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Cytogenetic Variability Analysis for *Allium cepa* L in Response to Green Synthesis Silver Nanoparticles

Rania Salah Abd-elhamed
Department of Botany, Faculty of Science, Zagazig University, Egypt and Biology Department, Jazan University, Kingdom of Saudi Arabia (Current Address).
*Corresponding author: *araniasalah@yahoo.com*

Abstract

The present study was carried out on *Allium cepa* root tip to evaluate the cytoxicity of different concentrations of green synthesis silver nanoparticles (AgNPs). For this the root tips were treated with different concentrations of AgNPs (0.0, 5, 10, 20 and 40mg/L). The percentage of mitotic index was found to be significantly decreased as the concentration of the AgNPs increased except in 5mg/L concentration, where the mitotic index is higher thancontrol. The chromosomal abnormalities were also estimated. AgNPs induced a wide range of mitotic disturbances in the *Allium* root tips when compared to control. According to present findings 5mg/L of the AgNPs was found to enhance the rate of cell division when compare to control. On the contrary, higher of the AgNPs showed negative effects on mitotic division in root tip cells of onion. The SDS-PAGE analysis of the protein profile in root samples of AgNPs-treated plants revealed major changes than control. This experiment showed that the changes in protein profile may interrelated chromosomal abnormalities leading to mitotic arrest.

Keywords: nanoparticles; cytoxicity; mitotic index; chromosomal abnormalities; protein profile.

1. Introduction

Nanotechnology is an important field of modern research dealing with design, synthesis, and manipulation of particle structures ranging from approximately 1-100 nm. Nanoparticles (NPs) have wide range of applications in areas such as health care, cosmetics, food and feed, environmental health, mechanics, optics, biomedical sciences, chemical industries, electronics, space industries, drug-gene delivery, energy science, optoelectronics, catalysis, single electron transistors, light emitters, nonlinear optical devices, and photo-electrochemical applications (1, 2, 3).

Nanobiotechnology is a rapidly growing scientific *field* of producing and constructing devices. An important area of research in nanobiotechnology is the synthesis of NPs with different chemical compositions, sizes and morphologies, and controlled dispersities. Nanobiotechnology has turned up as an elementary division of contemporary nanotechnology and untied novel epoch in the fields of material science receiving global attention due to its ample applications. It is a multidisciplinary approach resulting from the investigational use of NPs in biological systems including the disciplines of biology, biochemistry, chemistry, engineering, physics and medicine. Moreover, the nanobiotechnology also serves as an imperative technique in the development of clean, nontoxic, and eco-friendly procedures for the synthesis and congregation of metal NPs having the intrinsic ability to reduce metals by specific metabolic pathways (4, 5). At some point, it is assumed that all

the nanoparticles (NPs) in different items will end up in the soil, air or water (6). Even though some studies have reported the effects of nAg in the environment (7), their toxicity on crop plants is not yet well understood. Previous investigations indicate that the effects of nAg on seed germination vary with NP characteristics and plant species. Kumari et al. (8) reported that nAg decrease the mitotic index and cause multiple chromosomal breaks and cell disintegration in onion (*Allium cepa*). Stampoulis et al. (9) found that nAg at 500 and 100mg/L reduced plant biomass and transpiration in zucchini *(Cucurbita pepo*) by 57 and 41%, respectively. In another experiment, *C. pepo* sp. *Ovifera* was exposed in hydroponics to 0, 100, and 500mg nAg/L and corresponding bulk Ag(Ag powder). Musante and White (10) showed more negative effects in plants exposed to nAg than bulk Ag. Moreover, it was reported that biosynthesized AgNPs showed a significant effect on seed germination of *Bacopa monnieri* and induced the synthesis of protein and carbohydrate and decreased the total phenol contents and catalase and peroxidase activities (11).

The development of green methods for the synthesis of nanoparticles is evolving into an important branch of nanotechnology, because these methods are considered safe and ecologically sound the nanomaterials fabrication as an alternative to conventional methods (12, 13, 14).Sometimes the synthesis of nanoparticles using plants or parts of plants can prove advantageous over other biological processes.

