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Full Length Research Article

In vitro Multiple Shoot and Micro Tuber Production of *Dioscorea oppositifolia* L. and Green Silver Nano Synthesis of Phytocompound Diosgenin

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Abstract

In our present study, the *in vitro* micro tuber of *Dioscorea oppositifolia* L. was successfully produced with a combination of Benzyl Aminopurine (BA) 3.0 mg/l along with Gallic acid 3(GA3)- 3.5mg/l in MS medium produced the weight fresh weight micro-tubers of 456.2 ±1.0 mg. Now a days, recent interest for pure silver nanoparticles synthesis focused on tissue-cultured plant source. Eco- friendly synthesis of silver nanoparticles from *in vitro* plants plays a major role in the advancement of drug development research. In the present study, the preparation of green silver nanoparticles by using tuber extract of *in vitro* raised *Dioscorea oppositifolia* L. is the novel work. This is the first report of producing silver nanoparticles from *in vitro* micro tuber extract of *Dioscorea oppositifolia* L. The *in vitro* method of synthesized silver nanoparticles was characterized using UV–Vis spectroscopy, FTIR, Scanning Electron Microscopy (SEM), Transmission electron microscopy (TEM), Dynamic light scattering (DLS). The UV/Vis spectrum at 430 nm is the confirmation spectrum of silver nanoparticles. FT-IR peaks ranging from 1000-4000 cm^{-1} which showed the presence of alkaloid and steroidal saponin which act as a reducing agent required for the silver nanoparticles synthesis. The size of the silver nanoparticle was confirmed by Transmission Electron Microscopy (TEM) and the 3D structure of silver nanoparticles was predicted by Scanning Electron Microscope. SEM and TEM results infer that synthesized *in vitro* silver nanoparticles were predominantly spherical with an average size of 42.56 nm.

***Key words*:** *In vitro* tuber, *Dioscorea oppositifolia* L., Silver nanoparticles, FTIR, SEM, TEM

Dioscorea, belongs to Dioscoreaceae family consisting of more than 500 species, is commonly known as yam. They have high nutritional value, particularly as an alternative source of starch, and are also used widely for therapeutic purposes as well. The Dioscorea species contain many pharmaceutical compounds such as phenols, alkaloids, tannins, flavonoids, saponins, glycoside steroids, anthraquinones, etc. [52], [55]. In recent years, due to the development of the steroid hormone drug synthesis industry, the demand for diosgenin has been increasing, resulting in the over-exploitation of natural *Dioscorea sps* [43]. *Dioscorea oppositifolia* L. Common names: Chinese yam (English), cinnamon vine (English). *Dioscorea oppositifolia* L. is a twining vine that has more steroidal saponin Diosgenin as active principle so that *D. oppositifolia* is used as an alternative medicine a huge demand for production of bioactive compounds [4].

Medicinal plants are the rich source of bioactive compounds which plays a major role in development of novel nanomedicine [50]. Dioscorea is a promising source of many pharmacologically active compounds and has high efficacy to develop novel natural drugs for the treatment of dangerous and metabolic diseases [53], [56]. To the best of our knowledge, the *in vitro* micro tuber extract of *Dioscorea oppositifolia* L. has not been taken previously for the synthesis of silver nanoparticles. Therefore, in the present work, the green synthesis method was used to reduce Ag NPs using the extract of *Dioscorea oppositifolia* L *in vitro* raised micro tuber as a green reducer. The green synthesized Ag NPs were confirmed by Ultraviolet-visible (UV–Vis) spectroscopy, Fourier transform infrared (FT-IR), Transmission electron microscope (TEM) and Scanning Electron Microscope (SEM). *In vitro* culture could meet rhizomes demands of commercial uses without any seasonal constraints [6]. Since there are more attention has been focused for natural plant-derived compounds from *in vitro* tuber extract as an alternative supplement.

