon esculentum* | Tomato              | Solanaceae      | 10–40            |
| *Triticum aestivum*     | Wheat                | Poaceae         | 10–30            |
| *Vigna mungo*           | Black Gram           | Fabaceae        | 10–30            |

Table 1. Details of plant species used for exogenous generation of silver NPs by root system of intact plants.
medium consisting of 2500 mg L\(^{-1}\) KNO\(_3\), 250 mg L\(^{-1}\) MgSO\(_4\), 150 mg L\(^{-1}\) CaCl\(_2\), 150 mg L\(^{-1}\) Na\(_2\)PO\(_4\), 134 mg L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 10 mg L\(^{-1}\) MnSO\(_4\), 3 mg L\(^{-1}\) H\(_3\)BO\(_3\), 2 mg L\(^{-1}\) ZnSO\(_4\), 0.75 mg L\(^{-1}\) KI, 0.25 mg L\(^{-1}\) Na\(_2\)MoO\(_4\) and iron source (27.8 mg L\(^{-1}\) FeSO\(_4\) and 37.3 mg L\(^{-1}\) EDTA) \([37]\) were sterilized by autoclaving at a pressure of 1.06 Kg cm\(^{-2}\) and at temperature of 121°C for 30 min. Surface sterilized seeds were sown in these bottles under sterile conditions, in laminar air flow. For present investigations 20 day old plants of \textit{Lycopersicon esculentum} (Le), \textit{Brassica juncea} (Bj), \textit{Vigna mungo} (Vm), \textit{Cicer arietinum} (Ca), \textit{Ocimum sanctum} (Os), \textit{Catharanthus roseus} (Cr), \textit{Cynodon dactylon} (Cd), \textit{Cannabis sativa} (Cs), \textit{Phyllanthus fraternus} (Pf), \textit{Portulaca grandiflora} (Pg), \textit{Triticum aestivum} (Ta) and \textit{Vernonia cinerea} (Vc) under non-sterile (a–c) and sterile (d) conditions. Ctrl represents 0.1 mM DCPIP. Vertical lines on bars represent mean ± standard error, n = 5. Values designated by different letters above bars are significantly different between plant species at p ≤ 0.05 level (Duncan’s multiple range test).

**Determining root surface redox activity**

In order to determine reduction potential associated with root surface, roots of intact plants were submerged in 0.1 mM DCPIP solutions. Reduction potential of roots of intact plants was determined by monitoring decrease in the optical density at 600 nm due to reduction of DCPIP to DCPIPH\(_2\). Capacity of roots of intact plants to reduce DCPIP was expressed in terms of nano moles of DCPIP reduced h\(^{-1}\)g\(^{-1}\) root fresh weight. Reduction potential associated with roots of intact plants of different plant species was also determined using ferricyanide which is a membrane impermeable electron acceptor \([32–35]\). Potassium ferricyanide \(\text{K}_3\text{[Fe(CN)₆]}\) was used at 1 mM concentration for evaluating redox activity of roots. The reduction
Incubation of plants under strict sterile conditions with salt solutions

For evaluating potential of plants to generate Ag-NPs they were exposed to different concentrations (viz. 0.01, 0.05, 0.1, 0.25, 0.5, and 1 mM) of AgNO₃ prepared in sterile double distilled water. Plants were incubated at room temperature (25±2°C) with a 16/8 h light/dark cycle with a light intensity of 120 μmol m⁻² s⁻¹ photon flux density. For experiments carried under strict sterile conditions the stock solutions of filter sterilized Ag was used. Different concentrations were prepared from this stock using sterile double distilled water in laminar air flow. The glassware(s) used in these experiments were sterilized by autoclaving at a pressure of 1.06 Kg cm⁻² and at temperature of 121°C for 30 min. After washing roots 2–3 times with sterile double distilled water, the plants raised under sterile conditions were transferred to 25×200 mm sterile Borosil tubes with their roots immersed in sterile salt solutions, in laminar air flow. The exogenously generated silver nanoparticles were characterized through UV-Vis spectral studies, TEM investigations coupled with EDX and PXRD studies.

