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Silver nanoparticles induced effect on *in-vitro* callus production in *Bacopa monnieri*

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Abstract

Bacopa monnieri has been chosen as model medicinal plant to study the induced effect of Silver nanoparticles on *in-vitro* callus production. Distinct morphological changes on callogenesis were observed on stress application. The effect of Silver nanoparticles on callus induction and autoclaving were studied within MS media and without MS media. The MS medium with the lower concentration ($16X10^3$) of Silver nanoparticles capable for medium growth of callus as that control and simultaneously reducing the rate of contamination. Biosynthesis of Silver nanoparticles were carried out by using *Fusarium oxysporium* fungus and characterized by UV-Vis spectrophotometer and LM 20.

Key words : Silver nanoparticles, *Bacopa monnieri*, Callus, LM 20

INTRODUCTION

Bacopa monnieri commonly known as "Brahmi", the member of family Scrophulariaceae, is placed second in the priority list of Indian medicinal plants. It is commonly found on the banks of rivers and lakes. It has been used for centuries in folklore and traditional system of medicine as a memory enhancer, anti-inflammatory, analgesic, antipyretic, sedative and anti-epileptic agent [1-5]. The memory enhancing effects of this plant have been attributed to the active constituent bacosides A and B [6]. In addition to unique medicinal use, it has also been linked to phytoremediation programmes for the removal of heavy metals such as Cadmium and Chromium [7].

In 1990, the annual requirement of *Bacopa monnieri* was 2.7×10^6 Kg of dry biomass at a value of $34 million. With increasing demand for herbal drugs, the natural populations of this plant are threatened with over exploitation. Therefore, the International Union for Conservation of Natural and National Resources has a long time ago listed it as a threatened species [8].

The requirement of Brahmi is met solely from the natural populations, leading to their gradual depletion. Its seeds have poor propagule due to their short viability and frequent seedling death, which makes difficult to raise this plant from seeds. Its vegetative propagation is also slow [9]. Therefore, development of a rapid clonal multiplication protocol of this medicinally important herb has become imperative in order to reduce the existing pressure on natural populations and constant supply of plant material for pharmaceutical industry. There is a demand for further improvement in the tissue culture protocol for the mass multiplication of *Bacopa monnieri*, both for commercial farming system and later, if required for replanting in the natural habitat when the plant population declines. For that an innovative micropropagation protocol has been developed which has not been attempted so far in *Bacopa monnieri* [10].

MATERIALS AND METHOD

Callus induction:

All plant materials required for different experiments were selected from mother plant field station of Krishi Mitra Biotech, Arvi (Dist. Wardha) .Only elite disease free plants were selected for the experiments. All chemicals and plant growth regulators (PGR's) were purchased from Hi Media Laboratories, Mumbai. Glassware used for all these experiments were of Borosilicate purchased from Alka Scientific Corporation, Nagpur.

The basal medium employed in this study for culture of *Bacopa monnieri* was MS medium [11]. The concentrated stock solutions of the major salts, minor salts and vitamins were prepared, for preparation of the media and stored under refrigeration. Auxins and Cytokinins were dissolved in 1N NaOH and 2, 4-D was dissolved in Ethyl alcohol before making up the final volume with distilled water. The medium was prepared by adding appropriate quantities of the stock solutions and correct volume was made up with the distilled water. The pH was adjusted in all cases to 5.8 by using 1N NaOH and 1N HCl and CleriGel™ (HiMedia: PT079). Agar 0.8% (w/v) was used as solidifying agent. Before autoclaving, the media was poured into washed culture bottles (30-35ml) which were capped and labeled properly. These were then autoclaved at 121^0C for 15 minutes at 15-psi pressure and transferred to the inoculation room where they were stored under aseptic conditions till their use.

Explants for the initiation of callus induction were largely detected by the method adopted for *in vitro* clonal propagation. According to the procedure vigorously growing leaves of "*Bacopa monnieri*" were excised from proliferating shoots and their leaflets were used as explants for callus induction studies.

The selected explants were washed thoroughly with constant shaking with tap water for 5 minutes for the removal of external dust and contaminants. Thereafter, they were washed twice with distilled water and then with 10% Dettol, a liquid disinfectant

used for 3 minutes to avoid chance of any internal microbial contamination and again rewashed with distilled water for two times. Finally explants were kept in sterile distilled water bottle.

