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# Using *Alternaria solani* Toxin and Mycelia as a Stabilizer for Nanosilver Particles to Improve Acquired Immunity in *Solanum tubrusum* by *in vitro* technique

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**ABSTRACT**

The antimicrobial effects of silver ion (Ag) or salts are well known, but the mechanism of silver nanoparticles (AgNPs) on microorganisms and antimicrobial have not been revealed clearly. Stability of AgNPs nanoparticles were prepared and their shape and size distribution characterized by the transmission electron microscope [TEM]. The effect of nanosilver at 6 µM loaded on *Alternaria* fungus fragments and their toxins, were investigated against five *Alternaria solani* isolates (*AST-2, AST-5, AST-6, AST-7* and *AST-11*) during 2013 - 2015. These fungi were responsible for early blight disease in potato *Solanum tubrosum* cv. Sponta. In these tests, the best media to induce somatic embryogenesis of potato was MS medium containing 3 mg/l 2,4-dichlorophenoxy acetic acid (2, 4-D) on which (44%) of the explants produced somatic embryos, and these somatic embryos were used in the study. Ag nanoparticles at various concentrations and the somatic embryos were grown in a liquid media containing (1ml/l of 100% toxins). The results, revealed that fungi toxins inhibited growth of fungi in the toxin isolate *AST-2* (with dilution 50%) of Ag nanoparticles and increased the proliferation capacity of the somatic embryos to 33.33 %. But the growth-inhibitory effects on somatic embryogenesis were obtained by using dry a mycelium matter of fungi and Ag nanoparticals (0.0 %). These results suggested that Ag nanoparticles can be used as an effective acquired immunity inhibitor to the early blight pathogen and may be used in other various microorganisms, making them applicable to antimicrobial control systems. However, the isolation and identification testes of *Alternaia solani* carried out to the treated explants with toxins showed disease symptoms.

***Keywords:*** Potato, *Solanum tubrosum, in vitro,* somatic embryogensis, Silver-nanoparticles, early blight disease, *Alternaria solani* fungi

## Introduction

Silver has been valued throughout history for many of its properties that are useful to humans. It is used as a precious commodity in currencies, ornaments, jewelry, electrical contacts and photography, among others. One of the most beneficial uses of silver has been as a potent antibacterial agent that is toxic to fungi, viruses and algae. Silver has long been used as a disinfectant; for example, the metal has been used in treating wounds and burns because of its broad-spectrum toxicity to bacteria as well as because of its reputation of limited toxicity to humans (Katrina *et al.,* 2010).

In nanotechnology, a nano particle is defined as a small object or particle that behaves as a whole unit in terms of its transport and properties. Nanotechnology takes advantage of the fact that when a solid material becomes very small, its specific surface area increases, which leads to an increase in the surface reactivity and quantum-related effects. The physical and chemical properties of nanomaterials can become very different from those of the same material in larger bulk form. Nanomaterials (such as nanotubes and nanorods) and nanoparticles are particles that have at least one dimension in the range of 1 to 100 nm. Nanoparticles are classified solely based on their size, and may or may not exhibit size-related properties that differ significantly from those observed in bulk materials (ASTM, 2006; Buzea *et al*., 2007). Due to the properties of silver at the nanoscale, nanosilver is nowadays used in an increasing number of consumer and medical products. Nanomaterials are nanoparticles that have special physicochemical properties as a result of their small size (Buzea *et al*., 2007).

Nanosilver is an effective, fast-acting fungicide against a broad spectrum of common fungi including genera such as *Aspergillus*, *Candida*, and *Saccharomyces* (Wright *et al*., 1999). The exact mechanisms of action of silver nanoparticles against fungi are still not clear, but mechanisms similar to that of the antibacterial actions have been proposed for fungi (Wright *et al*., 1999). Silver nanoparticles (diameter 13.5 ± 2.6 nm) are effective against yeast isolated from bovine mastitis (Kim *et al*., 2007).

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Biosyntehsis of nanoparticles have been takes place extra cellular, intera cellular and surface of the cell wall. Extra cellular synthesis has been observed in *Fusarium oxysporum* with silver and gold–silver nanoparticles (Ahmad *et al.,* 2003). The reduction of silver ions by *Fu. oxysporum* strains has been attributed to a nitrate-dependent reductase and a shuttle quinine extracellular process. Bhainsa and D'Souza (2006) and Basavaraja *et al.* (2007) reported the extracellular synthesis of silver nanoparticles by the fungi, *Aspergillus fumigatus* and *Fusarium semitectum,* respectively. Many reports on the synthesis of metal and semi-conductor nanoparticles using yeast and fungi have been appeared. Silver nanoparticles in the range of 2-5 nm were synthesized extracellularly by a silver tolerant yeast strain MKY3 (Kawshik *et al.,* 2003). Investigations carried out on 20 different fungi reveals that fungi are extremely good candidates in synthesis of metal and metal sulphides nanoparticles (Ahmad *et al.,* 2003; Mukherjee *et al.,* 2001). The shift from bacteria to fungi as a means of developing natural "nano-factories" has the added advantage that downstream processing and handling of biomass would be much simpler (Sastry *et al.,* 2003). The fungi *Verticillum* sp., *Fu. oxysporum*, *Fu. semitectum* and *Aspergillus flavus* have shown the capability of the nanometal production either extra or intracellularly (Mukherjee *et al.,* 2008; Bhainsa and D'Souza, 2006). Latter, Gajbhiye *et al.* (2009) reported the extracellular biosynthesis of silver nanoparticles using a common fungus, *Alternaria alternata*. These silver nanoparticles were evaluated for their part in increasing the antifungal activity of fluconazole against *Phoma glomerata*, *Phoma herbarum*, *Fu. semitectum*, *Trichoderma* sp., and *Candida albicans*. The synthesis of silver particles using soil dwelled *Aspergillus niger* strains was investigated by Sadowski *et al.* (2008a,b). It was presented (Duran *et al.,* 2005) that enzyme hydrogenase is present in a filtrate broth which obtained from *Fu. oxysporum*. The silver nanoparticles production capacity has been depended on the reductase/electron shuttle relationships. The reduction of metal ions occurs on the surface by the enzymes presented in the cell wall (Mukherjee *et al.,* 2001). The extracellular enzymes such as naphthoquinons and anthraquinones showed excellent redox properties, they can act as electron shuttle in silver ions reduction. In 2009, Kathiresan and coworkers exploited the marine fungus, *Penicillium fellutanum* from mangrove sediment for synthesis of stable silver nanoparticles (Kathiresan *et al.,* 2009). In 2009, Balaji and co-workers synthesized stable silver nanoparticles using culture filtrates of *Cladosporium cladosporioides,* a commonly available fungus found in marshland regions (Balaji *et al.,* 2009).

Somatic embryogenesis being one of the *in vitro* techniques that produce high regeneration rates, serves for high frequency propagation, gene transfer and germplasm storage.

## Materials and Methods

*Plant material*

The potato cv. 'Sponta" was collected from disease free plant at the Experimental Potato Station at the Horticulture Researches Institute, Agricultural Researches Center, Egypt during 2013-2015 growth season and kept in cold for 2 weeks. After sprouting the micronodes were thoroughly washed in tap water with 5% detergent solution (Teepol) for 20 minutes, followed by 2–3 washes in sterile distilled water. The explants were cut into convenient sizes ( 2-3.5 cm in length) after removing the leaf sheaths. The cut pieces (micronodes) were surface sterilized with Colorex (Sodium hypochlorite) 30 % for 20 minutes and rinsed 4–5 times with sterilized double distilled water and then trimmed to 1.0–1.5 cm in length. The explants were transferred onto full strength MS free medium (Murashige and Skoog 1962) as a growing and callus medium. The grown *in vitro* aerial stem explants(micronodes), cultured on MS free medium were repeated cut into 1.0 –1.5 cm in length and transferred to the same medium for obtaining an sufficient number of *in vitro* stems and callus. Explants were maintained in an air conditioned room at 22 ± 2 °C and photoperiod regime of 16 hr light and 8 hr dark with a light intensity of 3000 lux, provided by Philips cool white fluorescent tubes.