Nanobiotechnology deals with the synthesis of nanostructures using living organisms [44]. Among the source for nanoparticle synthesis, *in vitro* plant extract have found to

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be more effective for synthesizing metal nanoparticle purely. Biological method with the use of plants for the synthesis of nanoparticles could be advantageous over other chemical methods [45]. Biosynthetic silver nano synthesis using *in vitro* tissue cultured plant extracts produced different shape and size nanoparticles. Mechanism of elucidation of plant-mediated silver nano synthesis is a very promising area of research [7].

To meet this demand, the cultivation of more medicinal plants through tissue culture for the production of bioactive compounds is essential [7]. *In vitro* regeneration using a combination of plant growth regulators (PGRs) for callus and shoot induction is considered one of the main factors for successful way for silver nano synthesis [2]. Thus, establishing an efficient *in vitro* micro tuber production for green synthesis of silver nanoparticles are essential for effective drug production for various diseases like cancer and mental disorder which was already proved in our previous studies [51].

MATERIALS AND METHODS

Plant material

Dioscorea oppositifolia L. was collected from the Kolli hills in Tamil Nadu, India (Fig 1) and was authenticated in Herbarium, Botanical Survey of India, Tamil Nadu Agriculture University Campus, Coimbatore.

In vitro micro tuber production of Dioscorea oppositifolia L.

Node and shoot tips of *Dioscorea oppositifolia* L. were used as explants for direct organogenesis and also for multiple shoot production. Explants were washed under running tap water for at least 1 - 2 hrs. and then by soaking in 5% (w/v) detergent solution (Teepol) for 10 min, followed by washing with sterile distilled water. Then subjected to 50% ethanol wash and are followed by 0.1% (W/V) aqueous mercuric chloride solution for 5 min, again followed by repeated washing with sterile distilled water. Shoot tips and nodal explants were placed on semisolid MS supplemented with different concentrations of BAP and GA_3 were tested for tuber production. The cultures were incubated under 16 hours light and 8 hours dark and maintained at a constant temperature of 25 ± 2°C.

Synthesis of silver nanoparticles

The *in vitro* tubers were cleaned with sterile water, oven-dried for 48 hours, and grind to make a fine powder. The fine powder of plant material (150 gm) was dissolved in 150ml of distilled water and the extracts were filtered by using Whatmann No.1 filter paper and then 0.1mm silver nitrate solution was added to the filtrate. The filtrate was heated at 65˚c for 25mins in the micro oven for color change and the UV spectrum range from 300-900 nm should be taken within 3 hrs of colour change for further analysis.

Characterization of silver nanoparticles (Ag-nps)

UV VIS spectrum analysis

The coloured solution was subjected to UV- Vis spectral analysis by using a UV-Vis spectrophotometer [16]. After 3hrs of incubation of the above filtrate, preliminary detection of Ag-nps was carried out by visual observation of color change of the plant filtrate. These samples were later subjected to optical measurements which were carried out by using a UV-Vis spectrophotometer (Lamda-25; Perkin Elmer; Waltham, Massachusetts) and scanning the spectra between 300 -900 nm at the resolution of 1nm.

Fourier transform infrared (FTIR) analysis

In Fourier transform infrared (FTIR) analysis, the FTIR spectrum of the condensed nanoparticle sample from the plant filtrate was recorded on a Perkin Elmer 1600 instrument in the range 450-4000 cm^{-1} at a resolution of 4 cm^{-1}.

Dynamic light scattering (DLS) measurements

Dynamic light scattering (DLS) is an analytical tool used for measuring the dynamic size of the synthesised silver nanoparticles in a liquid medium. Silver nanoparticles (Ag-nps) are subjected to their surface plasmon resonance wavelength. In this study, we demonstrate that DLS (Zeta sizer Nano-2590, Malvern Instruments Ltd, Worcestershire, UK) can be used as a very convenient and powerful tool for determining the size of the silver nanoparticles [10].

TEM analysis

The size of the bio-reduced silver nanoparticles produced from *in vitro* microtuber extract were determined using a transmission electron microscope (TEM, Tecnai 12 Cryo, FEI, Eindhoven, and The Netherlands).

SEM analysis

SEM image of *in vitro* microtuber extract of *Dioscorea oppositifolia* L., fabricated silver nanoparticles was done by using SEM (JEOLMODEL 6390). The morphology of the silver nanoparticles were also confirmed by a Scanning electron microscope.