UV-Vis spectral studies

UV-Vis absorption spectra of various test solutions in which roots of intact plants were submerged for different time intervals were recorded between 190–1100 nm using Specord 200 AnalytikJena UV-Vis spectrophotometer. The formation of Ag nanoparticles was indicated by the appearance of characteristic absorption peak generally at 410–480 nm.

Transmission electron microscopic (TEM) investigations

AgNO₃ solutions incubated with roots of intact plants for duration of ~24 h were collected and subjected to sonication for 30 min to minimize primary particle agglomeration. 10 μl of the resulting colloidal solution was drop coated onto a 200-mesh copper grid covered with an ultrathin continuous C film in a desiccator. The grids were viewed in transmission electron microscope of Technai G² T30 U-TWIN at a voltage of 300 kV. Hardware associated with the machine also allowed (i) energy dispersive X-ray (EDX spectra), and (ii) Selected area electron diffraction (SAED) pattern measurements. EDX spectra indicated the composition of isolated nanoparticles and SAED patterns indicated the crystalline/amorphous nature of the nanoparticles that were fabricated and released by the plants into the solutions.

Powder X-ray diffraction (PXRD) Studies

AgNO₃ solutions incubated with roots of intact plants was drop coated on Silica surface and dried. The PXRD pattern was collected using Rigaku Rotaflex RAD-B or Bruker or Pan Analytika instruments using Cu target CuKα at the rate of 0.020 step in 1.2 s in the 2 theta range 30–80°.

Detection of proteins, amino acids or phenolics in incubation medium

Roots of intact plants were incubated in sterile double distilled water for a duration of 24 h. This incubation medium was then tested for the presence of proteins, phenolics and amino acids using standard protocols [38–40].

Comparative study of roots of plants with sodium citrate

The efficiency of roots of plants was compared to one of the most widely used reductant viz. sodium citrate. The stock solution of AgNO₃ was filter sterilized and various concentrations viz. 0.01,
0.05, 0.1, 0.25, 0.5 and 1 mM were prepared using sterile double distilled water.

**Determination of dehydrogenase activity**

**In vitro.** Roots of (i) *Triticum aestivum* seedlings (4–5 d old) raised under sterile conditions; and (ii) *Portulaca grandiflora* plants raised in earthen pots were homogenized independently in 50 mM phosphate buffer (pH-7.2) containing 5 mM EDTA and 5% of polyvinylpyrrolidone and centrifuged at 16000 xg at 4°C. Supernatant was used as the crude enzyme extract for determining dehydrogenase activity following modified protocol of Yamauchi and co-workers [41]. The reaction mixture for determining the dehydrogenase activity consisting of 500 μl crude enzyme extract, 10 μmoles of 2,3,5-triphenyltetrazolium chloride and 2 μmoles of NADH in a final volume of 4 ml of 100 mM phosphate buffer (pH 7.6), was incubated at 37°C for 6 h. Subsequently, the reaction mixtures were centrifuged at 10000 xg for 20 min and the pellet was dissolved in 4 ml ethanol. The quantity of 1,3,5-triphenylformazan formed due to dehydrogenase activity was determined by taking absorbance at 484 nm. The dehydrogenase activity was finally expressed as nmoles of formazan formed g⁻¹ fresh weight of root h⁻¹.

Simultaneously, the potential of dehydrogenases present in crude enzyme extract to reduce Ag⁺ and form silver-NPs determined by incubating 500 μl of crude enzyme extract with 100 mM phosphate buffer (pH-7.6) containing 1 μmole AgNO₃ and 2 μmoles NADH in a final volume of 4 ml at 37°C for 6 h. The presence of silver-NPs in resultant reaction mixture was confirmed by (i) absorption spectra which showed Ag-NPs specific peak; (ii) transmission electron microscope.