*Callus induction and somatic embryogenesis*

Callus cultures derived from the aerial stem, leaves and roots explants were sub cultured onto MS medium containing 2,4-D or NAA at 2,3,4,5 and 10 mg/l. These cultures were keeping them without sub culturing for 60 days to induce embryogenic calli. The embryogenic calli obtained from leaves more than stem and roots were then transferred on to MS medium containing BAP, Kin and 2,4-D at (2, 3 and 4 mg/l, respectively) and finally at glutamine 50, 100 and 200 ppm for the induction of somatic embryos. The count of cells was calculated by BD Cell Quest Pro software on Mac. OS 9 as shown the next formula:

MC = [(FWM-DWM)/FWM]*100
Callugensis = [(count of explants formed callus/total explants cultured)]*100
Somatic embryo cells count = [(somatic cell count/total cell count)]*100 /HPF
Somatic embryo %= [(somatic formation count/total callus formation)]*100

*Fungi growth*

The early blight fungus, *Alternaria* sp., was isolated from seven Governorates, *i.e*. Behera, Ismailiya, Menufiya, Kafer El-Shaykh, Qalyubiya, Gharbia and Giza during 2013 - 2015 growing seasons

The fungi planted and maintained on potato dextrose agar (PDA) medium then incubated at 25±2°C for 5-7 days. Purified fungal isolates were identified microscopically at the Mycology and Plant Diseases Survey Research Dept., Plant Pathology Research Inst., ARC, according to the morphological characteristics, *i.e.*, average length and width of conidia, pick length and the ratio of the pick length to conidial length, as described by Barnett and Hunter (1972). Pure cultures were maintained on PDA slants at 4-6°C and sub cultured monthly.

Five isolates of *A. solani* were grown in Cazpek's Dox Medium(CDM) and incubated for 21days at 24°C in the dark without shaking. The contents of the flasks were filtered through the Whatman No. 4 filter paper. Culture filtrates were stored at 4°C in the dark up to 10 hours until used.

After incubation period, mycelia were separated from the culture broth by centrifugation (5000 rpm) at 10°C for 20 min and the settled mycelia were washed twice with sterile distilled water and dried by hot air at 50°C. Ten grams of the harvested mycelial powder mass was then re-suspended in 100 ml sterile distilled water in 500 ml Erlenmeyer flasks at pH 5.8.

The toxin of fungi was isolated at the end of incubation period, it was filtered by wattman paper No 4.0 and stored for nanosilver use.

*Nanosilver synthesis*

*Preparation*

Exactly 0.06 uM of $AgNO_3$ (common amounts, WHO, 2002) is dissolved in 100 mL distilled water and ten ml of both dried mycelium fungi solution or toxin are added to 10 ml of $AgNO_3$ solution for reduction of $Ag^+$ ions and stirring for 1000 rpm at 60℃ for 1h. The reduction of pure $Ag^+$ ions is monitored by change the color of solutions from clear to brown.

*Loading*

The somatic embryos produced from the previous trails were immersion in the five solutions of *Alternaria solani* isolates (*AST-2, AST-5,AST-6, AST-7 and AST-11).*

AgNPs were loading on dry mycelium or toxins as a stabilizer of $Ag^+$ ion to block any receptor of mycelium or toxin in cell surface by AgNPs, we used four concentrations 100, 50, 25 and 12.5% for toxins or dry mycelium.

The explants (macronodes explants) were soaked in nano-silver particles loaded on five fungi isolates (*AST-2, AST-5, AST-6, AST-7 and AST-11)* at three concentrations (100, 75 and 50%). All immersion treatments were incubated for three days in the room temperature.

The successive explants or somatic were cultured on MS containing 1ml/l of 100% toxins was added by filtration methods (Kashiwagi *et al.,* 2006).

*Materials and instruments characterization of synthesized silver nanoparticles*

UV-Vis spectra were recorded from 190 to 450 nm and absorbance was plotted on graph by graph pad prism 5.0 software. Silver nitrate (AgNO3), Sodium hydroxide (NaOH), sodium chloride (NaCl), methanol (MeOH) and hydrochloric acid (HCl) were purchased from Merck. Deionized water was used for synthesis of AgNPs. A digital pH meter model (Accument) equipped with a glass working electrode and a reference Ag/AgCl electrode was used. UV–vis spectra were recorded with a Shimadzu UV-240, Hitachi U-3200 spectrometer with a path length of 1 cm. Microscopic image of synthesized AgNPs was taken with a Zeiss Libra transmission electron microscope (TEM) operated at 120 keV. The suspensions were subsequently examined for the purpose of determining the average particle size. The examination was carried out with the use of the dynamic light scattering technique (DLS).

## Results and Discussions

## Callus induction

Data presented in Table (1 and Fig.1) reported that the callus induction was obtained from three parts of explants after cultured on MS medium with two additives plant growth regulators 2,4-D and NAA at 2,3,4,5 and 10 mg/l for each. According to the Table (1 and Fig.1b,c,d) callus induced from stem has highest frequency

quantity of callus (98.33%) after treated with 2,4-D at 3 mg/l compared with those induced from roots (80.5%) and stems (81.67% ) compared with a little amount of callus for control treatments in three parts stem, leaves and root (3.7, 1.11 and 1.11%, respectively).

**Table 1:** Effect of various concentrations of 2,4-D and NAA on callus percentage of three parts of explants stem, leaves and roots in *Solanum tubrosum* in vitro culture

| Treatments | Callus induction % | | |
|---|---|---|---|
| | Stem | Leaves | Root |
| Control | 3.703 e | 1.11 e | 1.11 e |
| NAA 2 mg/l | 24.07 d | 33.52 d | 26.44 cd |
| NAA 3 mg/l | 46.43 c | 45.55 cd | 37.22 cd |
| NAA 4 mg/l | 63.89 b | 37.03 d | 29.63 cd |
| NAA 5 mg/l | 53.70 bc | 52.16bcd | 45.23 bcd |
| NAA10 mg/l | 18.92 d | 30.18 d | 23.41 de |
| 2,4-D 2mg/l | 22.19 d | 44.54 cd | 48.33 bc |
| 2,4-D 3mg/l | 98.33 a | 81.67 a | 80.55 a |
| 2,4-D 4mg/l | 91.67 a | 76.67 ab | 63.89 ab |
| 2,4-D 5mg/l | 48.83 bc | 80.98 a | 44.86 bcd |
| 2,4-D 10mg/l | 22.22 d | 65.74 abc | 65.11 ab |
| LSD 5% | 15.21 | 27.14 | 22.51 |

**Growth characteristics of callus**

The medium containing NAA and 2,4-D had a positive effect of callus fresh weight (Table 2 and Fig. 1 ) measurements especially for roots cultured on MS medium containing 2,4-D at 3.0 mg/l, it graves the highest quantity of callus fresh weight (4.5 g) followed by the same hormone at 5 mg/l ( 4.2 g) compared with the root cultured on MS free medium. These amounts decreased to (0.228 g) for the same treatments (2,4-D 3 mg/l) for D.W., while the control treatment of root culture decreased to 0.005 g. These results explained the moisture contents of callus induced from roots have a non-significant effect between all treatments except control (64.81 %) but the moisture contents reached to the maximum 96.98 % for callus cultured on MS medium containing NAA 2 mg/l.

On the other hand, leaves cultured on MS medium containing 2,4-D at 4 mg/l produced high amount of fresh weight (3.38 g) compared with all other treatments and control (0.597 g), Also, dry weight of leaves callus decreased from 0.415 mg for NAA 2mg/l to 0.0079 mg for control. While the amounts of water contents for all treatments have non-significant effect except with control (65.58 %).

Finally, NAA at 4 mg/l scored 1.39 g of fresh weight for stem part cultured on MS medium containing previous treatment but NAA at 2 mg/l scored the highest weight of dry weight 0.147 g for the same part of explant. The moisture contents of all treatments have a non-significantly affect compared with control (33.0%)

These results showed the relation between effect of PGRs and amount of callus obtained from the various parts of explant indicated to ability of PGRs on cell division and increased the callus growth in presence of moisture contents, which relied with growth cell size not count.

