# Potential Environmental Pollution: Toxicity of AgNPs to Tobacco BY-2 Cells

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**Abstract**. The widespread use of nanomaterials results in the inevitable release of nanoparticles into the environment, including soil, water and plants, which are the main sinks for those nanoparticles. From the increasing social use of AgNPs, it is essential to understand their potential toxic effects on the environment. The objective of this study was to investigate the effects of AgNPs on the tobacco Bright Yellow-2 suspension-cultured cells. AgNPs exhibited inhibitory growth against BY-2 cells in a dose dependent manner. The specific growth rate (μ) was the first time to be used to explain the proliferation ability of cells in the nanoparticles toxicity studies. Results indicated that CAT seemed to be slightly more susceptible to high levels of oxidative stress comparison to SOD. AgNPs migrated steadily from cell wall to nucleus, which was related to the exposure time of AgNPs. The principal part of this study was focused on the ability of AgNPs to induce the processes of programmed cell death (PCD). Our results may fulfil the explanation of the toxic effect of these nanoparticles on plant cells.

## 1. Introduction

Nanotechnology manipulates matter at the nanoscale (1–100 nm) producing nanoproducts and nanomaterials that can have novel and size-related physicochemical properties differing significantly from those from larger particles. AgNPs have recently gained much popularity owing to distinctive physicochemical properties, including a high electrical and thermal conductivity, surface-enhanced Raman scattering, chemical stability, catalytic activity and non-linear optical behaviour [1-2]. But they may have a negative impact on human health or the environment. AgNPs are known to be potentially toxic to bacteria [3] algae [4] human cells [5], animal cells [6] and plants [7]. The research for toxicity in AgNPs in plant cells is becoming a crucial topic in toxicity studies. The results of Mazumdar indicated that AgNPs (25 nm) can penetrate through Oryza sativa root cells by damaging the cell wall as well as vacuoles [8]. AgNPs exhibited similar biological effects in causing lesser extent of cytotoxicity and greater extent of genotoxicity in the model of onion [9]. In this study, we sought to determine the toxicity of AgNPs in the plant cell model BY-2. Cells can be protected against the oxidative damage by a broad spectrum of radical-scavenger systems including antioxidant enzymes and a number of biologically active substances such as glutathione, carotenoids, ascorbate and phytochelatins that may prevent the free radical induced cellular damage [10]. Studies by He et al. [11] demonstrated that activities of SOD and CAT increased by 1.45 folds and 1.56 folds respectively, to protect tilapia Oreochromis niloticus

from short-term low temperature stress. Similar results were observed in Microcystis aeruginosa under linoleic acid (LA) stress [12].

In this study, we focuse on growth parameters of the plant cell model BY-2 suspension cells treated with AgNPs. To better evaluate cells proliferation exposed to nanoparticles, a common kinetic parameter named specific growth rate (μ) is referred in this study according to researches of microorganisms. To the best of our knowledge, there is no research involved the specific growth rate (μ) in nanoparticle toxicity studies. The dynamic changes of SOD and CAT and the pathway of AgNPs in cells have been also investigated. The principal goal of the work is to demonstrate the potential of AgNPs to induce programmed cell death in tobacco BY-2 cells. This study prompts us to further explore the oxidative response and potential mechanisms of cytotoxicity of nanoparticles in different cell lines.

## 2. Material and methods

### *2.1. Materials*

AgNPs was prepared according to the method from Wei et al. [13]. The silver particles show a narrow particle size distribution with a diameter range from 8 to 10 nm, as revealed by AFM (Fig. A.1.) and TEM (Fig. A.2.) (data is shown in the supporting information). Tobacco BY-2 cells are a gift of Prof. Zhang from laboratory of plant physiology in Shanghai University. Trypan blue dye was procured from Sigma Chemical Company. SOD and CAT assay kit were obtained from Nanjing Jiancheng Bioengineering Institute. All other chemical reagents are of analytical or higher grades, which are bought from traditional Chinese medicine company.

### *2.2. Effect of AgNPs on the proliferation and mortality in tobacco BY-2 cells*

Under constant shaking (150 rpm) at 27℃ in the dark in 250 ml Erlenmeyer flasks. The pH of the cultivation media was adjusted to 5.8 with KOH. AgNPs were added when cells were in the beginning of exponential growth phase, to create finial concentrations of 0, 1, 2, 3, 4 and 5 mg/L. The cell density of suspension culture was determined by using a Spectrophotometer (Bio Photometer, Germany). The specific growth rate (μ) is defined as the increase in cell mass per unit time, which was calculated using the following formula $\mu(h^{-1})$ =production rate of cells (cells $h^{-1}$)/total number of cells.

