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BIOSYNTHESIS OF SILVER NANOPARTICLES USING CELL FREE CALLUS EXUDATES OF *MEDICAGO SATIVA* L.

H. S. HEGAZY[1], LAMIS D. SHABAAN[1*], G. H. RABIE[1] AND DIANA S. RAIE[2]

[1]*Botany Department, Faculty of Science, Zagazig University, 44519, Zagazig, Egypt*
[2]*Process Design and Development Department, Egyptian Petroleum Research Institute, Nasr City, Cairo, Egypt.*
Corresponding author e-mail: lamis_shaaban@yahoo.com

Abstract

The present study is designed to investigate the biosynthesis of silver nanoparticles by using cell free callus exudates of *Medicago sativa* L. Explants are surface sterilized and then sub-cultured on MS medium supplemented with 3% sucrose, 2 mg/L 2,4D and 1 mg/L BA. Cultured tissues are derived from hypocotyls, and then soaked in sterilized deionized water (8 h) on dark. The cell free exudates incubated with aqueous silver nitrate, at room temperature, showed change in the color of the mixture from colorless to yellow indicating the silver nanoparticles synthesis. Silver nanoparticles are formed with different shape and variable size. Synthesis of silver nanoparticles is influenced by the pH variation of silver nitrate solution, at pH 5, the results showed significant and insignificant differences on shape and on size respectively. FT-IR absorption spectra conclude that the stabilizing agent could be a polyphenol with amide group. The reducing agent was supposed to be a member of antioxidants.

Key words: *Medicago sativa* L., Callus, Biosynthesis, Nanoparticles, Antioxidants.

Abbreviation: UV-Vis (ultraviolet-visible), TEM (transmission electron microscope), SPR (surface Plasmon resonance), FT-IR (Fourier transform infrared spectroscopy)

Introduction

The broad range of applications of nano-materials is a logical result of size-based exclusive characters. Nanotechnology is dealing with synthesis and application of nanoparticles with size up to 100 nanometers. Silver nanoparticles have charismatic interest because of their unique properties and their effective antimicrobial potential(Agarwal *et al.*, 2014).

Generally, nano-silver can be synthesized through numerous physical and chemical methods. Use of such methods becomes undesirable as some chemicals used are toxic, flammable, and non-degradable (Nabikhan *et al.*, 2010). Chemical synthesis methods lead to the presence of some toxic chemicals absorbed on the surface that, may lead to adverse effect in the medical applications (Jain *et al*., 2009). So, searching of green approach for nano-making becomes a necessity.

Recently green biosynthesis of nanoparticles depends on three major sources: bacteria (Saifuddin *et al*., 2009), fungi (Vala *et al.*, 2014), and plant extracts (Ghosh *et al*., 2012) due to their ability to provide bio-reducers and bio-stabilizers (Prabhu & Poulose, 2012). The stabilizing agent is a critical factor to control nanoparticles size, shape and stability (Lukman *et al.*, 2011).

However, using of plants for biosynthesis of nanoparticles is rapid, low coast, eco-friendly and in single step method. Cultured tissue is a continuous reservoir for plant biomass. It can also applied in large-scale for biosynthesis of nanoparticles. Many research reported the synthesis of silver nanoparticles using extracts from cultured tissues such as *Carica papaya* (Mude *et al.*, 2009), *Sesuvium portulacastrum* (Nabikhan *et al.*, 2010), *Citrullus colocynthis* (Satyavani *et al.*, 2011), *Costuss peciosus* (Malabadi *et al.*, 2012a), *Clitoria ternatea* (Malabadi *et al.*, 2012c), *Catharanthus roseus* (Malabadi *et al.*, 2012b), *Lycopersicon esculentum* (Asmathunisha & Kathiresan, 2013) and *Jatropha curcas* (Demissie & Lele, 2013). In these trails, extractions were done through grinding (Mude *et al.*, 2009, Satyavani *et al.*, 2011) and boiling the bio-mass in water (Nabikhan *et al.*, 2010, Malabadi *et al*., 2012 a,b,c, and Asmathunisha & Kathiresan, 2013).