**Table 2:** Effect of various concentrations of 2,4-D and NAA on fresh and dry weight of three parts of explants stem, leaves and roots in *Solanum tubrosum in vitro* culture

| Treatments | Stem callus | | | Leaves callus | | | Root callus | | |
|---|---|---|---|---|---|---|---|---|---|
| | F.W. | D.W. | M.C. | F.W. | D.W. | M.C. | F.W. | D.W. | M.C. |
| Control | 0.303 d | 0.003 c | 33.0 b | 0.597 e | 0.0097 b | 65.58 b | 0.363 e | 0.005 d | 64.81 b |
| NAA 2 mg/l | 1.150 abc | 0.147 a | 88.03 a | 2.243 cd | 0.415 a | 82.07 ab | 3.387 bcd | 0.101 c | 96.98 a |
| NAA 3 mg/l | 0.957 abc | 0.050 bc | 94.65 a | 3.060 ab | 0.167 ab | 94.49 ab | 3.213 cd | 0.220 a | 93.13 ab |
| NAA 4 mg/l | 1.390 a | 0.041 bc | 97.09 a | 3.143 a | 0.183 ab | 94.01 ab | 4.083 abc | 0.136 bc | 96.66 a |
| NAA 5 mg/l | 1.107 abc | 0.041 bc | 96.26 a | 3.293 a | 0.103 b | 96.87 a | 3.447 bcd | 0.127 c | 96.18 a |
| NAA10 mg/l | 0.727 cd | 0.013 c | 98.04 a | 3.347 a | 0.142 b | 95.83 a | 3.363 bcd | 0.107 c | 96.79 a |
| 2,4-D 2mg/l | 1.073 abc | 0.040 bc | 96.25 a | 2.107 d | 0.039 b | 98.23 a | 3.877 abc | 0.211 ab | 94.45 a |
| 2,4-D 3mg/l | 1.357 ab | 0.093 b | 92.89 a | 2.907 abc | 0.087 b | 97.16 a | 4.500 a | 0.228 a | 94.94 a |
| 2,4-D 4mg/l | 0.873 abc | 0.013 c | 98.57 a | 3.380 a | 0.102 b | 96.94 a | 3.220 cd | 0.130 c | 95.81 a |
| 2,4-D 5mg/l | 0.800 bcd | 0.011 c | 98.56 a | 2.390 bcd | 0.247 ab | 91.21 ab | 4.207 ab | 0.279 a | 92.96 ab |
| 2,4-D 10mg/l | 0.810 bcd | 0.034 c | 96.05 a | 2.017 d | 0.026 b | 98.80 a | 2.597 d | 0.141 bc | 94.52 a |
| LSD 5% | 0.5674 | 0.05386 | 29.04 | 0.7226 | 0.2526 | 29.71 | 0.914 | 0.07617 | 29.25 |

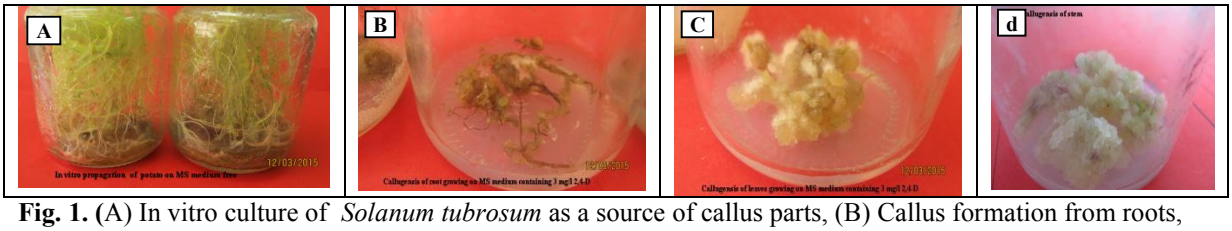



**Fig. 1. (**A) In vitro culture of *Solanum tubrosum* as a source of callus parts, (B) Callus formation from roots, (C) callus from leaves, (D) callus from stems.

**Somatic embryos intiation**

The callus cultures reflected morphological variations four weeks after culturing. The leaf explants showed swelling and expansion and a curvature indicating cellular somatic embryos growth while stem just showed swelling without differentiation. On the other hand, the root culture has not obtained any somatic cells. The induction of embryos was visible within 8 - 10 weeks. Somatic embryos were induced directly at the callus induced from the leaves and embryos were green, small, and globular, arising individually or in group (Fig. 2). MS medium fortified with 3 $mgl^{-1}$ 2,4-D showed faster response to induce indirect somatic embryos (44.0 %) as compared to other concentrations. The induction frequency of embryos was significantly high at medium concentration of the 2,4-D. The frequency of indirect somatic embryo induction was highest in the leaf explants (44 %) as compared to control callus cultured (11.0 %) on MS free medium (Table 3 and Fig. 2). These somatic embryos formed either individually or in groups, directly on service of callus formation. Most of the globular embryos gradually developed as bipolar structures resembling heart-, torpedo-, and cotyledon-stage embryos (Fig.2).

Some treatments produced high quantity of callus like control (89%) and BAP at 2.0 mg/l (77.67%) these amounts of callus were not important because these amounts decreased somatic embryogensis formation to 11.0 and 22.23 %, respectively. In this case, 2,4-D at 3.0 mg/l produce a little amounts of callus 56% but recorded 44 % of somatic from all callus formation compared with control 11.0%.

According to the data in Table (3 and Fig. 2) the differential count of somatic embryos were found that the relative count of somatic embryos have a non-significantly affect except with those treated with 2,4-D 3 mg/l and control 10.67 and 8.67 %, respectively. But only all somatic embryos formation on media containing 2,4-D at 3 mg/l were synchronized and differentiated to various somatic embryos stages at highest percentage 9.0%, 8.3% and 6.7 % for torpedo, heart and cotyledonary stage. Glutamine at 200 ppm increased relative count of globular stage 10.3 %.

**Table 3:** Effect of somatic embryogensis inducers on somatic embryogensis percentage and differential of leaves callus in *Solanum tubrosum in vitro* culture

| Somatic inducer treatments | Callugensis cell % | Somatic embryo % | Somatic cells /100 | Differential somatic cells % | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | G | T | H | C | Reg |
| Control | 89.00 a | 11.0 e | 8.67 c | 2.00 d | 0.33 c | 0.00 e | 0.00 c | 0.00 b |
| BAP 2 mg/l | 77.67 ab | 22.33 de | 17.00 abc | 2.67 d | 1.67 bc | 1.00 c-e | 0.00 c | 0.00 b |
| BAP 3 mg/l | 73.33 bc | 26.67 cd | 23.00 a | 2.33 d | 1.33 bc | 0.00 e | 0.00 c | 0.00 b |
| BAP 4 mg/l | 69.33 bcd | 30.67 bcd | 24.00 a | 3.67 cd | 1.67 bc | 0.33 de | 0.33 c | 0.00 b |
| Kin 2 mg/l | 66.00 cde | 34.00 abc | 22.67 a | 8.00 ab | 2.00 bc | 1.33 cde | 0.00 c | 0.00 b |
| Kin 3 mg/l | 67.67 bcd | 32.33 bcd | 25.67 a | 4.99 cd | 1.33 bc | 1.33 cde | 0.00 c | 0.00 b |
| Kin 4 mg/l | 68.33 bcd | 31.67 bcd | 21.33 a | 6.33 bc | 2.33 bc | 1.67 cde | 0.00 c | 0.00 b |
| Glut 50 mg/l | 68.33 bcd | 31.67 bcd | 17.33 abc | 8.67 ab | 2.33 bc | 2.00 bcd | 1.33 c | 0.00 b |
| Glut 100 mg/l | 66.67 b-e | 33.33 a-d | 20.33 a | 8.00 ab | 2.33 bc | 2.33 bc | 0.33 c | 0.00 b |
| Glut 200 mg/l | 63.00 cde | 37.00 abc | 19.33 ab | 10.33 a | 3.00 bc | 2.67 bc | 1.67 bc | 0.00 b |
| 2,4-D 2 mg/l | 61.33de | 38.67 ab | 18.00 ab | 7.00 abc | 4.67 b | 3.67 b | 4.00 b | 1.33 a |
| 2,4-D 3 mg/l | 56.00 e | 44.00 a | 10.67 bc | 7.67 ab | 9.00 a | 8.33 a | 6.67 a | 1.67 a |
| 2,4-D 4 mg/l | 59.67 de | 40.33 ab | 16.67 abc | 7.67 ab | 12.0 a | 1.67 cde | 2.00 bc | 0.33 b |
| LSD 5% | 11.54 | 11.54 | 9.336 | 3.433 | 4.134 | 1.897 | 2.511 | 0.6698 |

*G: Globular , T: Turbido, H: Heart, C: Cotyledonary*

**Nano-silver charactarzation**

UV-VIS spectrophotometric analysis is one of the simple and mostly used to characterize the presence of nanoparticles in extracts based on change in color. After giving various variation treatments, change in color from pale yellow to black were observed. This color showed the presence of silver nanoparticles or reduction of Ag+ of $AgNO_3$ to $Ag^0$ (Silver Nanoparticles). After observing changes in color of the extracts, we were scanned from 190-680 nm spectrophotometric analysis keeping toxin extract (without silver nitrate treatment) as blank and maximum absorbance at 450 nm due to Surface Plasmon Resonance (SPR) of silver nanoparticles. Fig. 3A presents spectrophotometric data obtained for the received AST-2 extract without $AgNO_3$. It is worth noticing that the space between 400 and 500 nm of wavelength remains empty (without any characteristic peak) so as to leave the possibility of the occurrence of the characteristic nanosilver peak. According to the spectrophotometric data (Fig. 3C), the presence of nanosilver particles in $AgNO_3$ was confirmed with the AST-2 diluted with 50%, 100% and 25% with $AgNO_3$. It is evidenced by the presence of the characteristic nanosilver peaks. With highest regeneration values, that was the reason why only AgNPs (Ag-AST-2) was qualified for further use in the study. On the basis of the data regarding nanoparticles size (Fig. 3B), it was decided to include only AgNPs (Ag-AST-2) small size of $Ag^+$ ranged from 15.6 to 25.6 nm (Fig. 3B) compared with $AgNO_3$ the more than 100 nm(Fig. 3D) .