To assess the cytotoxicity of cells exposed with AgNPs, Trypan blue assay was used as described by Rujanapun et al. [2]. Living cells do not take up the stain and retain their natural color because the stain cannot penetrate beyond the plasma membrane; cells damaged by AgNPs cannot keep the stain from penetrating and thus stain deep blue, readily distinguishable from viable cells upon microscopic examination. Exposed cells were collected and incubated with trypan blue 0.4% for 3 min. The percentage of stained cells (i.e. non-viable cells) was counted using a light microscope (CKX31, Japan) continuously over a 6 days period. Over 500 cells were counted for each treatment. Each experiment was conducted in triplicate and the mean value with standard deviation was reported.

### *2.3. Effect of AgNPs on activities of antioxidant enzymes in tobacco BY-2 cells*

To quantitate cellular oxidative stress induced by AgNPs, cells were collected every day during the culture period, and then used to examine the activities of SOD and CAT, respectively. Cells were made into cell homogenate (20%) with ultrasonication, after that cell homogenate (20%) was centrifuged at 3500 rpm for 10 min, the supernatant was used as crude enzymes to be measured. All reagents were added to the reaction system according to the assay kit. Then 50 μL of crude enzymes was added with a stir by Vortex mixer and in a water bath at 37℃ (for SOD). The plate was incubated for 10 min at room temperature, and the absorbance A550 and A405 was measured for SOD and CAT using a UV-vis spectrophotometer (U-3900H, Japan), respectively. In addition, three independent experiments were performed for each condition.

### *2.4. The migration of AgNPs in tobacco BY-2 cells*

To explore the toxicity mechanism of AgNPs on cells, the migration of AgNPs is verified by using CLSM (LSM710, Germany) according to the method of Shcherbakov et al. [14], which is more simple and intuitive. The observed cells treated with 5 mg/L of AgNPs were selected according to the mortality curves. Cells were centrifuged (5,000 rpm, 10 min), washed with 0.1 mol/L phosphate saline buffer (PBS, pH 7.4) in the culture time interval of 0, 6, 12, 24 h. The conditions used in CLSM were 405 nm for excitation wavelength, 472 nm for emission wavelength, 488Hz for sampling frequency, 5 min for record time and medium for scanning speed. This experiment was performed without exposure to light.

### *2.5. Effects of AgNPs on cytoplasm variations in tobacco BY-2 cells*

Tobacco BY-2 cells treated with 5 mg/L of AgNPs were incubated for 24 h. Cells cultures in the medium without adding AgNPs were taken as the control. The morphological changes of cell cytoplasm were observed under a light microscope (CKX31, Japan) in the culture time intervals of 0, 6, 12, 24 h as described by Huang et al. [15].

### *2.6. Effects of AgNPs on nucleus variations in tobacco BY-2 cells*

Morphological feature of the cell nuclei was studied by staining with DAPI [24], a fluorescent dye that can bind to DNA, which makes cell nuclei blue under the fluorescence microscope. The untreated cells and those exposed to 5 mg/L of AgNPs were incubated for 24 h and centrifuged at 10,000 rpm for 30s. Cells were fixed briefly in 70% (v/v) ethanol, incubated with 50 μmol/L of DAPI in PBS (pH 7.4) at room temperature for 15 min, and then subsequently rinsed twice with PBS. The morphological feature of stained nuclei was observed and photographed using a fluorescence microscope (LBD-65, Germany).

### *2.7. Effects of AgNPs on DNA variations in tobacco BY-2 cells*

To estimate the DNA fragmentation of cells treated with AgNPs, cell DNA was extracted by using a modified CTAB method according to Ghosh et al. [16]. The step was as following: the suspension cells were centrifuged, weighed and frozen dried, and then kept in 4℃ tills to be used. The cells were ground in extraction buffer (100 mM Tris buffer pH 8.0, 25 mM EDTA, 2 M NaCl, 3% CTAB, 3% PVP). After 10 min for incubation at room temperature, the phenol/chloroform/isoamyl alcohol (25:24:1) was added to the suspension cells and the mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was then transferred to a new test tube, to which 9 mL isopropyl alcohol was added. The DNA was precipitated by centrifugation at 10,000 rpm for 10 min. The resulting pellet was washed twice with 75% ethanol and dried under a Hybridization Oven until the edge of DNA was transparent. The isolated DNA was detected in agarose gel electrophoresis with 2% agarose gel after it was dissolved completely in TE buffer (10 mM Tris buffer, pH 8.0, 1 mM EDTA). DNA was stained with EtBr, visualized by exposing the gel to UV light and photographed.