**In vivo.** Roots of intact plants, of (i) *Triticum aestivum* (4–5 d old) raised under sterile conditions; and (ii) *Portulaca grandiflora* raised in earthen pots, were incubated in phosphate buffer (pH-7.6) containing either 2,3,5-triphenyltetrazolium chloride or 0.25 mM AgNO₃. After incubating for different time intervals (6 to 24 h), (i) resultant incubation medium was centrifuged at 10000 xg for 20 min and the pellet consisting of 1,3,5-triphenylformazan was dissolved in 95% ethanol; and (ii) root system of plants were excised, cut in to segments and dipped in 95% ethanol for 12 h to extract out 1,3,5-triphenylformazan. The formazan formed due to
dehydrogenase activity was determined by measuring absorbance at 484 nm. The dehydrogenase activity was finally expressed as nmoles of formazan formed g⁻¹ fresh weight of root h⁻¹.

Results and Discussion

It is well established that generation of silver nanoparticles similar to other metal nanoparticles primarily involves reduction of their ions [10,11]. Earlier, it had been unequivocally proven that root system of plant species possess strong reducing capacity [32–36].

During present investigations root system of intact plants of all 16 plant species, from 11 diverse families of angiosperms effectively reduced artificial electron acceptors (DCPIP) and ferricyanide within duration of 6-12 h. As is evident from Fig. 1 root system of plants reduced (i) blue colored DCPIP to colorless DCPIPH₂ and (ii) ferricyanide to ferrocyanide. Earlier, Rubinstein and co-workers recorded reduction of membrane impermeable ferricyanide to ferrocyanide in the solutions in which roots of intact plants were incubated [34–35]. Our findings similar to the findings of Rubinstein et al. [34] clearly established that root surface could exogenously reduce ferricyanide to ferrocyanide. This prompted us to test if root system of intact plants can exogenously reduce Ag⁺ to Ag₀ at the root surface and generate silver nanoparticles in aqueous phase under ambient conditions.

As anticipated, root system of intact plants of all plant species tested, altered color of clear AgNO₃ solution, turbid brown, within a duration of 6–12 h (Fig. 2a–c), probably due to reduction of Ag⁺ and formation of silver nanoparticles. It is known that clear AgNO₃ solutions turn brown upon generation of silver nanoparticles [11,42]. In general, the UV-Vis absorption spectra of these brown colored colloidal solutions showed absorption maxima between 380 to 450 nm (Fig. 3). Absorption peak in this range has been reported to arise due to surface plasmon resonance in silver NPs [11]. The intensity and position of absorption peak varied depending on the concentration of Ag⁺ and plant species used (Fig. 3).

Transmission electron microscopic (TEM) investigations confirmed the presence of distinct NPs in brown colloidal solutions that resulted due to incubation of root system of intact plants in AgNO₃ solutions (Figs. 4–6, Table 1). In general, the NPs were in the range of 5–50 nm and spherical (Figs. 4–6). These results are in accordance with the UV-Vis absorption spectra of the colored colloidal solutions that showed surface plasmon resonance (SPR) peak centered around 430 nm, implying the presence of spherical NPs [43]. Energy dispersive X-ray (EDX) studies confirmed the presence of Ag in these NPs (Figs. 4–6). The EDX pattern collected from these nanoparticles showed distinct peaks at 3.40 keV and 22 keV corresponding to Ag, while the peaks situated at binding energies of 8.06 and 1 keV correspond to Cu and C, respectively. The peaks of C and Cu arose due to their presence as an integral component of carbon coated copper grids.

Powder X-ray diffraction (PXRD) patterns of silver nanoparticles generated exogenously by root surface of intact plants showed presence of distinct peaks, corroborating their crystalline nature. Irrespective of the plant species used, the PXRD patterns show Bragg reflections characteristic of crystalline Ag₀ (Table 1, Figs. 4–6). The presence of distinct rings corresponding to Bragg reflections in selected area electron diffraction (SAED) pattern established that these silver NPs were crystalline (Figs. 4–6).