Therefore, in the present study an attempt has been made to evaluate the cytotoxic efficacy of green silver nanoparticles (AgNPs) prepared using leaves extract of *Solanum nigrum*. Moreover, *Allium cepa* root tip was employed as a model species to investigate the mitotic index; chromosomal abnormalities and protein profile in response to AgNPs.

2. Materials and Methods

2.1. Preparation of the leaf extract

Fresh and healthy leaves of *Solanum nigrum* L were collected locally and rinsed thoroughly first with tap water followed by distilled water to remove all the dust and unwanted visible particles, small pieces were cut and dried at room temperature. About 10 g of these finely incised leaves of the plant were weighed separately and transferred into 250 mL beakers containing 100 mL distilled water and boiled for about 20 min. The extracts were then filtered thrice through Whatman No. 1 filter paper to remove particulate matter and to get clear solutions which were then refrigerated (4°C) in 250 mL Erlenmeyer flasks for further experiments. In each and every steps of the experiment, sterility conditions were maintained for the effectiveness and accuracy in results without contamination.

2.2 Green silver nanoparticles (Ag NPs) synthesis

One millimole aqueous solution of silver nitrate ($AgNO_3$) was prepared and aqueous extract of leaf of *Solanum nigrum* used for the synthesis of silver nanoparticles according to (15). 10 mL of leaf extract was added into 90 mL of aqueous solution of 1 mM silver nitrate for reduction into Ag^+ ions and kept at room temperature for 5 h.(Fig. 1). In the meantime, the colour change of the mixture from faint light to yellowish brown to reddish brown to colloidal brown was monitored periodically (time and colour change were recorded along with periodic sampling and scanning by UV-visible spectrophotometry) for maximum 30 min. The reactions were carried out in darkness (to avoid photoactivation of $AgNO_3$ at room temperature. Suitable controls were maintained all through the conduction of experiments. Complete reduction of $AgNO_3$ to Ag^+ ions was confirmed by the change in colour from colourless to colloidal brown. After irradiation, the dilute colloidal solution was cooled to room temperature and kept aside for 24 h for complete bioreduction and saturation denoted by UV-visible spectrophotometric scanning. Then, the colloidal mixture was sealed and stored properly for future use. The formation of Ag NPs was furthermore confirmed by spectrophotometric analysis. The morphology of AgNPs was examined by means of T. electron microscopy (Fig. 2).

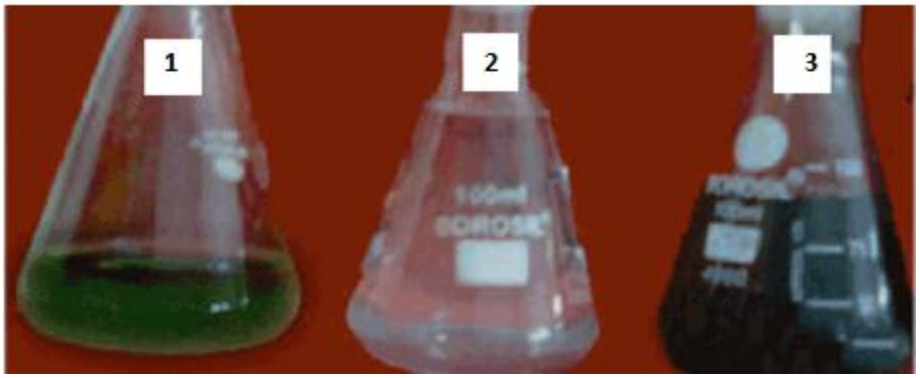


Fig.1: Grean synthesis of nanoparticle using silver nitrate and leaf extract of *Solanum nigrum* 1: Leaf extract, 2: 1 mM $AgNO_3$ without leaf extract and 3: 1 mM $AgNO_3$ and 10% leaf extract after five h