## 3. Results

### *3.1. Effect of AgNPs on cell proliferation and mortality in tobacco BY-2 cells*

A concentration range of 1-5 mg/L was selected to assess the toxicity threshold of AgNPs. AgNPs were introduced at the beginning of exponential growth phase of cells culture (cell density reached about $1.8\times10^5$ cells/mL). As can be seen from Fig. 1, the addition of AgNPs led to distinctive changes in cell growth and mortality. Cell density in all experimental groups increased gradually. The density of cell treated with lower concentrations of AgNPs (1 and 2 mg/L) showed a little inhibition compared to the control, untreated BY-2 cells. However, a dose of higher concentrations of AgNPs (4 and 5 mg/L) induced complete inhibition in cell growth (Fig. 1A).

Fig. 1B showed that the specific growth rate of cells increased steadily with the extension of culture time, which reached a maximum and then dropped down. The μmax obtained from cells treated with 5 mg/L of AgNPs was only 34.60% of the control. The μmax obtained in all experiment variants showed

regular changes, which suggested that the proliferation abilities of cells decreased with the increase of AgNPs concentrations.

In Fig. 1C, the results of the Trypan blue test after cells incubated with AgNPs were shown. No significant difference in cell mortality was detected in the lower concentrations of AgNPs groups (1 and 2 mg/L) compared to the control. Cell mortality reached approximately 40% on 3 mg/L of AgNPs exposure after 24 h. In the case of higher concentrations (4 and 5 mg/L) group, the increase in cell mortality compared to control was well evident, and the cells almost dead within 24 h treatment. The curve of cell mortality was corresponding with cells growth.

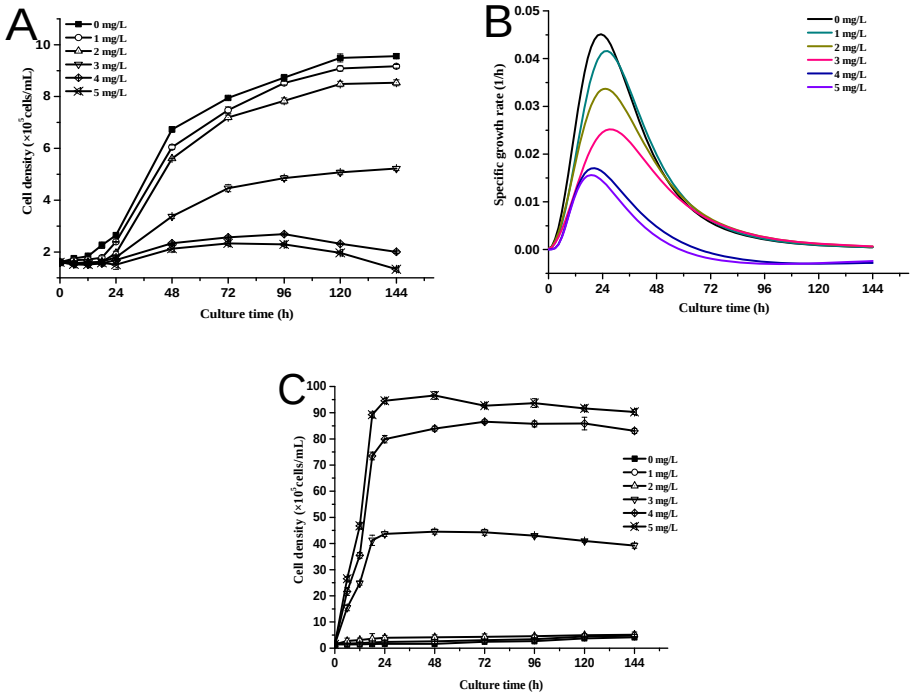


**Fig.1.** Effect of AgNPs on the growth (A), specific growth rate (μ) (B) and mortality (C) of the tobacco BY-2 cells. The data was expressed as mean±standard deviation (S.D.) of three-independent experiments.