These explants were taken inside the laminar air flow for further sterilization. Firstly, they were washed with sterile distilled water by continuous shaking for 5 minutes and this process was repeated two times. Then they were treated with 0.1% mercury chloride for approximately 3-4 minutes for the removal of contaminants. After that, explants were washed thoroughly two times with sterile distilled water so as to remove all traces of Mercury Chloride. Thereafter, surface sterilization was done with Sodium hypochloride for approximately 3-4 minutes. Then they were removed from the sterilizing solution and rinsed twice in sterilized distilled water so as to remove all traces of Sodium hypochloride. Finally the extra water was removed with the help of sterile tissue paper from the explants and then they were used for the inoculation.

Methods of inoculation and culture condition:

Sterilized explants were transferred aseptically from the bottles to sterile glass plates for giving a fresh cut on both sides. The forceps were rinsed in the 70% ethanol, flamed them and allowed to cool for while. The explants were placed inside the bottle on semisolid (MS) basal medium supplemented with different concentrations of PGRs using long forceps without touching the rim of the bottle. At least three explants were placed in each bottle. Cap of the bottle was carefully placed, tighten and then sealed with clean film. The same procedure was repeated for all available explants and finally bottles were kept in the rack of growth room, which maintained at $25 \pm 2^{o}C$, with 16 h photoperiod under cool, white fluorescent lamps and 8 hrs night break with (2000 Lux) light intensity.

I) Bio-synthesis and characterization of Silver nanoparticles:

In the present study Silver nanoparticles were bio-synthesized by *Fusarium* fungus. It was identified on the basis of their morphological characteristic and microscopic examination. The pure cultures of *Fusarium* were maintained on potato dextrose agar slant. For the synthesis of Silver nanoparticles, the biomass of fungus *Fusarium* was prepared by growing it aerobically in a liquid medium (potato dextrose broth containing infusion of 250 g potato and 20 g dextrose per liter of distilled water). The flasks were inoculated and then incubated on orbital shaker at 25 ± 2^{0} C and agitated at 120 rpm for 72 h. The biomass was harvested after complete incubation by filtering through filter paper followed by repeated washing with distilled water to remove any medium component from the biomass. About 20 g (wet weight) was brought in contact with 100 ml of sterilized double distilled water for 24 h at 25 ± 2^{0} C in a 250 ml Erlenmeyer flask and agitated again at 120 rpm. After the incubation, the cell filtrate was obtained by passing it through whatman filter paper No. 1. The filtrate was treated with aqueous 1 mM $AgNO_3$ solution in an Erlenmeyer flask and incubated at room temperature. After 2 hours of incubation, the absorbance of fungal filtrate containing nanoparticles was measured in the UV-Visible spectrophotometer by scanning the spectra. The color change of the medium from yellow to dark brown indicated the production of Silver nanoparticles.

Characterization of nanoparticles:

1. UV-Vis Spectrophotometric analysis:

The detection of Silver nanoparticles by a UV-Vis spectrophotometer was carried out after synthesis of Silver nanoparticles, the reaction mixture i.e. control- $AgNO_3$ and experimental solution of Silver nanoparticles were subjected to optical analysis and spectra were recorded using a UV-Vis spectrophotometer (Shimadzu UV-1700, Japan) at the resolution of 1nm from 200 to 800 nm for each sample.

2. Nanoparticles Tracking Analysis (NTA) through LM-20:

The nanoparticles tracking analysis was carried out by using the liquid sample of Silver nanoparticles prepared by diluting with the nuclease free water and 0.5 ml of diluted sample was injected into the sample chamber and observed through LM-20. Nanoparticles present within the laser beam path were observed by optical instrument (LM-20, NanoSight Pvt. Ltd., UK) having CCD camera and size of the nanoparticles was measured on the basis of Brownian motion of the particles.

Autoclaving of nanoparticles:

In these experiments, 5 ml of Silver nanoparticles were taken in its synthesized form (colloidal) in two separate test tubes. They were autoclaved at 121^{0}c and 15 lbs for 15 minutes. Thereafter, they were characterized by UV-Visible and LM 20 to check the thermal stability of nanoparticles.