Statistical analysis

All data were represented as the mean value for each treatment. Differences among the treatments were determined using analysis of variance (ANOVA). All statistical analyses were performed using SPSS 18.0 software and post-hoc testing was carried out using Duncan's Multiple Range Test. P values<0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

The *in vitro* tubers were produced on semisolid MS supplemented with the concentrations of BAP and GA_3 at 3.5 mg/l and 4.0 mg/l.The *in vitro* raised tubers were used for silver nanoparticle synthesis. Effect of sucrose concentration on *in vitro* tuber production with MS Medium supplemented with BA + GA_3 – 3.5 + 4.0 mg/l increases the weight of *in vitro* tuber (Fig 2). In the present study, we have found that with increasing the concentration of sucrose weight of the *in vitro* rhizome or *in vitro* tuber gets partially increased. The *in vitro* tuber filtrate was prepared and to the filtrate silver nitrate (0.1mM) was added and subjected to optical measurement by UV- Vis spectrophotometer; this analysis showed an absorbance peak at 410 nm (Fig 3), which was specific for the silver nanoparticles due to their surface plasmon resonance. The *in vitro* tuber extract has a large amount of phytocompound than the wild tuber.

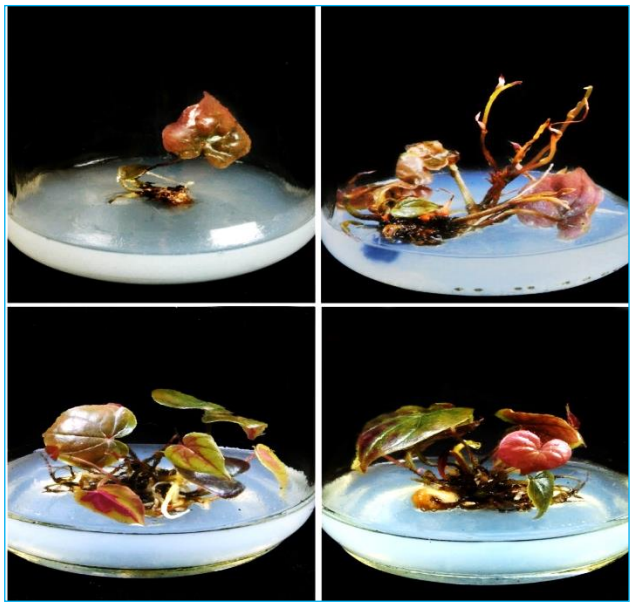
In vitro propagation is the best method to mass conserve the medicinally important plants like *Dioscorea oppositifolia* L. Moreover, shoot multiplication and rooting induction experiments were incubated on hormone-free MS medium within 35 g/l sugar and 9 g/l agar. Multiple shoot formation at hormonal concentration of kinetin and GA_3 - 2.0 + 1.5 mg/l producing shoot length of 5.0±0.5 cm. Multiple shoot formation at hormonal concentration of BA + NAA-2.5 + 2.0 mg/l producing shoot length of 5.9±0.5 and root length of 2.5±0.5 cm (Table 1). Large number of multiple shoots arising at hormonal concentration of BA and GA_3 -2.0 + 1.5 mg/l producing shoot length of 6.0±0.5 cm and root length of 4.2±0.1

cm. Micro tubers formation from multiple shoots at hormonal concentration of BA and GA_3 -3.5 ± 3.0 mg/l producing shoot length of 6.4±0.5 and root length of 7.5±0.5cm with micro tubers (Fig 1).