In the current study, we tested the ability of cell free callus exudates of *Medicago sativa* L. to biosynthesize silver nanoparticles. Effect of pH variation on size and shape of silver nanoparticles are also tested. We tried to characterize the reducing and stabilizing agents which mediate the biosynthesis of nano-silver.

Materials and Methods

Callus induction: Alfalfa (*Medicago sativa* L.) seeds have been kindly provided by Forage Crops Research Section, Field Crops Research Institute of the Agricultural Research Centre, Giza- Egypt. Under aseptic conditions, seeds are sterilized with ethyl alcohol (70%) for 5 seconds and washed with sterilized distilled water. They were soaked in sodium hypochlorite (20%) for 10 min and washed several times with sterilized distilled water. Surfaces sterilized seeds were transferred to sterilized Petri dishes containing moist Whatman No. 3 filter paper then incubated in dark at 25ºC for germination. After 7 days, cut hypocotyls of 3-5 mm were cut and planted on the callus induction medium. Cultured explants were incubated in growth chamber at constant temperature of 25± 2ºC. After three weeks, three callus sub-cultures were maintained under the same conditions used in callus initiation.

Callus was initiated and sub-cultured on MS medium, supplemented with 3% sucrose, 2mg/L 2, 4 D and 1 mg/L BA. The pH of medium was adjusted at 5.8 ± 0.2 by (0.1 N) HCl and (0.1N) NaOH and followed by the addition of agar (8 g/L). The medium was autoclaved for 20 min at 121°C for sterilization (El-Hattab *et al.*, 2005).

Preparation of callus exudates and biosynthesis of silver nanoparticles: Four grams of fresh callus of *Medicago sativa* were soaked in 40 ml non-aerated deionized water for 8 h at room temperature in darkness. The mixture was centrifuged at 30000 rpm for 40 min. The supernatant separated contained the cell free exudates. Two ml of cell free callus exudates was mixed with equal volume of 0.1 M aqueous silver nitrate and incubated at room temperature for 24 h in dark (Lukman *et al.,* 2011). The pH of silver nitrate solution was adjusted at 2, 5, 7, 9, 10, and 11 using ammonium hydroxide or nitric acid (Gorup *et al*., 2011). The mixture was centrifuged after 24 h at 18,000 rpm for 20 min, the precipitate was separated washed three times by deionized water, dried and then subjected to analysis of its contents from silver nanoparticles. The reducing agents content in the supernatant are analyzed.

Characterization of silver nanoparticles

UV-Vis spectrophotometer: The appearance of a yellowish color in solution was an initial observation of nano-silver formation. The synthesis of silver nanoparticles was subjected for optical measurements using UV-Vis spectrophotometer through examining the spectra between 250 to 800 nm (Lukman *et al.*, 2011).

Transmission electron microscopy: The morphology of silver nanoparticles is characterized using transmission electron microscopy (TEM) by casting onto carbon-coated gold grids (Lukman *et al*., 2011).

Identification of possible reducing and stabilizing agents: The possible reducing agents were determined in free nano-silver supernatant by calorimetrical methods. The total proteins, carbohydrates, phenols and flavonoids were determined according to Lowry *et al.*, 1951, Dubois *et al.*, 1956, Slinkard & Singleton, 1977, and Zhishen *et al.*, 1999, respectively. Silver nanoparticles are processed for FT-IR (Fourier transform infrared spectroscopy) analysis for determination the stabilizing agents (Demissie and Lele, 2013).

Statistical analysis: The data obtained is subjected to ANOVA test to estimate the difference (LSD) at $p \leq 0.05$ to compare the variation between shape and that of size of nanoparticles at different treatments. Samples analyzed in triplicates.

Results and Discussion

Callus is obtained from hypocotyls of *M. sativa* (Fig. 1). Biomass initiation and subculture were on MS medium. This culture medium was supplemented with 2 mg/L of 2,4 D and 1mg/L BA (El-Hattab *et al.*, 2005). A known fresh weight of cultured tissue was soaked with water to induce exudation. Cell free exudates of callus are subjected to biosynthesis of silver nanoparticles through pH adjusted and not adjusted of silver nitrate solution. This conversion of bulk material into nanoparticles is due to the nucleation of ions to nanoparticles by reducing agent, and adsorption or chemically binding to the surface of nanoparticles by a stabilizing agent (Lukman *et al.*, 2011).