Figure 4. TEM images (a–c), SAED (d–f) and EDX (g–i) of silver NPs synthesized exogenously by root system of intact plants of Tagetes erecta (a, d, g), Catharanthus roseus (b, e, h), Euphorbia hirta (c, f, i). SAED show Bragg reflections characteristic of crystalline Ag₀ (*) and Ag₂O (†). doi:10.1371/journal.pone.0106715.g004

Powder X-ray diffraction (PXRD) patterns of silver nanoparticles generated exogenously by root surface of intact plants showed presence of distinct peaks, corroborating their crystalline nature. Irrespective of the plant species used, the PXRD patterns...
Figure 5. TEM (a–c), SAED (d–f) and EDX (g–i) of silver NPs synthesized exogenously by roots system of intact plants of *Brassica juncea* (a, d, g), *Cicer arietinum* (b, e, h), and *Phyllanthus fraternus* (c, f, i). SAED show Bragg reflections characteristic of crystalline Ag⁰ () and Ag₂O (*).

Figure 6. TEM (a–d), SAED (e–h) and EDX (i–l) of silver NPs synthesized exogenously by root system of intact plants of *Cynodon dactylon* (a, e, i), *Portulaca grandiflora* (b, f, j), *Lycopersicon esculentum* (c, g, k) and *Vernonia cinerea* (d, h, l). SAED show Bragg reflections characteristic of crystalline Ag⁰ () and Ag₂O (*).

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of silver nanoparticles formed exogenously by root of intact plants matched with the standard diffraction pattern of Joint Committee on Powder Diffraction Standards (JCPDS) No. 89-3722, confirming the face-centered cubic (fcc) structure of Ag\(_0\), and 76–1393 characteristic of Ag\(_2\)O with cubic geometry (Fig. 7). These PXRD results revealed that the silver nanoparticles formed by the root surface of plant species are constituted of Ag\(_0\) and Ag\(_2\)O. During earlier investigations also we recorded susceptibility of Ag\(_0\) NPs to oxidation and accordingly, recorded formation of a mixture of Ag\(_0\)/Ag\(_2\)O NPs by yeast extract, mannitol as well as yeast extract-mannitol medium [11]. Levard and co-workers [44] also reported the susceptibility of Ag\(_0\) NPs to oxidation under ambient conditions [44]. Therefore, we believe that some of the Ag\(_0\) NPs formed by reduction of Ag\(^+\) at the root surface were oxidized to Ag\(_2\)O NPs in aqueous phase under ambient conditions. Recently, we also reported that the root surface of plants possesses potential to form iron oxy-hydroxide NPs [36].

Possible mechanism of generation of silver nanoparticles at the root surface

Earlier, two plant species namely, Medicago sativa (alfalfa) and Brassica juncea (mustard) which are considered to be metallo-phytes have been reported to synthesize silver nanoparticles within their cells [29–31]. Gardea-Torresdey et al. [29] reported that alfalfa roots absorb Ag\(_0\) from agar medium and transfer it to shoot in the same oxidation state, where these silver atoms (Ag\(_0\)) arrange themselves to form nanoparticles. However, these researchers neither addressed the mode of reduction of Ag\(^+\) to Ag\(_0\) in agar medium nor discussed about any factor (if any) responsible for arrangement of Ag\(_0\) to form nanoparticles in the shoots. Harris and Bali [30] noted formation of silver nanoparticles within the cells of plants of M. sativa and B. juncea exposed to AgNO\(_3\) solution, but these researchers also remained silent on mode of reduction of Ag\(^+\) and generation of Ag nanoparticles. Subsequently, Beattie & Haverkamp [31] reported synthesis of silver nanoparticles in cells of B. juncea, predominantly in chloroplast. The later researchers believed that the reducing sugars could play a role in reduction of Ag\(^+\) and generation of silver NPs. However, plant cells besides having reducing sugars also possess number of other biomolecules such as phenolics, organic acids, amino acids etc. which have been shown to be potent reductants for reduction of Ag\(^+\) to generate silver nanoparticles [22,27,45].