Table 1 Multiple shoot formation and micro tuber formation from callus explants

MS+Growth regulators (Mg/L)	Frequency %	Shoot length 1 (Cm)	Root length 1 (Cm)
Basal MS	0.0	0.0	0.0
GA_3 + Kinetin			
0.5+0.1	80.0	2.5±0.5d	0.0
0.1+0.5	84.6	2.3±1.2e	0.0
1.5+1.0	83.3	4.0±0.5c	0.0
2.0+1.5	90.5	5.0±0.5a	0.0
2.5+2.0	89.0	4.5±0.5b	0.0
BA + NAA			
0.5+0.1	80.0	2.5±0.5d	0.5±0.5e
0.1+0.5	84.6	2.3±1.2de	1.0±0.3d
1.5+1.0	83.3	4.0±0.5c	1.5±0.5c
2.0+1.5	89.3	5.0±0.5b	2.0±0.3b
2.5+2.0	91.3	5.5±0.5a	2.5±0.5 a
3.0+2.5	88.0	4.9±0.5b	2.0±1.0 b
BA + GA_3			
0.5+0.1	98.6	2.5±0.5d	2.0±0.2e
0.1+0.5	98.0	2.3±0.5e	2.7±0.2d
1.5+1.0	99.0	4.0±0.5b	3.5±0.2b
2.0+1.5	99.0	6.0±0.5a	4.2±0.1a
2.5+2.0	98.6	3.4±0.5 c	3.0±0.3 c

Values are expressed in mean ± SE of superscripts which are not marked with same superscript are significantly different at α=0.05 using Duncans multiple Range test (Post hoc analysis)



A. Multiple shoot formation at hormonal concentration of kinetin and GA_3-2.0+1.5 mg/l producing shoot length of 5.0±0.5 cm
B. Multiple shoot formation at hormonal concentration of BA + NAA-2.5+2.0 mg/l producing shoot length of 5.9±0.5 and root length of 2.5±0.5 cm
C. Large number of Multiple shoots arising at hormonal concentration of BA and GA_3 -2.0+1.5mg/l producing shoot length of 6.0±0.5 cm and root length of 4.2±0.1 cm
D. Micro tubers formation from Multiple shoots at hormonal concentration of BA and GA_3 - 3.5±3.0 mg/l producing shoot length of 6.4±0.5 and root length of 7.5±0.5 cm with micro tubers

Fig 1 Multiple shoot formation and micro tuber formation from *Dioscorea oppositifolia* L

FTIR spectrum of the nanoparticles obtained in the present study is presented in (Fig 2). Among them the absorption bands are observed in the range of 600- 4000 cm^{-1} is 671.41, 1650.03**,** 2092.06, and 3402.65 cm^{-1} (Fig 4) The band at 1650.03 has been identified as amide II. These peaks correspond to amide II and amide III aromatic rings. The bands at 2092.06 have been identified as COOH –Acid group (Table 1). At particle sizes below 100 nanometers the optical scattering efficiency drops rapidly with particle size. DLS report gives the average particle size of the nanoparticle is around 110 nm (Fig 5). TEM image showed the size of the nanoparticle is 42.56 nm which will be more suitable for biomedical activity (Fig 6). SEM image shows spherical nanoparticles were successfully synthesized from *in vitro* raised *Dioscorea oppostifolia* L. micro tuber (Fig 7).

D. oppositifolia, a traditional medicinal plant with curative effect against various ailments which is having nanobiotechnological potential and its tuber extract synthesized both AuNPs and AgNPs within 5 h which is considered to be a rapid, efficient, and environmentally benign route for the synthesis of metal nanoparticles [31]. Effect of sucrose concentration on *in vitro* tuber production with MS Medium supplemented with BA + GA_3 – 3.0+3.5 mg/l increases the weight of *in vitro* tuber in our study. Auxin and cytokinin have been used for the shoot regeneration in *Dioscorea deltoidea* for the production of pharmaceutically active compounds. The hormonal combination of 1.5 mg/l Kn + 1 mg/l BAP + 0.5 mg/l NAA increased the stem height along with the broadest *Dioscorea alata* leaves at diameter of 1.5750 cm and 1.3969 cm respectively [47].