### *3.2. Effect of AgNPs on activities of SOD and CAT in tobacco BY-2 cells*

To evaluate the effect of AgNPs on antioxidant enzymes in cells, the levels of SOD and CAT were examined. In the controls, the activities of SOD and CAT showed hardly changed during the culture of BY-2 cells (Fig. 2). However, remarkably increases in levels of SOD and CAT as a response to AgNPs were observed in experimental variants of higher concentrations (3 and 4 mg/L). Compared to the control, the activities of SOD (Fig. 2A) and CAT (Fig. 2B) increased from 47.46 and 11.44 U/mg. prot to 141.94 and 100.41 U/mg.prot at 48 h with 5 mg/L AgNPs treatment, respectively. Cells incubated with lower concentrations of AgNPs (1 and 2 mg/L) had a small but significant increase in activities of SOD compared to CAT. Noteworthy, Levels of SOD and CAT showed a tendency to decrease after 48 h of cultivation, which was correspond with the mortality curves (Fig. 1C).

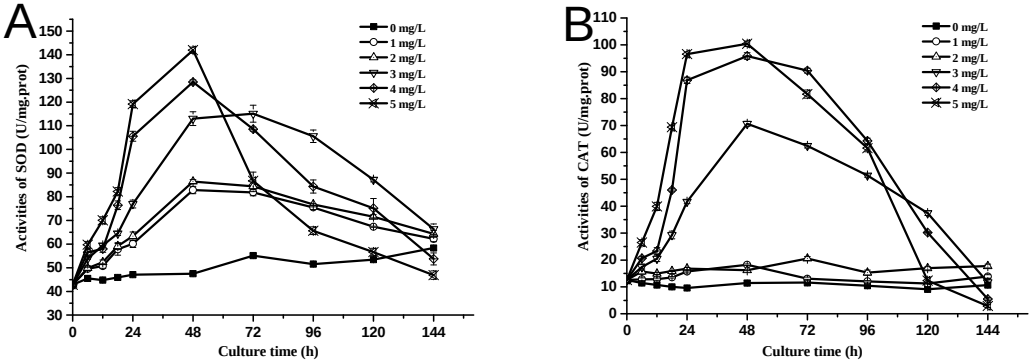


**Fig.2.** Activities of SOD (A) and CAT (B) in tobacco BY-2 cells with AgNPs treatment. The cells were treated in different days post AgNPs (0, 1, 2, 3, 4, 5 mg/L) treatment. The data's were expressed as mean±standard deviation (S.D.) of three-independent experiments.

*3.3. The migration of AgNPs in tobacco BY-2 cells*

According to the mortality curves, cells exposed to 5 mg/L of AgNPs were almost dead in 24 h (Fig. 1C), so in order to better understand the mechanisms of cytotoxicity of AgNPs in cells, migration of AgNPs in tobacco BY-2 cells was traced in the culture time intervals of 0, 6, 12 and 24 h.

CLSM is an important technique in the biomedical sciences to image in situ cellular material to micro- and nanoscale resolution [17]. The excitation (405 nm) and emission wavelength (472 nm) used in CLSM has been investigated by Fluorescence Spectrophotometer (data not shown). AgNPs attached on the surface of cells subsequently with the addition of AgNPs in cell cultures (Fig. 3A). After 6 h treatment, cells had taken up several silver nanoparticles, which typically accumulated in cytoplasm (Fig. 3B). Upon exposure to AgNPs for 12 h treatment, a massive AgNPs internalization was observed, causing significant changes in cellular morphology that can be obviously observed in Fig. 3C. Serious plasmolysis was occured in cells after 24 h, and few bright green spots were observed inside cells, indicating less nanoparticles internalization (Fig. 3D). Interestingly, AgNPs migrated steadily from cell wall to nucleus, which was correlated with the exposure time of AgNPs.

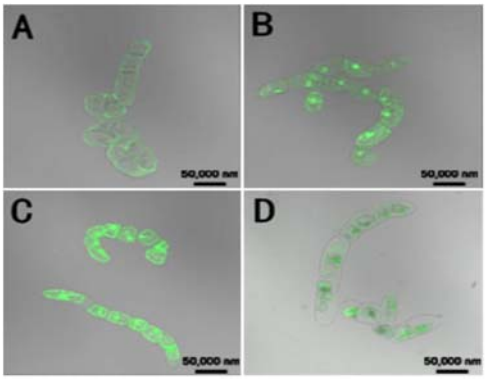


**Fig.3.** The migration of AgNPs in tobacco BY-2 cells, with exposure time of 0 h (A), 6 h (B), 12 h (C), 24 h treatment (D). The magnification factor was 10×20. The green fluorescence was imaged by excitation (405 nm) and emission wavelength (472 nm).