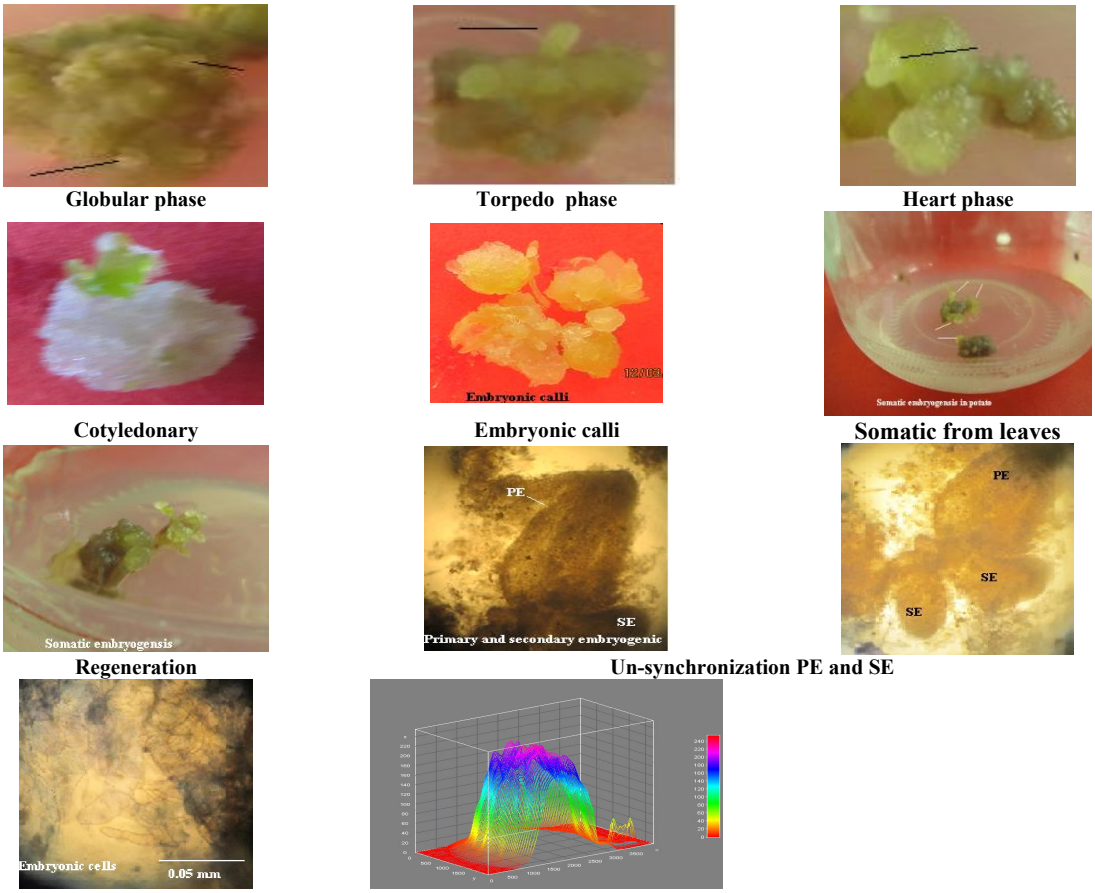



**Fig. 2:** (Upper right to left ) In vitro somatic embryogensis stages of *Solanum tubrosum* globular, torpedo, heart, Cotyledonary and embryonic calli . (down right to left) somatic embryos obtained from callus leaves , regeneration of somatic, primery and secondary somatic and un-synchronization between embryonic cell growth.

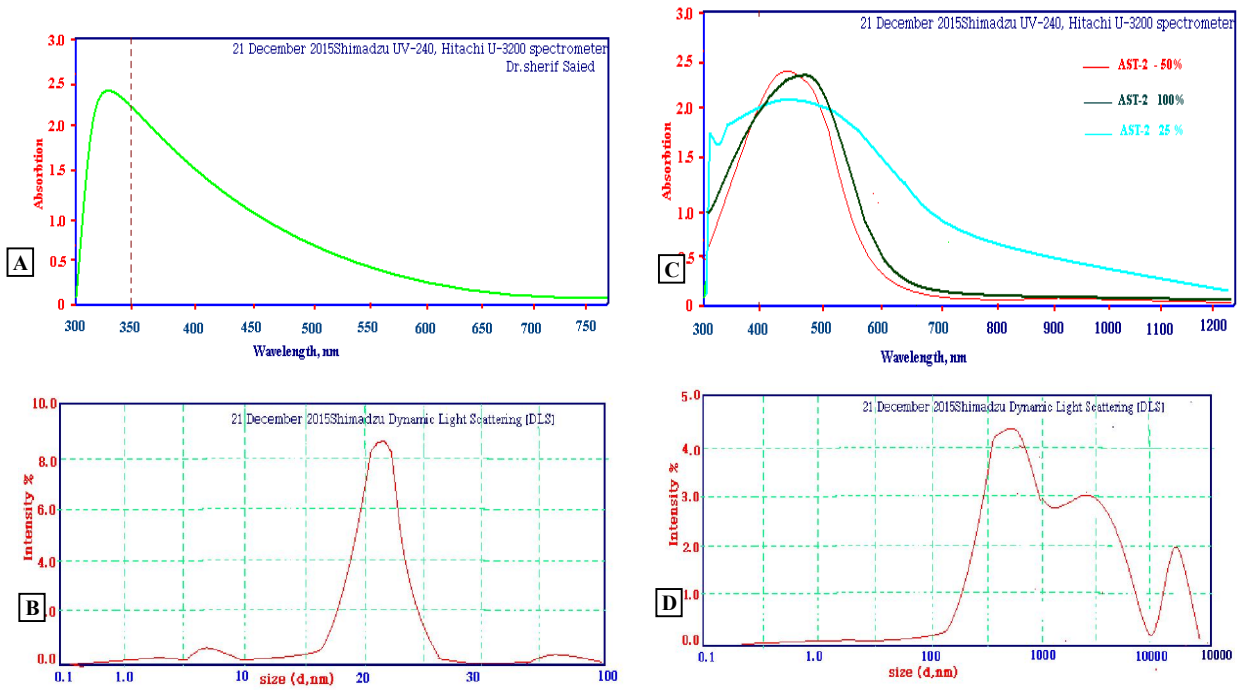



**Fig. 3 :**UV. Spectrophotometric shows wave length of a new band of AgNPs (a-c) and size (b-d)

## 1. Improvement systemic acquired immunity

*Somatic embryos*

The somatic embryos produced from the previous trails were exposed to nano-silver particles materials. The dried mycelium and toxins of five isolates of *Alternaria solani* (*AST-2, AST-5,AST-6, AST-7 and AST-11)*were using as a stabilizer of $Ag^+$ ion to block any cellular receptor bind with mycelium of toxin by silver it was used at four concentrations (100, 50, 25 and 12.5% for each) Table (4). After soaking the somatic embryogensis in solutions for 3 days and washing then cultured on regeneration media and comparative all treatments with control (that soaked in MS medium free). The data in Table (4 and Fig. 4) show that the second concentration (50%) of AST-2 toxin scored the highest survival rate as control (without any treatments) 100%. These survived somatic embryos scored relative percent 75 % of embryonic calli followed by AST.7 at 50% and initiated protochorms 95.07 % and 88.8%, respectively. The regeneration rate initiate scored 33.33 %. for AST2 50% treatment.