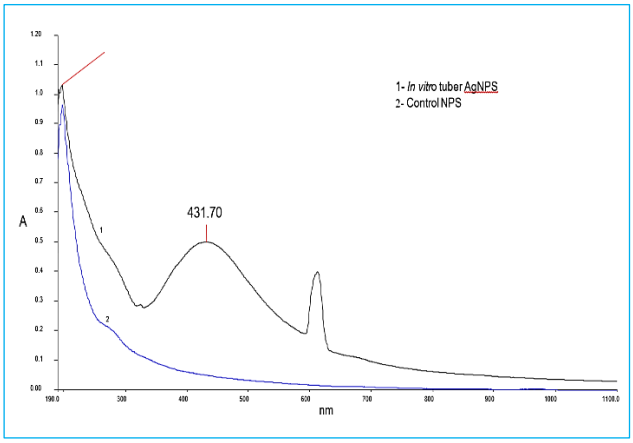


Fig 2 UV- Visible spectrum of *Dioscorea oppositifolia* L *in vitro* tuber filtrate containing silver nanoparticles (0.1mM)

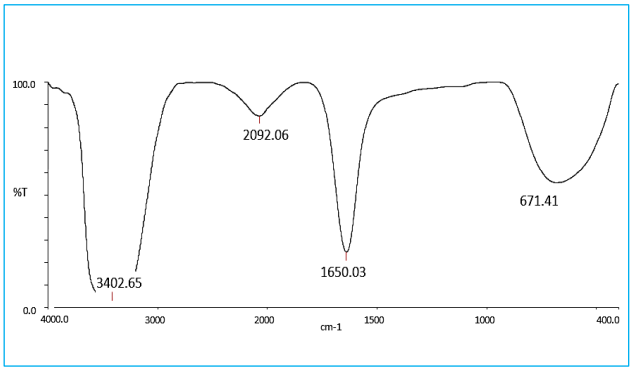


Fig 3 FTIR spectra recorded from silver nanoparticles synthesized using *Dioscorea oppositifolia* L *invitro* tuber

The amide groups and COOH groups are acts as a ligand for nanoparticle synthesis. At particle sizes below 100

nanometers the optical scattering efficiency drops rapidly with particle size. In this size region, the scattering is described by Rayleigh scattering where the scattering is the Efficiency per unit volume of particles is proportional to the particle diameter cubed, D_3 [18], [19]. DLS report gave the average particle size of the nanoparticle as around 100 nm. TEM image showed the size of the nanoparticle is 46 nm which will be more suitable for drug production. UV-Vis absorption spectra show peaks characteristic of the surface plasmon resonance of nanosized particles [4], [6], [13], [16-17]. The silver nanoparticles were found to possess potent antibacterial activity against both Gram-negative and Gram-positive bacteria [46].

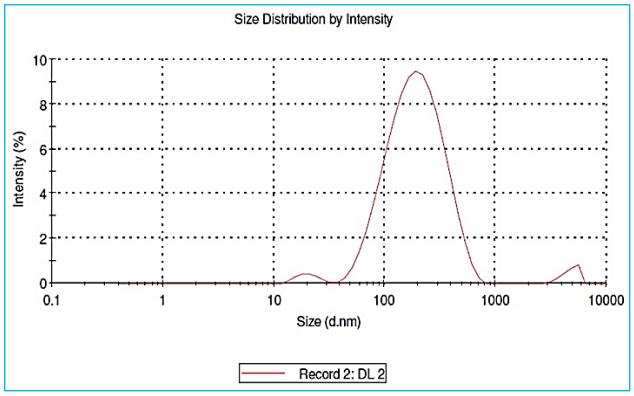


Fig 4 DLS - Dynamic light scattering- The size of the nano particle is around 100nm, which is confirmed by DLS (Dynamic light scattering)

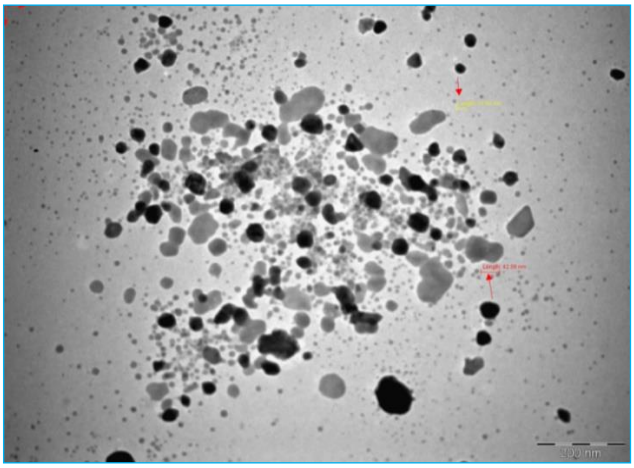


Fig 5 TEM image of silver nanoparticles produced from *in vitro* tuber extract of *D. oppositifolia* L. size of the nanoparticle - 42.56 nm

