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Research article

Taxanes content and cytotoxicity of hazel cells extract after elicitation with silver nanoparticles☆

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ABSTRACT

The toxicity of silver nanoparticles (AgNPs) has been attributed to the generation of Ag^+ ions as well as production of ROS. The latter can elicit defensive response of plant cells in different ways e.g., enhancement of secondary metabolite productions. In the present study this hypothesis was evaluated by assessment of taxanes production by suspension-cultured hazel (*Corylus avellana* L.) cells after treatment with AgNPs. The cells were treated with different concentrations of AgNPs (0, 2.5, 5, and 10 ppm), in their logarithmic growth phase (d7) and were harvested after 1 weak. The growth of cells and their membrane integrity decreased but extracellular electro conductivity and total dissolved solids increase by AgNPs (probably due to loosening of cell membrane). Treatment of hazel cells with AgNPs (in particular of 5 ppm) rapidly and remarkably increased the yields of two major taxanes, i.e., Taxol and baccatin III; so that 24 h of the treatment their contents reached to 378% and 163% of the control, respectively. Increase of Taxanes was accompanied by the increase of total soluble phenols. The extracts of AgNPs-treated cells were able to inhibit the growth of cancerous HeLa cells and reduce their viability to 60% of the control. The results suggest the elicitation of suspension-cultured hazel cells with AgNPs as a procedure for rapid enhancement of anticancer taxanes biosynthesis by the cells.

1. Introduction

The use of nanoparticles is gaining impetus as they posse large surface area to volume, resulting in defined chemical, optical and mechanical and remarkable antibacterial properties (Rai et al., 2009). AgNPs are one of the most commonly used in the field of nanoparticles (Oukarroum et al., 2012). The most application of AgNPs in biology and medicine comes from its antimicrobial properties (Radzig et al., 2013). As a new approach, application of AgNPs on intact plants or cell cultures is growing. For example due to their effects on the metabolism, respiration and reproduction of microorganism, AgNPs are suggested to extend maintenance period of cut flowers and leaves (Lok et al., 2006). It has been shown that very low concentrations of AgNPs (<1 ppm) could be toxic to seedlings of *Arabidopsis thaliana* (Ma et al., 2010). AgNPs of 20 nm–80 nm clearly stunted the growth and their phytotoxicity was concentration and particle size dependent. Moreover, the root tip were turned light brown and this was attributed to the adsorption of AgNPs either itself or in conjunction with cell wall materials or secondary metabolites produced by root tips, although the exact mechanisms are yet to be elucidated (Ma et al., 2010). Krishnaraj et al. (2012) found that biologically synthesized AgNPs exerted a slight stress condition on the growth and metabolism of a rapid proliferating species of wetland ecosystem, *Bacopa monnieri* L. They assumed that the toxic effects induced by AgNPs to the plant systems might be due to the toxic compounds adsorbed on the surface of nanomaterials. The toxicity of silver nanoparticles (AgNPs) has been attributed to the generation of Ag^+ ions as well as production of ROS. It is not clear to which degree the toxicity of AgNPs results from released silver ions and how much toxicity is related to the AgNPs per se (Beer et al., 2012). Although AgNPs toxicity effect was partly explained by the release of Ag+, a direct or indirect cause of AgNPs toxicity is still in debate. Kim et al. (2009) suggested that the toxicity of AgNPs is mainly due to oxidative stress and independent of silver ions. Analysis of total phenol

Abbreviations: AgNPs, silver nano particles; DMEM, Dubecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EC, electro conductivity; EtOH, ethanol; FBS, fetal bovine serum; IAA, indole-3-acetic acid; MDA, malondialdehyde; MeOH, methanol; MTT, 3-[4,5-dimethylthiazol-2-yl]-2-5-diphenyltetrazolium bromide; NAA, α-naphthalene acetic acid; PAL, phenylalanine ammonia-lyase; PAM, phenylalanine aminomutase; PBS, phosphate buffered saline; ROS, reactive oxygen species; SA, salicilic acid; TBA, Thiobarbituric acid; TCA, trichloroacetic acid; TDS, total dissolved solids.

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content in the plants however, showed a shift towards secondary metabolism. Therefore, it can be suggested that the interaction of AgNPs with the cellular components is mediated by ROS and oxidative stress has been cited to be one of the most important mechanisms of toxicity related to nanoparticle exposure (Kaur and Tikoo, 2013).

Elicitors are chemicals or biofactors from various sources that can induce physiological changes of the target living organism. In a broad sense, elicitors, for a plant refer to chemicals from various sources that can trigger physiological and morphological responses and phytoalexin accumulation.

Most biologically active compounds of medicinal plants are defensive metabolites that can be induced by chemical elicitors. It is well known that treatment of plants with elicitors, or attack by incompatible pathogens, causes an array of defense reactions, including the accumulation of a range of plant defensive secondary metabolites such as phytoalexins in intact plants or in cell cultures (Zhao et al., 2005). Taxol is a useful secondary metabolite and an anticancer agent with outstanding activities against breast, ovarian and non-small cell lung cancer. Taxol is mainly produced by different species of Taxus which is an environmentally protected and one of the slowest growing plant species. Chemical synthesis and partial synthesis of Taxol from its precursors are also very expensive and time consuming. Extraction of Taxol from cell cultures of hazel (*Coryllus avellana* L.) was a great progress in the biotechnology of Taxol production and cancer research (Rezaei et al., 2011; Bemani et al., 2012). Sustainable production of Taxol and related taxanes by hazel cell culture is the most promising method and can be improved by elicitation. This is evaluated in the present study through elicitation of hazel cells by silver nanoparticles.