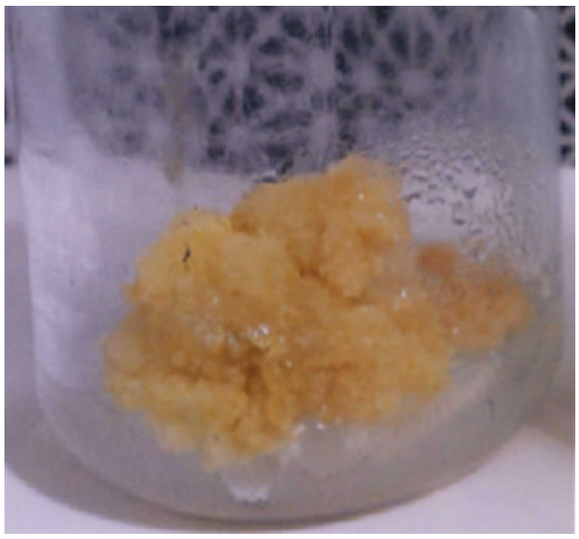


Fig. 1. Optical photo of hypocotyls-derived callus of *Medicago sativa* L.

Fruitfully, yellowish color developed after 15 min of the reaction, except at pH 2 (Fig. 2). This color indicates nano-silver formation. It is stimulated due to excitation of surface plasmon resonance (Lukman *et al.*, 2011). At pH 2, the mixture still colorless even after 24 h of incubation, this means that no reaction proceeds. The darkest yellowish color appeared at pH 5 indicted the silver nanoparticles formation. Samples are subjected to UV-Vis spectrophotometer to confirm the formation of nanoparticles (Fig. 3). All broad spectra are a sign of large particle size distribution nanoparticles (Lukman *et al.*, 2011). TEM images (Fig. 4) proves nano-silver formation and confirm the results obtained by UV-Vis spectrophotometer. In non-pH adjusted case, size ranges from 10 to 50 nm. At pH 5, size ranges from 5 to 60 nm. Spherical, disk and irregular shapes are observed in both cases of pH adjusted and non-adjusted. In addition, there is insignificant difference between mean of size of nanoparticles produced. This variation in size and shape may be due to the reducers and/or stabilizers bio-molecules present in the exudates in an insufficient quantity (Lukman *et al.*, 2011). This may be resulted from incomplete metabolite pathways due to its dedifferentiated callus cells. Biomolecules stabilization of nanoparticles is detected using FT-IR spectrum. Fig. 5A, shows peaks at 3430, 2925, 1628 and 1027 cm^{-1} corresponding to OH of polyphenol, C-H of alkanes, C=C of alkenes and C-N of aliphatic amine respectively. These results confirm that the compounds attached with silver nanoparticles could be polyphenols with amide group acting as stabilizing agent, which also act as a capping agent to prevent the agglomeration and provide stability to the medium (Demissie & Lele, 2013). After the separation of silver nanoparticles the analysis of supernatant showed that the cell free callus exudates contain biomolecules which act as reducing agents mediating the reduction of silver ions to nanoparticles. These reducing agents are protein, carbohydrates, phenol and flavonoid (Fig. 5B).