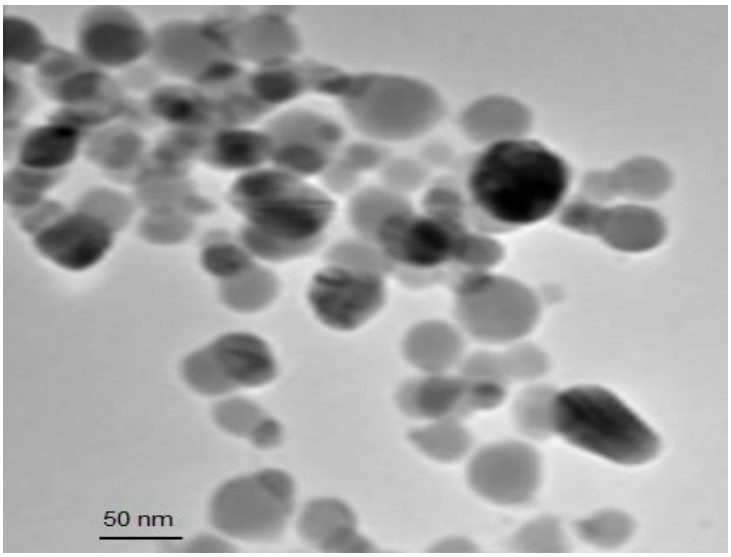


Fig. 2.Silver nanoparticles (AgNPs) under TEM.

2.3. The *Allium* test

Twenty *A. cepa* bulbs of equal size were divided into five groups (4 bulbs in each group, the last group being the control). The bulbs were then rooted for 96 h then the root tips were treated with different concentrations of silver nanoparticles (0.0, 5, 10, 20 and 40 mg/L) for 12 h. Five root tips from each bulb were harvested and fixed in ethanol; glacial acetic acid (3:1) and slides for microscopic studies were prepared from these fixed rot-tips using the acero-orcein squash technique. Another roots sample from control and treated bulbs were taken for protein measure.

The number of cells at division phase, abnormal cells and chromosomal aberrations were noted in each concentration and mitotic index was calculated using the formula:

$$MI = \frac{\text{Total number of cells in division}}{\text{Total number of cells observed}} X100$$

2.4. Protein electrophoresis (SDS-PAGE)

Total protein was extracted from the control and treated bulbs of onion plants. Then, it was ground to fine powder with pestle and mortar. Ten mg of powered flour was homogenized thoroughly with 400 µl extraction buffer using vortex. The extraction buffer was prepared by dissolving 0.6 g Tris base, 0.2 g Sodium Dodecyl Sulfate (SDS) and 30 g of urea in 50 ml of double distilled water. One ml of β-mercaptoethanol was added and then the solution was diluted to 100 ml with double distilled water. The mix was kept overnight at 4°C and then centrifuged at 13000 rpm for 10 minutes at room temperature (Sigma 3K 18 Bench Top centrifuge).

For qualitative analysis of protein, 20 µl of the extracted protein was boiled in a water bath for 3-5 min and loaded on Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) containing 12.5% resolving gel and 4% stacking gel **(16**) using 3 µl bromophenol blue as tracking dye. The samples were then loaded in equal amounts (15 µl) and a molecular weight marker standard (5 µl) (Prism Ultra Protein Ladder) were loaded at on to each well. Electrophoresis was carried out at 150 V and 25 mA until tracking dye reached to the bottom of the gel. The gels were distained in solvent composed of 40 ml of methanol, 10 ml glacial acetic acid and 50 ml distilled water. The gel was stained overnight in 25 ml of Coomassie Brilliant Blue (R-250) staining buffer. The gels were photographed and the molecular weights of the polypeptide bands were estimated by correlating position of the molecular weight marker standards.

2.5. Statistical analysis

The data was subjected to statistical analysis using SPSS package (ANOVA) Ver.16 according Tukey's HSD significant test at 5% level.