During present investigations, root system of all plant species tested possessed potential to exogenously reduce Ag\(^+\) and form

Figure 7. PXRD pattern of silver NPs. The PXRD pattern of silver NPs synthesized exogenously by roots system of intact plants of various plant species showing Bragg reflections characteristic of crystalline face-centred cubic structure of Ag\(_0\) () and cubic structure of Ag\(_2\)O (*). doi:10.1371/journal.pone.0106715.g007
silver nanoparticles when incubated with AgNO₃ solution. Roots of plants are known to exude a variety of biomolecules viz. organic acids, amino acids, phenolics etc. [46] which have been shown to possess capacity to reduce Ag⁺ and generate silver nanoparticles [11,47,48]. To evaluate if capacity of root system of plants to reduce Ag⁺ and generate Ag nanoparticles exogenously, recorded during present investigations is due to biomolecules that are exuded by the roots, 2 sets of investigations were carried. In the first set of investigations, we tested for presence of phenolics, amino acids and proteins in the medium in which roots of intact plants were incubated for 24 h, but, did not find any detectable levels of these biomolecules. Subsequently, the incubation medium in which roots of intact plants were incubated for 24 h, was tested directly for its potential to generate silver nanoparticles. Neither color change nor presence of any silver nanoparticles was recorded. However, as stated above roots of intact plants incubated in AgNO₃ solution could reduce Ag⁺ and generate silver nanoparticles within a duration of 6–12 h. Infact, earlier researchers also categorically demonstrated that the reduction of membrane impermeable ferricyanide to ferrocyanide is not due to any of the lechates from the roots [34,35]. Therefore, our findings unequivocally demonstrated that dehydrogenases present in the enzyme extract possess potential to reduce Ag⁺ to Ag⁰ and promote formation of silver-NPs.

Establishing potential of dehydrogenases to generate silver-NPs

Roots of intact plants of intact plants of (i) Triticum aestivum (raised under sterile conditions); and (ii) Portulaca grandiflora reduced clear 2,3,5-triphenyltetrazolium chloride to colloidal pink-purple 1,3,5-triphenylformazan at their surface (Fig. 11) confirming the presence of dehydrogenases in association with root surface cells. Reduction of clear 2,3,5-triphenyltetrazolium chloride to colloidal pink-purple 1,3,5-triphenylformazan is comparable to the conversion of clear AgNO₃ solution colloidal brown by the root system of intact plants (Fig. 11).

Root enzyme extracts of both Triticum aestivum and Portulaca grandiflora reduced triphenyltetrazolium to triphenylformazan in presence of NADH, confirming the presence of dehydrogenase activity (Table 2; Fig. 11). Root enzyme extracts of these plant species turned reaction mixture (phosphate buffer, pH 7.6) containing AgNO₃, colloidal brown within a duration of 3 to 6 h at 37°C, in presence of NADH (Fig. 11). Absorption spectra of these colloidal brown solutions showed presence of silver-NPs specific plasmon resonance peak. Transmission electron microscopic investigations revealed presence of silver-NPs in these solutions. These findings unequivocally demonstrated that dehydrogenases present in the enzyme extract possess potential to reduce Ag⁺ to Ag⁰ and promote formation of silver-NPs.
Figure 9. TEM images (a–b), SAED (c–d), EDX (e–f) and PXRD (g–h) of silver NPs synthesized exogenously by root system of intact plants of *Vigna mungo* (a, c, e, g) and *Triticum aestivum* (b, d, f, h). Bragg reflections characteristic of crystalline face-centred. doi:10.1371/journal.pone.0106715.g009