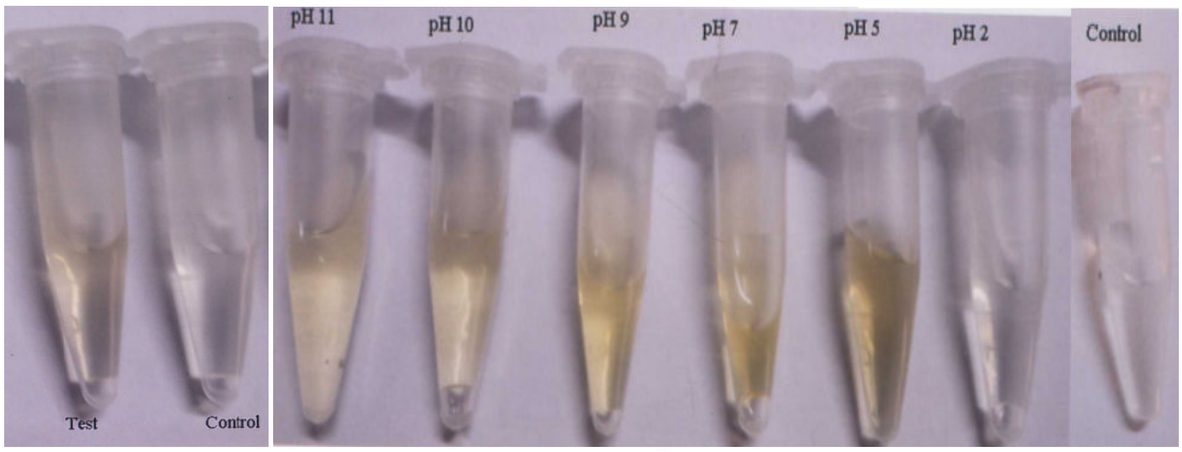


Fig. 2. Yellowish color of nano-silver biosynthesized by cell free callus exudates.
A. Without pH adjustment. B. At different pH

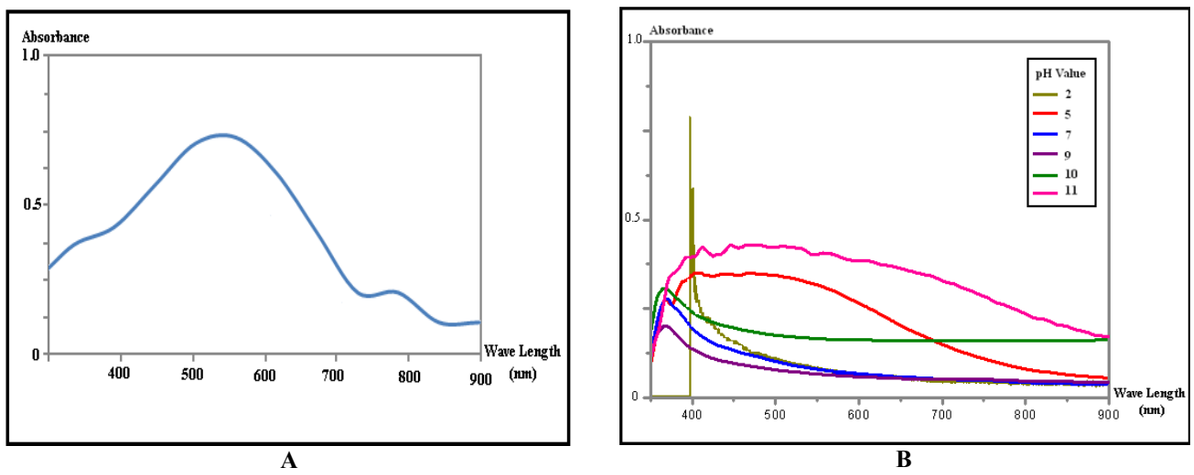


Fig. 3. UV-visible spectra of nano-silver biosynthesized by cell free callus exudates.
A. Without pH adjustment. B. At different pH.