3. Results and Discussion

Mitotic index is a best biomonitor to assess the effects of various chemicals (nanoparticles) on cell division (17). Moreover, the A. cepa test system provides important information to evaluate action mechanisms of an agent about its effects on the genetic material. The mitotic index was observed for more than 2000 cells. The silver nanoparticles a wide range of mitotic disturbances in the *Allium* root tips when compared to control. It was found that the mitotic index decreased with the increasing concentration of the nanopartles samples except at low doses 5 and 10mg/L when compared to control (10.63) and had a significant decrease in mitotic index in high doses, 20 and 40mg/L (Table 1). These findings are in agreement with those observed after treatments of *Allium cepa* root tip cells with silver nanoparticles (18, 19).

Table 1: Effect of different concentration of AgNPs on mitotic index.

AgNPs (mg/L)	**Total no. of Cells**	**No. of dividing Cells**	**Mitotic Index**
Control	4921	520	10.63 ± 0.36^{c}
5	5144	594	11.36 ± 0.35^{a}
10	4834	503	10.24 ± 0.06^{c}
20	4564	421	09.25 ± 0.04^{d}
40	3936	298	07.71 ± 0.15^{d}

Mean ± SE

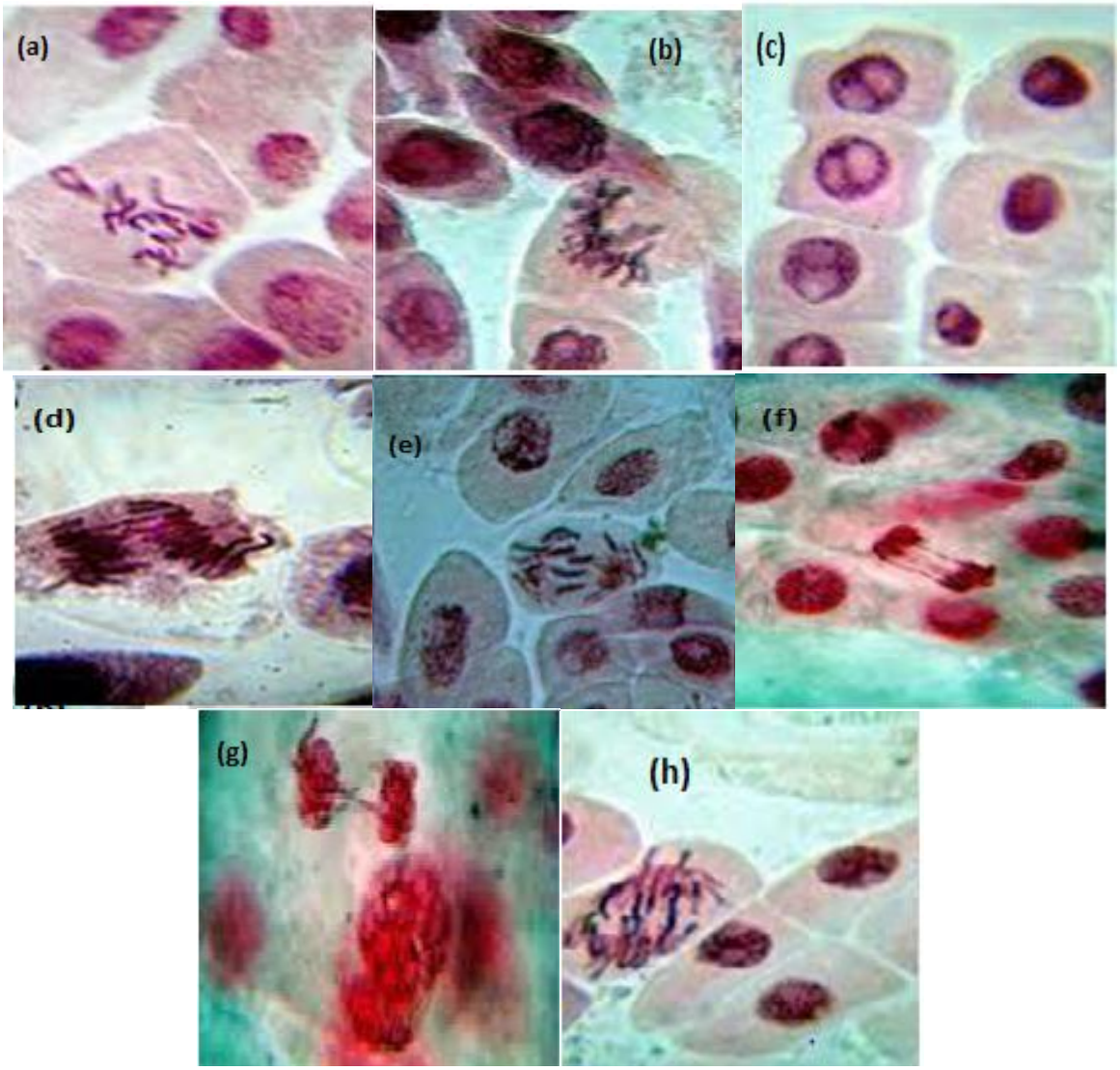
The present study showed that the chromosomal aberrations were observed in all the concentrations except control. Chromosomal aberration showed a maximum at 40mg/l AgNPs test solution when compared to the others and the lowest value was recorded at 5mg/AgNPs (Table.2). The observed chromosomal abnormalities include precautious movements, bridge formation, laggards, late anaphase, sticking of chromosomes, multinucleolated condition and denatured cells (Fig. 3-a-h).Our results generally were in similar with those observed by many investigators (20, 21).