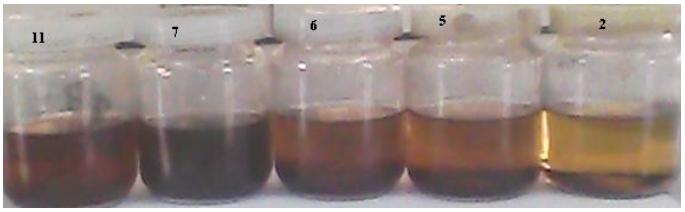
On the other hand, all dried mycelium treatments were died except with two treatments (AST-2 and AST-7 at 50 % for each) without any regeneration. (data not show) Fig. 4.

The callus exposed to various concentrations of *Alternaria solani* fungi gave a protochorms like somatic embryos these characters are a primary initiation somatic and differentiate cells from callus cells to somatic calli. Both of AST-2 at 50% and AST-7 at 50% gave the highest value of protochorms (95.07 and 88.88 %, respectively). These results have relation with embryonic calli formation, so the previous treatments scored the highest value of embryonic calli percentage (75 % and 66.66%, respectively) Fig. 5.

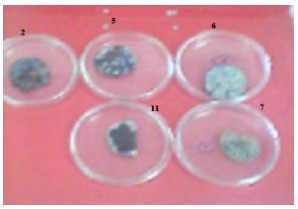
**Table 4:**Effect of nano silver particles loaded on *Alternaria solani* toxins isolates on somatic embryogensis tolerant infection and differential of leaves callus in *Solanum tubrosum* in vitro culture

| | Survival % | Callus characterization % | | | | | Regeneration |
|---|---|---|---|---|---|---|---|
| | | Calluogensis % | Embryonic calli % | Protochorms % | Color | Type | |
| Control | 100.0 | 75.0 | 0.0 | 10.86 | G | Granular | 0.0 |
| AST.2 100% | 3.330 | 25.0 | 53.0 | 75.61 | g/w | Soft | 15.0 |
| 50 % | 100.0 | 25.0 | 75.0 | 95.07 | g | Granular | 33.33 |
| 25 % | 66.66 | 11.11 | 55.0 | 33.54 | g | Granular | 11.11 |
| 12.5 % | 11.11 | 85.0 | 0.0 | 4.98 | w | Soft | 0.0 |
| AST.5 100% | 3.330 | 25.0 | 45.0 | 52.38 | w | Soft | 0.0 |
| 50 % | 66.66 | 25.0 | 55.0 | 44.12 | w/g | Granular | 11.11 |
| 25 % | 33.33 | 11.11 | 45.0 | 23.91 | b/g | Smooth | 2.0 |
| 12.5 % | 3.33 | 100.0 | 2.2 | 0.00 | b/g | Granular | 0.0 |
| AST.6100% | 3.330 | 22.22 | 55.0 | 22.43 | w/g | Granular | 0.0 |
| 50 % | 22.22 | 11.11 | 23.0 | 54.23 | w/g | Smooth | 0.0 |
| 25 % | 66.66 | 11.11 | 44.0 | 33.67 | b | Soft | 0.0 |
| 12.5 % | 3.33 | 100.0 | 3.3 | 6.98 | b | Granular | 0.0 |
| AST.7100% | 3.330 | 33.33 | 22.0 | 23.56 | b | Granular | 0.0 |
| 50 % | 75.00 | 22.22 | 66.6 | 88.88 | b | Smooth | 0.0 |
| 25 % | 95.00 | 11.11 | 33.3 | 33.87 | b | Soft | 0.0 |
| 12.5 % | 3.33 | 75.0 | 3.3 | 15.64 | b | Granular | 0.0 |
| AST.11100% | 3.33 | 11.11 | 44.0 | 34.23 | g | Granular | 0.0 |
| 50 % | 75.66 | 11.11 | 22.0 | 33.96 | g | Soft | 0.0 |
| 25 % | 66.66 | 11.11 | 55.0 | 23.01 | g | Granular | 0.0 |
| 12.5 % | 22.22 | 50.0 | 33.3 | 22.50 | b | Granular | 0.0 |
| AgNPs 0.06uM | 11.11 | 25.0 | 44.0 | 10.54 | b | soft | 0.0 |
| LSD 5% | 6.433 | 8.655 | 12.433 | 11.334 | | | 1.433 |

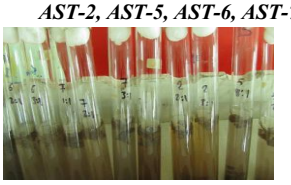
**AST:** *Alternaria solani toxin (*number of isolate 2,5,6,7 and 11)




***AST-2, AST-5, AST-6, AST-7 and AST-11*** **mycelium on nano -silver**



**Dried mycelium**





**Somatic soaked in AgNPS**

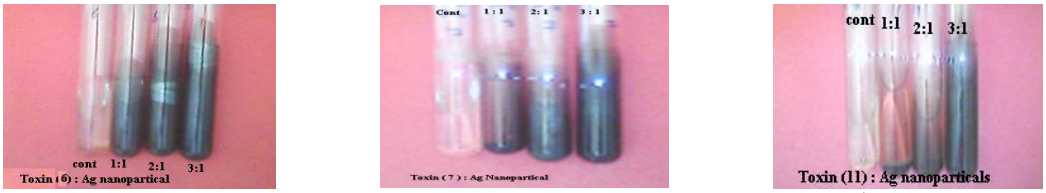



**Fig. 4:** (Upper right to left ) dried mycelium of five isolates dried mycelium loaded on $Ag^{+}$ and before use, somatic embryos soaking in nano solutions- down right to left five isolates toxins loaded on $Ag^{+}$ and somatic embryos were soaked for three days .