*3.4. Effects of AgNPs on cytoplasm variations in tobacco BY-2 cells*

To examine the variation of cytoplasm in the migration process of AgNPs in cells, cells were collected from experimental plates at 0, 6, 12, 24 h following 5 mg/L AgNPs exposures. The results were showed in Fig. 4. The variations of cytoplasm were correlated with the exposure time of AgNPs. The untreated

cells showed engorged and intact cytoplasm with nearly round nuclei. The first morphological apoptotic hallmarks consisting of high levels of cytoplasm denser and cell shrinkage were observed in samples obtained within the first 6 h (Fig. 4B)., The shrinkage of cell protoplast was obviously observed with formation of stretched nuclei from 6 h to 12 h (Fig. 4B and 4C). Upon exposure to AgNPs for 24 h, the nucleus become fuzzy gradually, cytoplasm condensed in the central of cells which was one of the phenotypic alterations that were related to apoptosis (Fig. 4D).

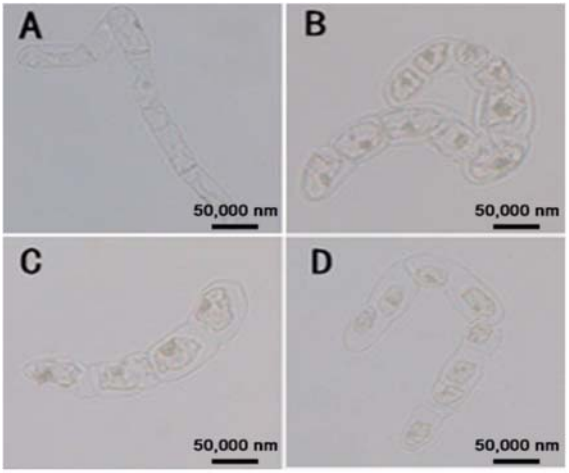


**Fig.4.** Tobacco BY-2 cells visualized under light microscopy after treatment with AgNPs (10×20). Cells were treated with AgNPs for 0 h (A), 6 h (B), 12 h (C) and 24 h treatment (D), respectively.

*3.5. Effects of AgNPs on nucleus variations in tobacco BY-2 cells*

For the evaluation of nuclear morphology in cells treated with AgNPs, DAPI staining was performed. Nuclei in untreated cells were round with very high and uniform electronic density (Fig. 5A). In the 5 mg/L AgNPs group, the nucleus became diffusing, chromatin became condensed and migrated to the edge of the nucleus in the first 6 h (Fig. 5B). At 12 h post AgNPs treatment, chromatin fragments were partly observed in the nucleus partly and the nuclear membrane was distorted (Fig. 5C). The chromatin in the nucleus became a mass of irregular fragments and the morphological of nucleus has been largely cleft in places, which indicated nucleus dissolution within 24 h post AgNPs treatment (Fig. 5D). All variations of cytoplasm (Fig. 4) and nucleus in cells were corresponded with the location of AgNPs (Fig. 3)

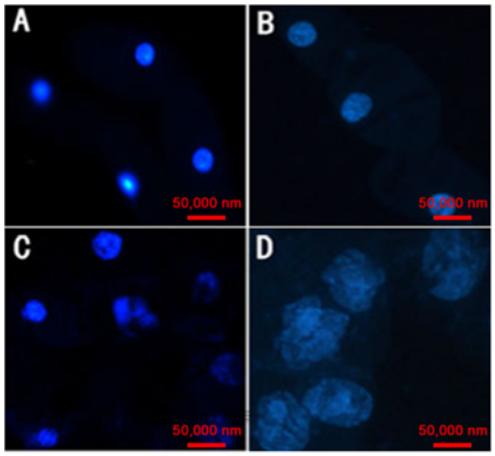


**Fig.5.** Nucleus morphology of BY-2 cells using DAPI staining after treatment with AgNPs(10×20)for 0 h (A), 6 h (B), 12 h (C) and 24 h treatment (D) respectively.

### *3.6. Effects of AgNPs on DNA variations in tobacco BY-2 cells*

Genomic DNA fragmentation to nucleosomal fragments is an integral part of apoptosis for plant systems and serves as a reliable indicator for apoptosis [18]. The DNA profiles of the cells treated with AgNPs (5 mg/L) for 24 h and untreated cells were analysed. The result was shown in Fig. 6. While the negative control set showed a thick band on the agarose gel (lane 1 and 2, about 5 kbp), which indicated that genomic DNA was undamaged. Cells treated with AgNPs exhibited extensive breaks. Light and shade fragments were observed obviously, which may be due to the AgNPs interactions with the purine and pyramidine groups of DNAs, thereby yielding a ladder appearance (lane 4 and 5) confirming the apoptosis. It can be speculated that DNA laddering detection coincided in time with the appearance of the highest quantity of nuclei with apoptotic chromatin morphology (Fig. 5).