The high frequency of chromosomal aberrations reflects that AgNPs act primarily on mitotic spindle, which results in disorientations of chromosomes at various stages of cell cycle. Chromosome bridges is either due to chromosome stickiness producing abnormal anaphase separation or may be attribute to unequal translocation or inversion of chromosome segments and also due to breakage and fusion of chromosome or chromatids (22). Among the aberrations observed, chromosomal bridge formation was found to be the most frequent change effected in all concentrations other than the control. The of high frequency of chromosomal aberrations reflects that AgNPs act primarily on mitotic spindle, which results in disorientations of chromosomes at various stages of cell cycle. The present study also showed that the percentage of number of abnormalities per cell was also increased in all the treatment concentrations (Table 2). It can be explained by the fact that the highest concentration (40mg/L) drastically diminished the existence of dividing cells as the cells became arrested at interphase stage, most probably due to interphase chromatin condensation, which was evidenced by the presence of highly condensed state of nucleus as compared to the control (Fig 4). The results of the present investigation suggest the phototoxicity of nanoparticles may be attributed to

its extremely small size and large surface to volume ratio, which lead to both chemical and physical differences in their properties. It is interested to mention that, the nano-metals induced chromosomal abnormalities directly correlate its cytogenotoxicity. It was suggested that silver nanoparticles are the most abundant substances which can inhibit root growth and cell division, thus causing growth retardation, multiple chromosomal breaks and finally cell disintegration (20, 23).

Table2: Effect of different concentrations of AgNPs **on types of aberrations observed in *Allium cepa* root tips.**

Chromosomal aberrations	Concentration of AgNPs (mg/L.)				Control
	5	10	20	40	
Early movements	1	1	3	2	-
Bridge formation	2	6	8	9	-
Laggards	2	2	2	3	-
Sticking of chromosomes	1	1	1	3	-
Multinucleolated condition	5	3	5	8	-
Total No. of aberrations	11	13	19	25	-



Figs. 3 (a-h):Showing abnormalities induced by AgNPs in roottip cells of *Allium cepa*.
a. Early movement of chromosomes,b. Indirect orientation of metaphase chromosomes, c. Multinucleolate condition, d. Anaphase with bridges,e. Lagging chromosomes, f. Clumping with bridge formation, g. Late anaphase and h. Multibrige formation.