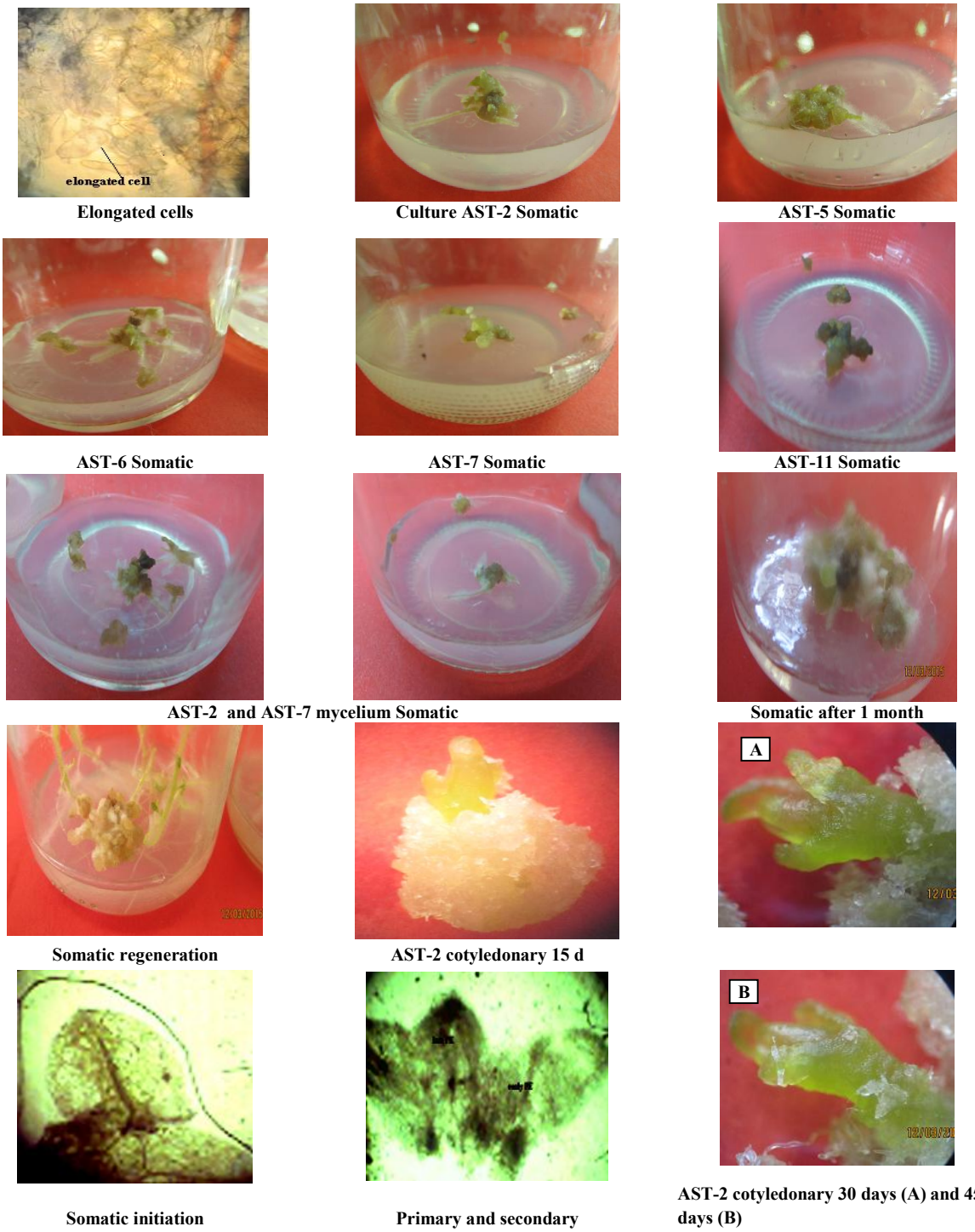



**Fig. 5:** (Upper right to left ) Elongated cell in callus, five isolates toxin with Ag treated somatic embryos . (down right to left) somatic embryos obtained from callus leaves , regeneration of somatic, maturation of somatic embryos

*Macronodes explants*

The explants (macronodes) were soaked for three days in nano-silver solution loaded on toxins of five fungi isolates, toxin free nano-silver and $AgNO_3$ after that the explants were cultured on growth medium MS supplemented 1ml/l toxins for observed the growth and contamination percentage. All positive control (toxin free nano-silver and silver free toxin) were died and contaminated 100% Fig 6A. But all the other treatments were survived at various percentages, the highest one (negative control and AST-2 at 75%) scored 100%. All survived explants are very healthy, stem thickness, leaves have dense chlorophyll amounts Fig 6B. While the explants exposed to $AgNO_3$ free were turned to black color and died Fig 6A.

On growth morphology, the concentration 75 % of both AST-2 and AST-7 were gave the highest number of shootlets/explants 4.31 and 4.01 for each AT 100% survival. While the lengths of the shootlets were 3.26 cm for AST-7 75% compared with control. Also, the leaves no/explant and rooting percentage were 6.75 and 6.31 %, respectively for AST-2 at 75 % compared with control Fig 6B.

*Disease severity (%)*

According to the data in Table (5) and Fig. (6A) the disease severity (%) showed that all positive control of the explants soaked in toxins of both five isolates *AST-2,AST-5,AST-6,AST-7* and *AST-11* have a significant effect where they appearing various characteristics of disease severity ranged for 46.3, 65.83, 53.3, 34.5 and 23.0 %, respectively. On the other hand, both of explants soaked in $AgNO_3$ and MS medium free have no disease severity but the explants soaked with silver turned into black color and died.

The best results were observed in this treatments for the explants soaked in both toxin loaded on nanosilver at 75 and 50 % which scored 0.0 % disease severity without any contamination symptoms, healthy characters and forty growth.

**Table 5:** Effect of nano silver particles loaded on *Alternaria solani* toxins isolates on macronodes explants tolerant infection and morphological characterization in *Solanum tubrosum* in vitro culture

| | Survival % | morphological characterization % | | | | Contamination | Disease severity (%) |
|---|---|---|---|---|---|---|---|
| | | Shoot no | Shoot length | Leaves no | Rooting % | | |
| Control (-) | 100.0 | 3.02 | 2.34 | 6.10 | 8.21 | 0.0 | 0.0 |
| AST.2 100% | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 100.0 | 46.3 |
| 75 % | 100.0 | 4.31 | 3.21 | 6.75 | 6.31 | 0.0 | 0.0 |
| 50% | 66.66 | 3.62 | 3.12 | 5.67 | 5.23 | 0.0 | 0.0 |
| AST.5 100% | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 100.0 | 65.83 |
| 75 % | 88.88 | 3.42 | 2.44 | 6.56 | 2.33 | 0.0 | 0.0 |
| 50 % | 66.66 | 3.21 | 2.34 | 5.43 | 1.43 | 3.33 | 0.0 |
| AST.6100% | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 100.0 | 53.3 |
| 75 % | 33.3 | 3.40 | 2.29 | 5.10 | 3.44 | 11.11 | 0.0 |
| 50 % | 66.66 | 3.12 | 3.01 | 6.03 | 2.45 | 3.33 | 0.0 |
| AST.7100% | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 100.0 | 34.5 |
| 75 % | 66.66 | 4.01 | 3.26 | 5.93 | 1.97 | 11.11 | 0.0 |
| 50% | 66.66 | 3.25 | 3.19 | 6.43 | 2.41 | 0.0 | 0.0 |
| AST.11100% | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 100.0 | 23.0 |
| 75 % | 75.00 | 2.58 | 2.33 | 4.76 | 3.12 | 33.33 | 0.0 |
| 50 % | 75.00 | 3.09 | 3.07 | 4.23 | 2.16 | 11.11 | 0.0 |
| AgNPs 0.06uM | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.0 | 0.0 |
| LSD 5% | 21.105 | 0.3106 | 1.071 | 0.2651 | 0.9610 | 1.433 | 12.302 |

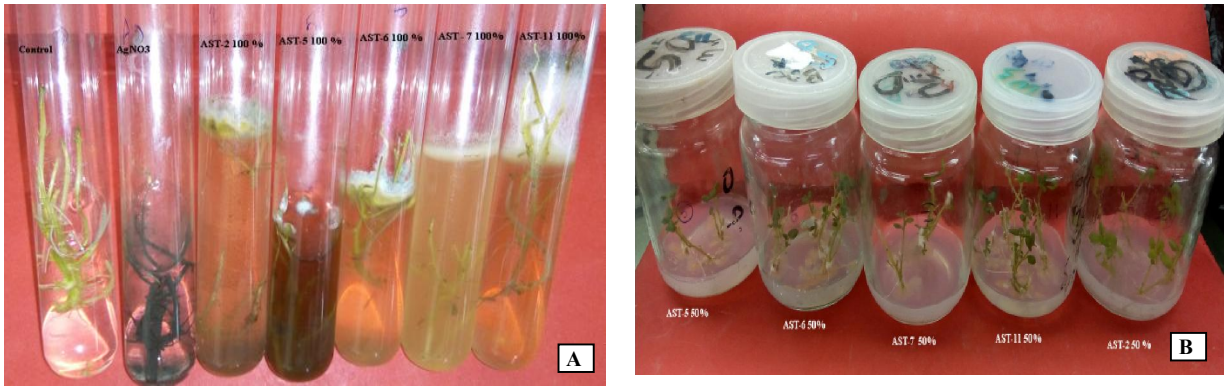



**Fig. 6:** (A) right to left in vitro explants (negative control), silver nitrite and five fungi isolates without nanosilver of *Solanum tubrosum*. (B) Five successive treatments after soaking in five toxins alternaria isolates loaded in silver.

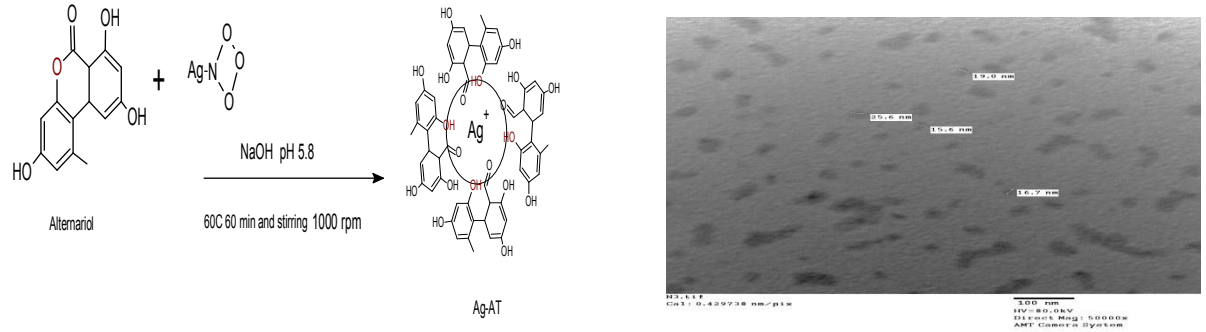



**Fig. 7:** Mechanism of Silver Nano-particle formation with alternariol and AgNPs embedded in AST-2 and their Size – TEM shows various size of nanoparticles

## Discussion

In *Solanum tubrosum*. cv. Sponta, the embryogenic culture was initiated and the various stages of embryo development were studied in detail. Our results indicated that the rate of embryogenesis was quite high in *Solanum tubrosum* as was reported in many other plant groups (Klimaszewska *et al.,* 2001 and Pullman *et al.,* 2003). The initiated cultures continued to proliferate for a period of two years or more making the culture a useful stable embryogenic source for future research purposes. Application of exogenous PGRs was found to be essential for the induction of callus, embryogenic culture establishment, proliferation, maturation and germination of embryos into plantlets. Although calli production was noted to be maximum on medium amended with BAP + NAA + 2,4-D, individual application of 2,4-D was also very effective in inducing callus from basal clove explant.