Figure 10. Schematic representation of the mechanism involved in the reduction of Ag\(^{+}\) and formation of silver NPs at the root surface of live plants. doi:10.1371/journal.pone.0106715.g010
Figure 11. Evidences demonstrating - the presence of dehydrogenases in association with root surface cells (a, b); and the potential of dehydrogenases to reduce triphenyltetrazolium to triphenylformazan and Ag⁺ to Ag⁰ (which in turn generate Ag⁰/Ag₂O-nanoparticles) (c). (a) and (b) displays root system of intact plants of *Portulaca grandiflora* incubated in phosphate buffer (pH 7.6) in absence (A) and presence of triphenyltetrazolium chloride (B) or AgNO₃ (C). Note colour change due to formation of triphenylformazan (B) and silver-nanoparticles (C). (c) depicts tubes with reaction mixture (100 mM phosphate buffer pH 7.6) containing - root enzyme extract with NADH (D); root enzyme extract with triphenyltetrazolium chloride (E); root enzyme extract with triphenyltetrazolium chloride and NADH (F); AgNO₃ with NADH (G); AgNO₃ with root enzyme extract (H); and AgNO₃ with root enzyme extract and NADH (I), 6 h after incubation at 37°C. Please note colour change due to formation of triphenylformazan (F) and silver nanoparticles (I). (d) shows silver nanoparticles from tube (I) formed due to dehydrogenase activity in presence of NADH. (e) depicts absorption spectra of the reaction mixtures from tubes (G), (H) and (I). Note silver nanoparticle specific absorption peak that intensified due to dehydrogenase activity in presence of NADH.

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Table 2. Dehydrogenase activities recorded at the surface of roots of intact plants (in vivo) and in root enzyme extracts (in vitro).

| S. No. | Plant Species       | Dehydrogenase Activity (nmoles g fresh weight⁻¹ h⁻¹) |
|--------|---------------------|-----------------------------------------------------|
|        |                     | **In vivo**                                          |
| 1.     | *Portulaca grandiflora* | 108.37±11.22                                         |
| 2.     | *Triticum aestivum*  | 898.86±7.67                                          |
|        |                     | **In vitro**                                         |
| 3.     | *Portulaca grandiflora* | 35.81±2.91                                           |
| 4.     | *Triticum aestivum*  | 572.4±6.18                                           |

Root systems of intact plants were incubated in 100 mM phosphate buffer (pH 7.6) containing triphenyltetrazolium chloride at 37°C. Root enzyme extracts were incubated with triphenyltetrazolium chloride and NADH at 37°C. Dehydrogenase activity was determined in terms of nmoles of triphenylformazan formed. Values represent mean ± standard error (n = 5).

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Advantages of employing root system of intact plants for generation of silver NPs

Biological methods for synthesis of nanoparticles are considered to be superior over various physical and chemical methods [18]. Successful generation of silver NPs using variety of microorganisms and plant extracts has been most widely reported. However, due to limitations none of these methods could be used for commercial production of silver NPs. Use of microorganism requires special facilities for their maintenance and safety measures [26]. Amongst microorganisms, even pathogenic microorganisms such as Klebsiella pneumoniae, Bacillus subtilis, Micrococcus luteus, Serratia marcescens, Aspergillus etc. have been employed for generation of silver NPs [21]. However, the serious concern for the use of such pathogenic microorganisms would be likely emergence of new pathogenic strains resistant to silver NPs that are otherwise widely used as antimicrobial agents. Although, synthesis of silver NPs using extracts of various plant materials was reported to be superior, it is difficult to precisely identify the biomolecule(s) amongst the cocktail of molecules in these extracts that are responsible for generation of silver NPs.

Similarly, the potential of live plants to synthesize silver NPs intracellularly cannot be exploited for commercial production due to practical difficulties in their extraction. Moreover, due to the presence of wide variety of biomolecules (having reducing strength) in the cells, intracellularly formed NPs would be of broad size range. Accordingly, Beattie and Haverkamp [31] observed nanoparticles in the range of 2–100 nm in cells of B. juncea plants exposed to silver salt solutions. In contrast, during present investigations potential of root system of intact plants to exogenously generate silver NPs was realized. Moreover, the silver NPs formed exogenously by root system were in narrow size range. Most importantly, these exogenously generated silver NPs can be harvested with ease.