Autoclaving of Silver nanoparticles within liquid MS medium:

For this study two test tubes were used for Silver nanoparticles. Nine ml MS medium and one ml Silver nanoparticles were added in each of them. One of them was autoclaved at 121^{0}c and 15 lbs for 15 min. Until autoclaving of the first tube, the second tube was kept for incubation in refrigerator. Thereafter, both tubes of each nanoparticle (autoclaved and incubated) were characterized by UV-Visible and LM 20 and observed the spectra.

Inoculation of callus in medium supplemented with nanoparticles:

Early developed callus of *Bacopa monnieri* with initiating stages was placed in MS medium supplemented with different concentrations of nanoparticles i.e. 10^{10}, 10^{5} and 10^{3}. For each concentration four tubes were prepared for experimental set and four tubes for control set.

RESULT

1. **Characterization of nanoparticles:**

The UV spectra showed that Silver nanoparticles have absorbed maximum wavelength at 250 nm (Figure 1). The effect of autoclaving on Silver nanoparticles by UV-Vis spectra shows that the wavelength of nanoparticles increases as the size of Silver nanoparticles decrease.

Silver nanoparticles were characterized by nanoparticles tracking analysis through LM-20. Silver nanoparticles were tracked in the range of 10 to 290 nm wavelength in 1 ml sample. The mean, mode size, concentration and size distribution of Silver nanoparticles were measured. The temperature was $22^{0}C$ and viscosity was 0.95 cP. (Table 2).

DISCUSSION

The lower concentration ($16X10^{3}$) of Silver nanoparticles showed contamination on callus culture. However, higher

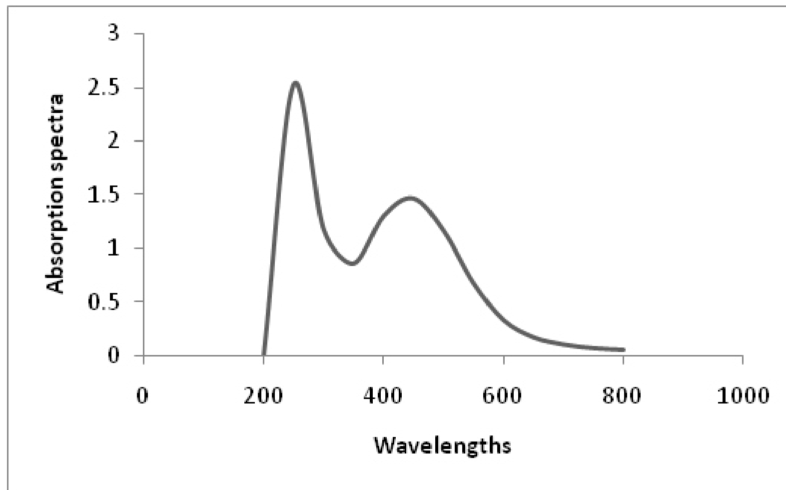


Figure 1: UV-Vis absorption spectra of Silver nanoparticles at various wavelengths.

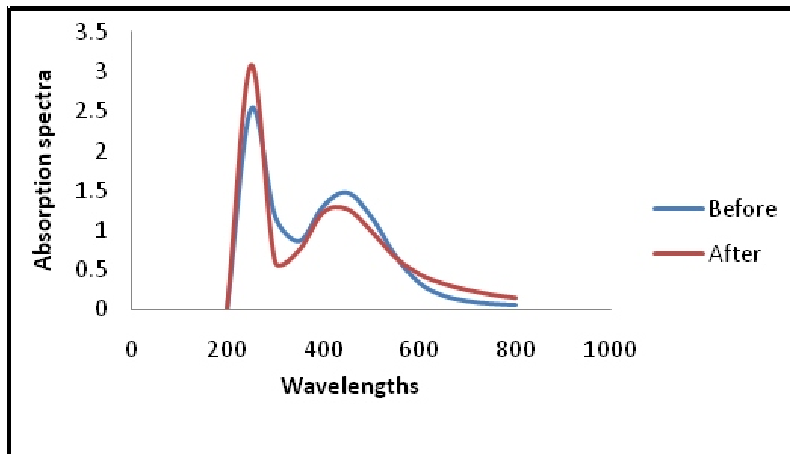


Figure 2: Effect of autoclaving on Silver nanoparticles characterized through UV-Vis absorption spectra at various wavelengths