Generally, 2,4-D is considered to be one of the most important PGRs that regulate somatic embryogenesis *in vitro* (Zhang *et al.,* 2007). During induction into the medium, 2,4-D increased explant's endogenous auxin level, one of the crucial signals that determine cultured cells' fate to become embryogenic (Victor 2005). Becwar *et al.,* (1988) earlier reported that compared to higher concentrations, low levels of 2,4-D were more effective when combined with BAP for inducing embryogenic tissue. In auxin amended medium, cultured cell or tissue produce more ethylene than the auxin free cultures, which suppresses embryo development as the tissue multiplication continues to proceed without much check, the embryonic clumps develop into mature embryos only on medium amended with a very low level of 2,4-D (ranging 1-5 mg) (Razdan 1993). These observations support our present study that the auxin (2,4-D) has no significant effect on induction of embryos rather it has a considerable positive effect on callus production during dedifferentiation stage. Similar observation was noted in other plants like Melia where embryos were formed from pre-embryogenic determined cells, and did not depend on 2,4-D requirement (Evans *et al.,* 1981, Litz & Schaffer 1987, Lipavska & Konradova 2004, Robichaud *et al.,* 2004).

Microscopic techniques such as scanning electron microscopy, transmission electron microscopy and atomic force microscopy are mainly used for morphological studies of nanoparticles. Before morphological studies, there is need to standardize the synthesis of nanoparticles using plants or their extracts. The formation of various nanoparticles from their different salts gives characteristic peaks at different absorptions that can be monitored using UV-vis spectroscopy Fig. 3 and 7. For example, silver nanoparticles formation from silver ions show an absorption peak around 450 nm, while gold nanoparticles show an absorption peak around 550 nm. Similarly, several other metal nanoparticles show characteristic absorption peaks. A progressive increase in the characteristic peak with increase in reaction time and concentration of plant extracts with salt ions is a clear indicator of nanoparticle formation. UV-vis absorption spectra show peaks characteristic of the surface plasmon resonance of nanosized particles (Armendariz *et al.,* 2002; Gardea-Torresdey *et al.,* 1999; 2003; Shankar *et al.,* 2003; 2004; 2005; Amkamwar *et al.,* 2005a; 2005b; L'opeza *et al.,* 2005; Duran *et al.,* 2005; Ghule *et al.,* 2006; Chandran *et al.,* 2006; Rodriguez *et al.,* 2007; Sharma *et al.,* 2007; Huang *et al.,* 2007; Haverkamp *et al.,* 2007).

There have been relatively few studies on the applicability of silver nanoparticles to control plant diseases. Kim *et al.* (2008), studied the antifungal effectiveness of colloidal nano silver (1.5 nm average diameter) solution, against rose powdery mildew caused by *Sphaerotheca pannosa v*ar. *rosae.* It is a very wide spread and common disease of both green house and outdoor grown roses. It causes leaf distortion, leaf curling, early defoliation and reduced flowering. The effects of silver nanoparticles were investigated for its antifungal activity against sclerotium-forming phytopathogens especially *Rhizoctonia solani, Sclerotinia sclerotiorum* and *S. minor* by (Min *et al.,* 2009). They found that the antimicrobial efficiency of the silver nanoparticles observed among the fungi on their hyphal growth in the following order, *R. solani* > *S. sclerotiorum* > *S. minor*. In particular, the

sclerotial germination growth of *S. sclerotiorum* was most effectively inhibited at low concentrations of silver nanoparticles. A microscopic observation revealed that hyphae exposed to silver nanoparticles were severely damaged, resulting in the separation of layers of hyphal wall and collapse of hyphae. This study suggests the possibility to use silver nanoparticles as an alternative to pesticides for scleotium- forming phytopathogenic fungal controls.

## References

Ahmad, A., P.Mukherjee, S.Senapati, I.Mandal, I.M. Khan, R. Kumar and M.Sastry, 2003. Extracellular biosynthesis of silver nanoparticles using the fungus Fusarium oxysporum, Colloids Surf B: Biointerfaces, 28:313-318.

Ankamwar, B., M. Chaudhary, and M.Sastry, 2005a. Gold nanotriangles biologically synthesized using tamarind leaf extract and potential application in vapor sensing. Synth React Inorg Metal-Org Nano- Metal Chem., 35: 19–26

Ankamwar, B., C. Damle, A. Ahmad, and M. Sastry, 2005b. Biosynthesis of gold and silver nanoparticles using *Emblica officinalis* fruit extract, their phase transfer and transmetallation in an organic solution. J nanosci Nanotechnol., 5: 1665–1671.

Armendariz, V., J. L. Gardea-Torresdey, M. Jose-Yacaman J. Gonzalez, I. Herrera and J.G. Parsons, 2002. Gold nanoparticles formation by oat and wheat biomasses, in Proceedings –Waste Research Technology Conference at the Kansas City, Mariott-Country Club Plaza July30–Aug1.

ASTM Standard E2456, 2006. Standard terminology relating to Nnaotechnology. ASTM International, West Conshohocken, PA, 2006, DOI: 10.1520/E2456-06.

Balaji, S.D., S. Basavaraja, R. Deshpande, B. D. Mahesh, K. B. Prabhakar and A.Venkataraman, 2009. Extracellular biosynthesis of functionalized silver nanoparticles by strains of *Cladosporium cladosporioides* fungus. Colloids Surfaces B: Biointerfaces, 68: 88-92.

Barnett, H. L. and B. B. Hunter, 1972. Illustrated genera of imperfect fungi. 3rd Edition Burgess Publishing Co., 263 pp.

Basavaraja, S., S.D.Balaji, A. Lagashetty, A. H. Rajasab, A. Venkataraman, 2008. Extracellular biosynthesis of silver nanoparticles using the fungus fusarium semitectum. Mater. Res. Bull., 43, 1164-1170.

Becwar, M.R., S. R.Wann, M. A. Johnson, S.A. Werhagen, R.P. Feirer and R. Magmai, 1988. Development and characterization of in vitro embryogenic systems in Conifers. In: Ahuja MR (ed) Somatic cell genetics of woody plants. Kluwer Academic Publishers, Dordrecht, p. 1-18

Bhainsa, K.C. and S.F. D'Souza, 2006. Extracellular biosynthesis of silver nanoparticles using the fungus *Aspergillus fumigatus*, Colloids Surf B: Biointerfaces, 47:160-164.

Buzea, C., I. I. Pacheco and K. Robbie, 2007.Nanomaterials and nanoparticles: Sources and toxicity, Biointerphases, 2(4): MR17-MR71,.

Chandran, P.S., M. Chaudhary, R. Pasricha, A. Ahmad, and M. Sastry, 2006. Synthesis of gold nanotriangles and silver nanoparticles using *Aloe vera* plant extract, Biotechnology Prog., 22: 577-583.

Duran, N., P. D. Marcato, O. L. Alves, G.I.H. De Souza, and E. Esposito, 2005. Mechanistic aspects of biosynthesis of silver nanoparticles by several *Fusarium oxysporum* strains. J Nanobiotechnol., 3: 8–14.

Evans, D.A., W.Sharp and C. Flick, 1981. Growth and behavior of cell cultures. In: Thorpe TA (ed) Plant Tissue Culture - Methods and Application in Agriculture, Academic Press, Orlando, Florida, USA, p. 45-113.

Gajbhiye, M., J. Kesharwani, A. Jngle and A. Gade and M. Rai, 2009. Fungus mediatedsynthesis of silver nanoparticles and their activity against pathogenic fungi in combination with fluconated. Nanomedicine, 5(4): 382 – 386.