The SDS-PAGE analysis of the protein profile in root samples of AgNPs-treated plants showed great variations than control. In response to AgNPs, the treated (5 and 10 mg/L) roots showed two new polypeptides (147.8 and 131.6 kDa, respectively). Peptide like 75.4 kDa expressed slightly in 10 mg/L of AgNPs treatment and 26.9 kDa of protein totally disappeared from all treated plants. Similar results were obtained in higher concentration that, a group of polypeptides were completely disappeared in 20 and 40 mg/L of treatment (Fig. 4). Through previous analyzes in the study, it is clear that the disappearance and reappearance of some proteins and de novo synthesis of others in response to AgNPs exposure indicated a direct relationship of nanoparticles induced proteomics. Similar observations were also reported by Ewais et al, (14) under AgNPs in *Solanum nigram* plants. Since proteins were newly synthesized under nanoparticles, it appears to have a role in the mechanism of nanometals tolerance which allows making biochemical and structural adjustments that enable the plant to cope with stress conditions. Since proteins were newly synthesized under nanoparticles, it appears to have a role in the mechanism of nanometals tolerance which allows making biochemical and structural adjustments that enable the plant to cope with stress conditions.Our results generally were in similar with those observed by other investigators Abdel-Azeem EA and Elsayed(17) who reported that engineered AgNPs induce a progressive decrease in nucleic acids as well as the total soluble protein contents. In this respect, it has been reported that silver engineered particles (SEPs) have the ability to damage the genetic material, since the engineered nanoparticles ENPs are able to cross cell membranes and reach the cellular nucleus causing DNA damage (24).

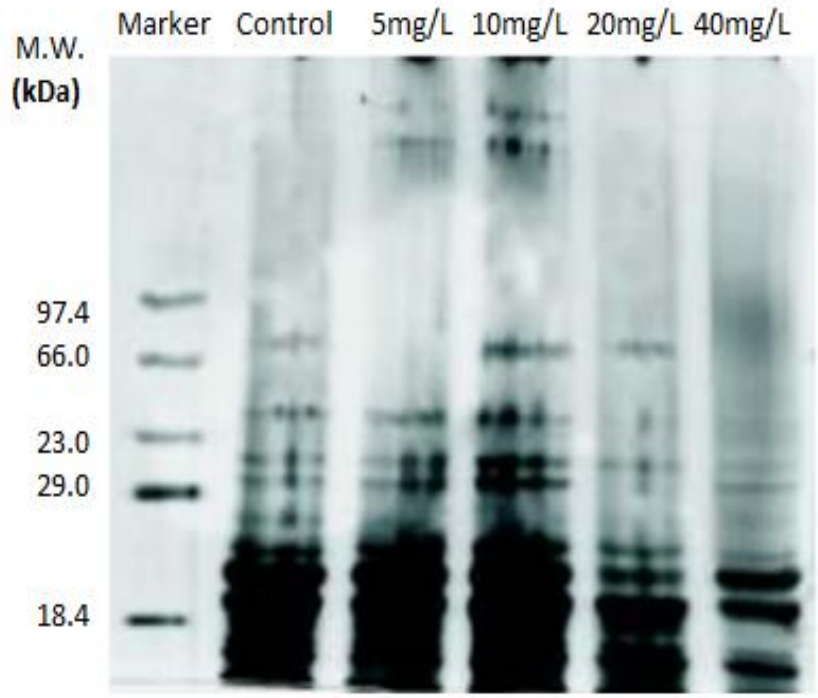


Fig. 4. Effect of different concentrations of AgNPs (5, 10, 20 and 40mg/L) on protein profile in root samples of onion.

4. Conclusion

The current study showed that silver nanoparticles (AgNPs) induced cytotoxic and genotoxic effects on *Allium cepa* root tip cells. The treatment of root tips in of different concentrations of AgNPs showed a gradual decrease in mitotic index, with increasing concentration of the nanoparticles. This antimitotic effect may be due to the arrest of cell division due to changes at in the chromosomal morphology or spindle orientation. A thorough screening of the chromosomal abnormalities showed that the total number of abnormal cells increased with increasing concentrations of nanoparticles in a dose dependent manner. There was a good correlation between protein contents and cytological parameters. Nucleolus being the centre of protein synthesis has a strategic role in cell division. Consequently, the changes in protein profile may reflect chromosomal abnormalities leading to mitotic arrest. The present study reveals that the research on NPs, essentiality for plants, is in the beginning; more rigorous works are needed to understand physiological, biochemical, and molecular mechanisms of plants in relation to NPs.

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