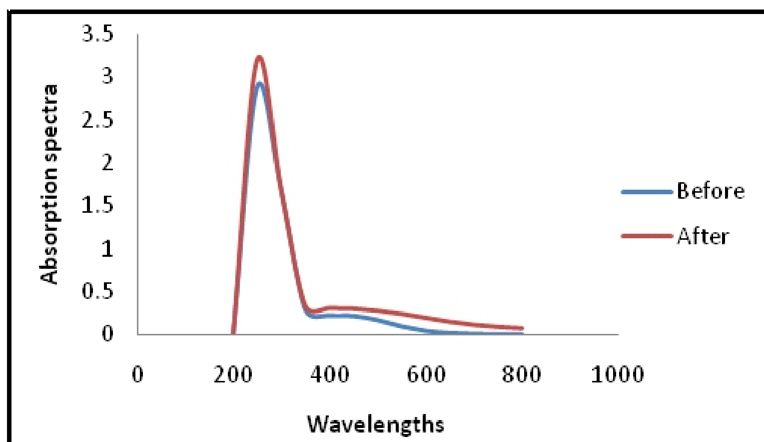


Figure 3: Effect of autoclaving on Silver nanoparticles (within MS medium) characterized through UV-Vis absorption spectra at various wavelengths.

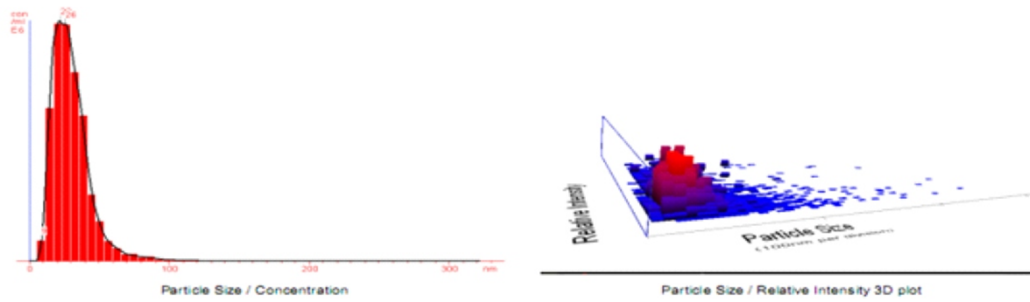


Figure 4: Characterization of Silver nanoparticles through LM-20

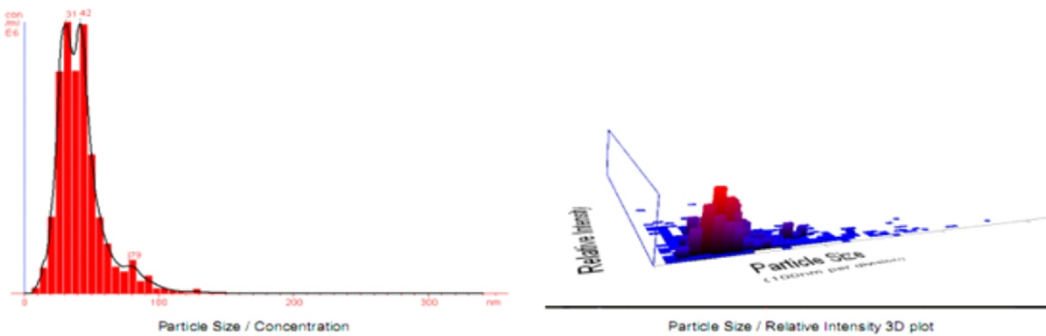


Figure 5: Characterization of Silver nanoparticles within MS media through LM-20 (Before autoclaving)