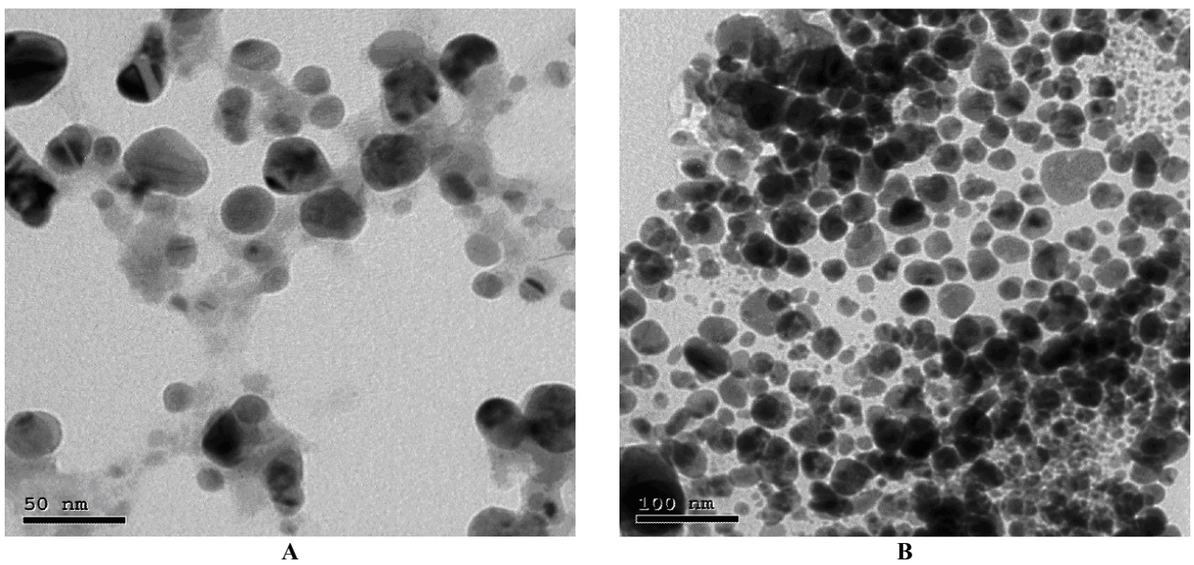
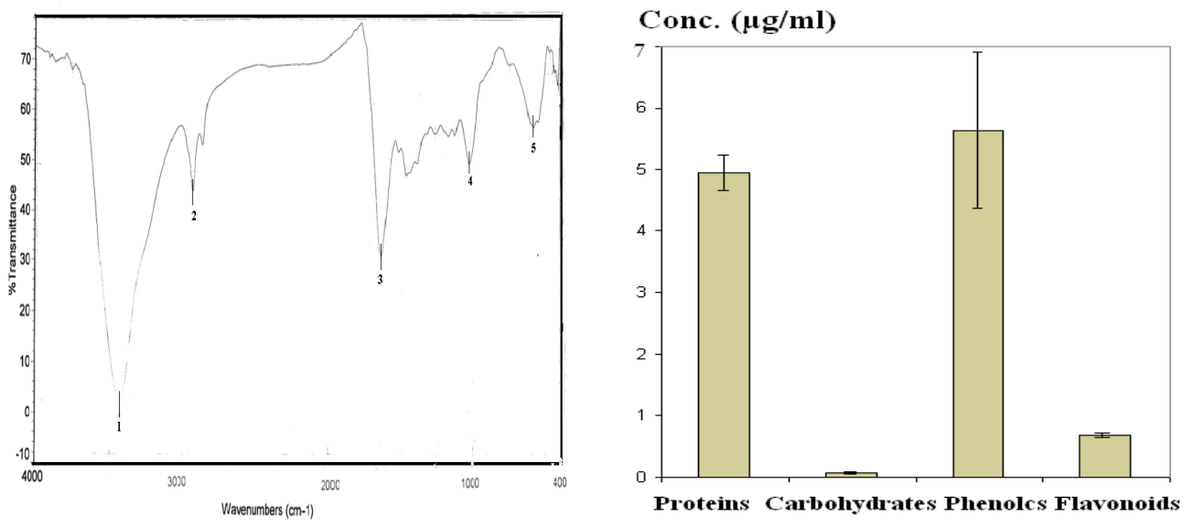


Fig. 4. TEM images of nano-silver biosynthesized by cell free callus exudates
A. Without pH adjustment. B. At pH 5.



A B

Fig. 5. **A.** FT-IR spectrum of cell free exudates bio-molecule adsorbed on surface of nanoparticles as a possible stabilizer; **B.** Total contents of anti-oxidants present in cell free exudates of callus; as probable reducers for silver nitrate

Conclusion

Cell free exudates from *Medicago sativa* L. callus can provide reducers and stabilizers for silver nanoparticles synthesis. Nano-silver particles are biosynthesized in poly-shaped and different size formed at room temperature. Although, pH has a steric effect on the biosynthesis process and it can control the size of nanoparticles. The stabilizer could be a polyphenol. The reducing agent is supposed to be a member of antioxidants.

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