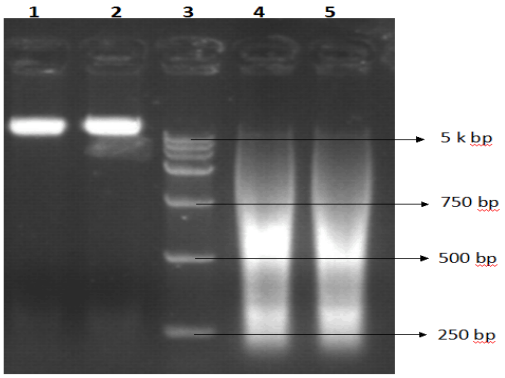


**Fig.6.** Analysis of genomic DNA fragmentation after 24 h AgNPs treatment. Lane 3, Standard DNA markers; lane 1 and lane 2 (parallel groups), untreated control BY-2 cells; lane 4 and lane 5 (parallel groups), AgNPs (5 mg/L) treated BY-2 cells.

## 4. Discussion

The results showed that the maximum specific growth rates were cross to concentrations of AgNPs. We also observed reduced growth in all experimental groups compared to the control (Fig. 1A).

Our results showed that SOD and CAT activities increased significantly under higher concentrations of AgNPs (4 and 5 mg/L) stress in the time of 48 h but decreased to the level of the control or even less during long-term exposure, which may have suggested that the ROS was increased under AgNPs stress and the collapse of defense system registered as a decrease in SOD and CAT activities. The maximum activities of SOD and CAT increased by 1.82 folds and 1.41 folds compared to the control with 2 mg/L AgNPs treatment, respectively, which ran counter to the groups of higher AgNPs concentrations (increased by 2.99 folds and 8.78 folds with 5 mg/L AgNPs treatment, respectively). The results suggested that CAT was more sensitive than SOD to high levels of oxidative stress in the tobacco BY-2 cells exposure to AgNPs.

Our results showed that AgNPs migrated gradually from cell wall to nucleus in tobacco BY-2 cells, which was correlated with the exposure time of AgNPs (Fig. 3). Similar results were observed in the adsorptions of AgNPs on *Oryza sativa* root cells. Studies of AgNPs on C26 colon carcinoma cells illustrated that AgNPs can not only transferred into cells but also persisted the SERS signal related to molecular vibrations of biogenic compounds [19]. The migration of AgNPs in cells can provide sufficient evidence for the toxicity of AgNPs.

The results of staining with Trypan blue showed that the high-concentration groups were lethal to cells (Fig. 1C), while it cannot distinguish the mechanisms of cell death. Light microscopy revealed that death of cells was accompanied by cell shrinkage away from the cell wall (Fig. 4). Fig. 5 showed that the most striking morphological changes were the fragmented nucleus. These typical ultrastructural changes were evidently compatible with cell apoptosis and similar to the changes observed in cells treated with inducers of plant cell apoptosis such as cadmium11 and aluminium oxide nanoparticles [20].

Many engineered NPs including AgNPs have been found to cause genotoxic effects. DNA fragments were observed in tobacco BY-2 cells with AgNPs treatment, which was correspond with the results of Asare et al. [21], who presented that AgNPs appeared to cause DNA-strand breaks in NT2 cells.

## 5. Conclusion

This work presents a new knowledge about the systematic effects of AgNPs on a model plant cell, tobacco BY-2 cells of a suspension culture. The maximum specific growth rates excellently illustrated that the proliferation abilities of cells decreased with the increase of AgNPs concentrations. More important is that CAT seems to be slightly more susceptible to high levels of oxidative stress in comparasion to SOD. AgNPs migrated gradually from cell wall to nucleus in tobacco BY-2 cells, which was correlated with the exposure time of AgNPs. We give sufficient evidence (shrinkage of cytoplasm, changes in the nuclear architecture, fragmentation of the DNA) to confirm that tobacco BY-2 cells exposed to AgNPs died through apoptosis. The results also suggest the need of a more detailed research for the assessment of nanoparticles toxicity.

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