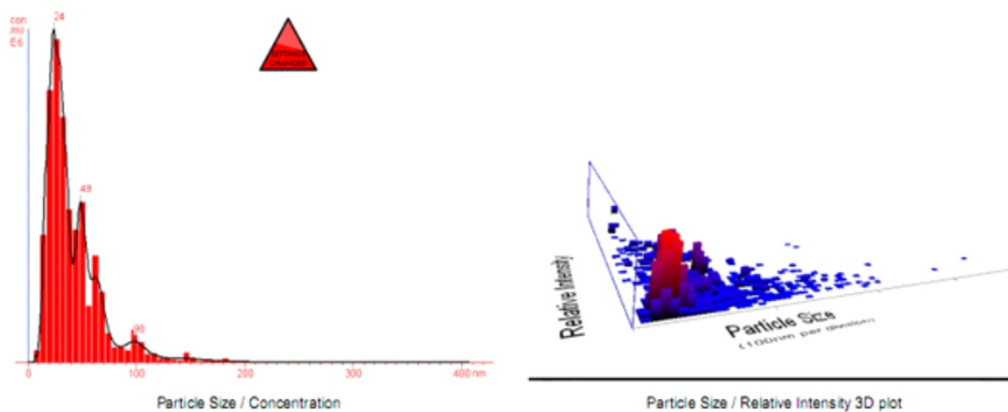


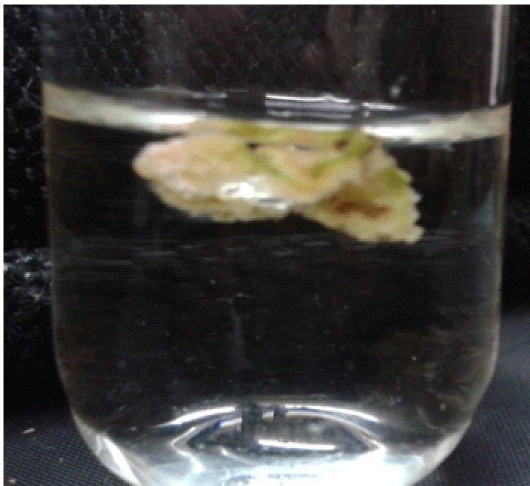
Figure 5: Characterization of Silver nanoparticles within MS media through LM-20 (After autoclaving)

concentration ($16X10^5$ and $16X10^{10}$) have not shown any contamination. (Table 1).

The NTA through LM-20 for Silver nanoparticles before autoclaving showed that the mean, mode, concentration and size distribution were 31 nm, 22 nm, 31 E^6 particles/ml and 290 nm respectively while, after autoclaving these values were observed as 27 nm, 21 nm, 15 E^6 particles/ml and 190 nm respectively. The NTA through LM-20 for Silver nanoparticles within media before autoclaving showed that the mean, mode, concentration and size distribution were 43 nm, 42 nm, 14 E^6 particles/ml and 290 nm, respectively, while after autoclaving these values were observed as 33 nm, 29 nm, 16 E^6 particles/ml and 250, respectively. The callus grown on the MS medium containing $16X10^3$ and $16X10^5$ concentrations of Silver nanoparticles showed the medium and minimum growth, respectively. However, no growth was observed in $16X10^{10}$ concentrations. The color of callus was not changed in $16X10^3$ concentrations. However, it was changed from green to light brown in $16X10^5$ concentrations and from green to dark brown in $16X10^{10}$ concentrations. The lower concentration ($16X10^3$) of Silver nanoparticles incorporated in MS medium was capable for medium growth of callus as that of control and simultaneously reducing the rate of contamination. However, their higher concentrations ($16X10^5$, $16X10^{10}$) have prevented the contamination and inhibited growth completely.

Table 1: Effect of Silver nanoparticles within MS media on growth, color and contamination of callus

Sr. No.	Concentration	Days after inoculation	Response of callus		
			Growth	Colour Change	Contamination
1.	Control	14	Medium growth (++)	No change Green to Green	More Contamination
2.	$16X10^3$	14	Medium growth (++)	No change Green to Green	Less Contamination
3.	$16X10^5$	14	Minimum growth (+)	Change Green to Light Brown	No contamination
4.	$16X10^{10}$	14	No growth	Change Green to Brown	No contamination



(a) Callus growth after 14 days (without Silver nanoparticles)

(b) Callus growth after 14 days (with Silver nanoparticles, dilution 103)

(c) Callus growth after 14 days (with Silver nanoparticles, dilution 10^5)

(d) Callus growth after 14 days (with Silver nanoparticles, dilution 10^{10})

CONCLUSION

The present investigator has observed the effect of Silver nanoparticles incorporated in the MS medium for callus induction in *Bacopa monnieri*. The growth of callus was seen at the lowest concentration of Silver nanoparticles i.e. $16X10^{3}$. Whereas in $16X10^{5}$ and $16X10^{10}$ concentrations the callus has not showed any positive response. Initially the color of shoot was green. However, it was changed subsequently to brown in $16X10^{10}$ dilutions and remained same for longer duration.

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