Gardea-Torresdey, J.L., E. Gomez, J. R. Peralta-Videa, J.G. Parsons, H.Troiani, and M. Jose-Yacaman, 2003. Alfalfa sprouts: a natural source for the synthesis of silver nanoparticles. Langmuir, 19:1357–1361.

Gardea-Torresdey, J.L., K. J.Tiemann, G. Gamez, K. Dokken, S. Tehuacanero and M. Jos´e-Yacam´an, 1999. Gold nanoparticles obtained by bio precipitation from gold (III) solutions. J Nanopart Res 1:397– 404.

Ghule, K., A.V. Ghule, J.Y. Liu, and Y.C. Ling, 2006. Microscale size triangular gold prisms synthesized using Bengal gram beans (*Cicer arietinum* L.) extract and HAuCl4 × 3H2O: a green biogenic approach. J Nanosci Nanotechnol, 6: 3746–3751

Haverkamp, R.G., A.T. Marshall, and Van D.Agterveld, 2007. Pick your carats: nanoparticles of gold–silver–copper alloy produced *in-vivo*. J Nanopart Res., 9: 697–700.

Huang, J., Q. Li, D. Sun, Y. Lu, Y. Su, X. Yang, H. Wang, Y. Wang, W. Shao, N. He, J. Hong, and C. Chen, 2007. Biosynthesis of silver and gold nanoparticles by novel sundried *Cinnamomum camphora* leaf. Nanotechnology, 18: 105104-105114.

Kashiwagi, Y., M. Yamamoto, M. Nakamoto, 2006. Facile size-regulated synthesis of silver nanoparticles by controlled thermolysis of silver alkylcarboxylates in the presence of alkylamines with different chain lengths. J. of Colloid and Interface Science, 300: 169–175.

Kathiresan, K., S. Manivannan, A. M. Nabeel and B. Dhivya, 2009. Studies on silver nanoparticles synthesized by a marine fungus *Penicillu fellutanum* isolated from coastal mangrove sediment. Colloids Surfaces B: Biointerfaces, 71: 133-137.

Katrina, V., O. Task, E. Amro, F. David, S. Jessica, R.Battelle, and R. Venkatapathy, 2010. Scientific, Technical, Research, Engineering and Modeling Support Final Report State of the Science Literature Review: Everything Nanosilver and More. U.S. Environmental Protection Agency Office of Research and Development Washington, DC 20460. Final Report. PP 221.

Kim, H.S., H. S. Kang, G. J. Chu, and S.B. Hong, 2008. Antifungal effectiveness of nanosilver colloid against rose powder mildew in greenhouses. Solid state Phenomena, 135: 15-18.

Kim, J.S., E. Kuk, K. N.Yu, J. H. Kim, S.J. Park, H. J. Lee, S. H. Kim, Y. K. Park, Y.H. Park, C.Y.Hwang, Y. K. Kim, Y. S. Lee, D. H. Jeong and M.H. Cho, 2007. Antimicrobial effects of silver nanoparticles, Nanomedicine, 3:95-101.

Klimaszewska, K., J. S. Park, C.Overton, I.Maceacheron and J.M. Bonga 2001. Optimized somatic embryogenesis in *Pinus strobus* L. In vitro Cellular and Developmental Biology -Plant 37: 392-399.

L'opeza, M.L., J. G. Parsonsb, J.R. Peralta Videab and T. L. Gardea-Torresdey, 2005. An XAS study of the binding and reduction of Au(III) by hop biomass. Microchem J 81:50–56.

Lipaska, H. and H. Konradova, 2004. Somatic embryogenesis in conifers: the role of carbohydrate metabolism. In Vitro Cellular and Developmental Biology - Plant 40: 23-30.

Litz, R and B.Schaffer, 1987. Polyamines in adventitious and somatic embryogenesis in mango (*Mangifera indica* L.). Journal of Plant Physiology 128: 251-258.

Min, J.S., K.S. Kim, S.W. Kim, J. H. Jung, K. Lamsal, S.B. Kim, M. Jung, and Y.S. Lee, 2009. Effects of Colloidal Silver Nanoparticles on Sclerotium-Forming Phytopathogenic Fungi. Plant Pathol. J., 25(4): 376-380.

Mukherjee, P., A. Ahmad, D. Mandal, S. Senapati, R. S. Sainkar, I. M. Khan, R. Parishcha, , V.P.Ajaykumar, M. Alam, R. Kumar and M. Sastry, 2001. Fungus-mediated synthesis of silver nanoparticles and their immobilization in the mycelia matrix. A novel biological approach to nanoparticle synthesis. Nano Letters, 1(10): 515-519.

Mukherjee, P., M. Roy, B. P.Mandal, G.K.Dey, P. K. Mukherjee, J. Ghatak, A. K.Tyagi, S.P. Kale, 2008. Green synthesis of highly stabilized nanocrystalline silver particles by a nonpathogenic and agriculturally important fungus *T. asperellum*, Nanotechnology, 19:075103 (7pp), doi: 10.1088/09574484/19/7/075103.

Murashige, T and F.Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plant 15: 473-497.

Pullman, G.S., K. Namjoshi and Y.Zhang, 2003. Somatic embryogenesis in loblolly pine (*Pinus taeda* L.): improving culture initiation with abscisic acid, silver nitrate and cytokinin adjustments. Plant Cell Report 22: 85- 95.

Razdan, M.K., 1993. Somatic embryogenesis. In: Razdan MK (ed), An introduction to plant tissue culture, Oxford & IBH Publi Co Pvt Ltd., New Delhi, p. 87-102.

Robichaud, R.L., V.C. Lesser and S. A. Merkle, 2004. Treatments affecting maturation and germination of American chestnut somatic embryos. Journal of Plant Physiology 161: 957-969.

Rodriguez, E., J. G. Parsons, J. R. Peralta-Videa, G. Cruz-Jimenez, J. Romero- Gonzalez, and B.E. Sanchez-Salcido, 2007. Potential of *Chilopsis linearis* for gold phytomining: using XAS to determine gold reduction and nanoparticle formation within plant tissues. Int J Phytoremed, 9: 133–147.

Sadowski, Z., H. I. Maliszewska, B. Grochowalska, I. Polowczyk and T. Kozlecki, 2008b. Synthesis of silver nanoparticles using microorganisms, Materials Science-Poland, 26(2): 419-424.

Sadowski, Z., I. Maliszewska, I. Polowczyk, T. Kozlecki, and B. Grochowalska, 2008a. Biosynthesis of colloidal-silver particles using microorganisms, Polish J. Chem., 82: 377-382.

Sastry, M., M. Ahmed, M. I. Khan and R. Kumar, 2003. Biosynthesis of metal nanoparticles using fungi and actinomyvete. Cur. Sci., 85: 162 – 170.

Shankar, S., A. Ahmad, and M. Sastry, 2003. Geranium leaf assisted biosynthesis of silver nanoparticles, Biotechno. Prog., 19: 1627 1631.

Shankar, S., A. Rai, A. Ahmad and M. Sastry, 2004. Rapid synthesis of Au, Ag and bimetallic Au core-Ag shell nanoparticles using Neem (*Azadirachta indica)* leaf broth. J. Colloid Inter. Sci., 275: 496-502.

Shankar, S.S., A. Rai, A. Ahmad, and M. Sastry, 2005. Controlling the optical properties of lemongrass extract synthesized gold nanotriangles and potential application in infrared-absorbing optical coatings. Chem Mater., 17: 566–572.

Sharma, N.C., S.V.Sahi, S. Nath, J.G.Parsons, J.L. Gardea-Torresdey and T. Pal, 2007. Synthesis of plant-mediated gold nanoparticles and catalytic role of biomatrix-embedded nanomaterials. Environ Sci Technol., 41: 5137–5142.

Victor, M.J., 2005. Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis.Plant Growth Regulation 47: 91-110

WHO, World Health Organization, 2002. Silver and silver compounds: Environmental aspects. (Concise international chemical assessment document; 44). International Programme on Chemical Safety II. Series. http://www.who.int/ipcs/publications/cicad /en/cicad44.

Wright, J.B., K. Lam, D. Hansen and R.E. Burrell, 1999. Efficacy of topical silver against fungal burn wound pathogens. Am. J. Infect. Control, 27:344-350.

Zhang, C.X., Q. Li and L.Kong (2007). Induction, development and maturation of somatic embryos in Bunge's pine (Pinus bungeana Zucc. ex Endl.). Plant Cell Tissue and Organ Culture 91: 273-280.