2. Materials and methods

2.1. Cell culture and AgNPs treatment

Previously selected rapid growing cell line of hazel (*Corylus avellana* L. cv. Gerd Eshkevar) was grown in a modified LS liquid medium without glycine and supplemented with 3 mg L^{-1} NAA and 3 mg L^{-1} IAA. The cells were maintained at 25 °C in dark with shaking at 110 rpm and were sub-cultured every 7 days (Safari et al., 2012). Spherical AgNPs solution was purchased from US Research Nanomaterial Inc, USA. According to the manufacturer the diameter of AgNPs was 30–50 nm and its purity was 99.99%. Yet, the nanoparticle size was measured before application by monitoring the change in hydrodynamic diameter using a Zetasizer Nano ZS dynamic light scattering (DLS) instrument (Malvern 3000 HSA, UK). The data were analyzed by Zetasizer software, version 6.12 (Malvern, UK). Same analysis was conducted in order to characterize the absorbed AgNPs in the extract of hazel cells. Characterization of the absorbed AgNPs was also achieved by TEM analysis (Zeiss - EM10C 80 KV, Germany). Addition of AgNPs to the cultures was accomplished under sterile conditions after filter sterilization of AgNPs (0.2 μm, Milipore). A series of preliminary experiments was conducted using concentrations of AgNPs which were applied for stimulation of secondary metabolites in intact plants (Ghanati and Bakhtiarian, 2013). The results of these experiments showed that concentrations higher than 10 ppm dramatically affected hazel cells growth. Therefore, seven day old cells (in their logarithmic growth phase) were treated with 0, 2.5, 5, and 10 ppm of AgNPs. The effect of AgNPs on the growth of the cells was monitored by weighing them 1 week. The cells were harvested under reduced pressure and the extracellular media were collected in order to detect EC (electro conductivity), TDS (total dissolved solids), and pH.

The harvested cells were washed thoroughly with deionized water and then were extracted for DLS and TEM analysis. The extraction was achieved in deionized water in order to avoid AgNPs aggregation since cations in electrolyte solutions could influence the aggregation of AgNPs (Zhang and Wang, 2013). Grate attempts were made in order to shortening the gap between extraction and observation. Moreover, a part of the cells were directly observed by TEM and the shape and size of particles in the cells and the extracts were compared.

2.2. Growth and membrane lipid peroxidation assay

The growth of hazel cells was monitored by differences between their fresh weight before and after the treatment.

The level of peroxidation of membrane lipids was assessed by measuring MDA as a final product of lipid peroxidation. Aliquots of frozen samples (0.2 g) were homogenized in TCA (10%). The homogenate was centrifuged at 10,000×*g* for 15 min and 1 mL TBA 0.5% was added to 1 mL of the supernatant. The mixture was incubated at 70 °C in water bath for 30 min, and was immediately cooled in ice–water bath. The absorbance of MDA was read at 440, 532 nm and 600 nm using spectrophotometer (Cintra 6, GBC, Australia). The amount of MDA-TBA complex was calculated from the extinction coefficient of 157 mM^{-1} cm^{-1} (Hodges et al., 1999).

2.3. Measurement of total soluble phenols, anthocyanins, and flavonoids

Total content of soluble phenolic compounds were quantified according to Pirie and Mullins (1976). In brief, 3 mL of methanolic extract of the cells was mixed with 3 mL ethyl acetate and centrifuged at 12,000×*g* for 15 min. Ethyl acetate phase was collected and evaporated. The residue was dissolved in EtOH (75% v/v). The absorbance of the supernatant was measured at 280 nm. For extraction and measurement of anthocyanins, 0.2 g of cells were homogenized in a mixture of MeOH: HCl (99:1 v/v) and centrifuged at 12,000×*g* for 15 min. The supernatant was kept in darkness overnight. Anthocyanin content was determined by measuring the absorbance at 550 nm using extinction coefficient of 33,000 cm^{-1} M^{-1}. Flavonoids were extracted in a mixture of EtOH: HCl (99:1 v/v), followed by centrifugation at 12,000×*g* for 15 min. The supernatant was separated, incubated in a water bath (80 °C, 10 min), and its absorbance was read at 270, 300, 330 nm. Flavonoid content was determined using extinction coefficient of 33,000 cm^{-1} M^{-1} (Krizek et al., 1998).

2.4. Analysis of Taxol and baccatin III

The cells were filtered and homogenized in 10 volume of MeOH followed by centrifugation at 5000×*g* for 10 min. The supernatant was used in order for assay intracellular taxanes. Equal volumes of aqueous methylene chloride (50%) were added either to the aforesaid supernatant or to the cell filtrate (for extraction of extracellular taxanes) followed by vigorous shaking and centrifugation at 5000×*g* for 10 min. The supernatant was collected, evaporated under a stream of filtered air, and finally resolved in 150 μL of MeOH before injection to HPLC (Ghanati et al., 2015). Quantification of Taxol and baccatin III was accomplished by comparing their retention time and peak area with reference standards.

2.5. Evaluation of cytotoxicity of hazel cell extracts by MTT assay

HeLa cell line (cervical carcinoma) was obtained from the