In our previous studies, silver nanoparticles synthesis and characterization by using Diosgenin and its axyolytic activity [51]. The extract incubated with $AgNO_3$ showed a color change of the extract from greenish to reddish brown with intensity increasing during the period of incubation (35). In our recent publications, silver nanoparticles (Ag-nps) are widely used for their applications in medicine for neurological disorders. Silver nanoparticles were synthesized from *Dioscorea alata* showed high bactericidal activity and optical limiting behavior [48].

In this present investigation, a large number of multiple shoots arising at a hormonal concentration of BA and GA_3 (4.0±3.5) mg/l producing shoot lengths of (6.0±0.5) mg/l and root lengths of 9.0±0.5cm and micro tubers formation from multiple shoots at a hormonal concentration of BA and GA_3 (3.5±3.0) mg/l producing shoot length of 6.4±0.5 and root length of 7.5±0.5cm with micro tubers. *D. bulbifera* in solid DKW medium; similarly *in vitro* propagation of other yam species is tried using solid medium [47]. In Anacardium occidentale leaf extract silver nano particles, UV spectrum were observed 430 nm similarly in our present work silver nanoparticles by *in vitro* tuber extracts of *Dioscorea oppositifolia* L silver nanoparticles, UV spectrum were observed 431 nm [50]. This is the first report for *in vitro* micro tuber production from *D. oppositifolia* L. multiple shoots and the synthesis of silver nanoparticles from *in vitro* tuber extract for effective biomedical applications. The morphology of *in vitro* rhizome-mediated silver nanoparticles was observed as spherical using SEM (JEOLMODEL 6390).

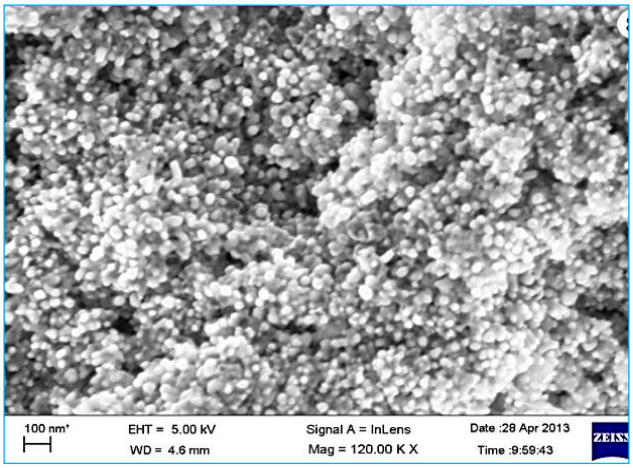


Fig 6 SEM image of silver nanoparticles of *in vitro* tuber-*Dioscorea oppositifolia* L.

CONCLUSION

In vitro tubers can be efficiently used in the synthesis of silver nanoparticles as a greener route. Control over the shape and size of nanoparticles seems to be very easy with the use of plants, especially *in vitro* plants. In the present study, we found that *invitro* tuber can be a good source for the synthesis of silver nanoparticles of different shapes like triangles and spherical shapes. This approach toward the synthesis of silver nanoparticles has many advantages such as the ease with which the process can be scaled up, economic viability, etc. This is the first report, on the synthesis of silver nanoparticles using *D. oppositifolia* L. *in vitro tuber*. Such nanoparticles produced using plants, especially from *in vitro* plants have been used in various applications for human benefit. However, the mechanism of such nanoparticle synthesis by aseptic plants (*in vitro* plants) is used in the new way of drug production for various dangerous diseases, which is prevailing as antibiotic resistance.

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Conflict of interest: None

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Ethics statement: None

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