Amongst chemical methods Lee Miesel protocol for synthesis of silver nanoparticles using sodium citrate continues to remain a standard method, with which newly invented methods are compared [16]. In this popular Lee Miesel method 10 ml of 1% sodium citrate is added to 500 ml of boiling AgNO3 solution, and the resultant mixture is boiled for 1 h for synthesis of silver NPs. The major limitation of this method is the production of silver NPs in broad size range i.e. 20–600 nm [15,16]. In contrast, during present investigations, roots of intact plants of all 16 plant species tested synthesized silver NPs at room temperature within the size range of 5–50 nm.

The potential of root system of intact plants of V. mungo and T. aestivum to generate silver NPs was compared with that of sodium citrate at room temperature and under strict sterile conditions. 0.02% sodium citrate (i.e. used for generation of silver NPs in the Lee Miesel method) failed to show any alteration in color of the AgNO3 solution or silver NPs specific SPR peak in the absorption spectra even after incubating for duration of 12 h at room temperature. As is evident from Fig. 8 root system of intact plants of V. mungo and T. aestivum incubated under ambient and sterile conditions altered the color of clear AgNO3 solution colloidal brown within 6 h and the absorption spectra of these colloidal solutions showed silver NPs specific SPR peak. Although, sodium citrate when used at a concentration 50 times higher than that used in Lee Miesel method, turned clear colorless AgNO3 solutions greyish colloidal, the intensity of color and silver NPs specific SPR peak in the absorption spectra of these colloidal solutions was significantly less than that recorded when root system of intact plants were used, under ambient conditions (Fig. 8). The dynamic light scattering (DLS) studies showed that the mean particle size of silver NPs synthesized using 1 mM AgNO3, by the root system of intact plants of V. mungo and T. aestivum was ~20 nm, while those synthesized in presence of 1% sodium citrate was ~35 nm under ambient conditions. These findings, clearly demonstrate that root system of intact plants can be employed for rapid synthesis of silver NPs under ambient conditions. To the best of our knowledge, the novel method of using root system of intact plants for bulk synthesis of silver NPs under ambient conditions would be most green, simple and economically viable.

Conclusions

The findings presented in this manuscript demonstrated for the first time that root system of intact plants possess immense potential to reduce Ag0 and form Ag0/Ag2O NPs. Using 16 plant species from 11 diverse families, it has been demonstrated that the root system of all angioperms possess reducing strength that can be aptly used for reduction of metal ions like Ag0. Root system of plants could reduce Ag0 and generate Ag0/Ag2O NPs even under strict sterile conditions, establishing that root system alone possess immense reducing strength. Efficacy of root system of these plant species to reduce membrane impermeable ferri cyanide to ferrocyanide and triphenyltetrazolium to triphenylformazan established that plasma membrane bound dehydrogenases/reductases are responsible for reduction of Ag0 at surface, and exogenous generation of Ag0/Ag2O NPs. Dehydrogenases in root enzyme extract possessed potential to reduce Ag0 to Ag0 and generate Ag0/Ag2O NPs in the presence of NADH. As the plasma membrane bound dehydrogenases seems to be a single major component for reduction of Ag0 the NPs generated were relatively more or less spherical and in narrow size range. The findings presented in this communication also established that synthesis of Ag0/Ag2O NPs by root system of intact plants is superior over all the biological methods reported so far. To the best of our knowledge, this novel method for generation of silver NPs is most green, simple, and economically viable.

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Author Contributions

Conceived and designed the experiments: PPS. Performed the experiments: PPS KY PS TP SN JS RN KSR. Analyzed the data: PPS PS SN JS RN. Contributed reagents/materials/analysis tools: PPS KSR. Wrote the paper: PPS PS SN JS RN KSR.

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