



## Application of Silver Nanoparticles for Production of *Sesamum* Plants Free of Phytoplasma Disease in Tissue Culture

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**S**ESAMUM phyllody is a severe disease that displays various symptoms such as floral virecence, phyllody, proliferation, yellowing, cracking of seed capsules, and the formation of dark exudates on foliage. Infected plants were found to have blue areas in the phloem region using Diane's stain, and phytoplasma structures in the phloem sieve elements were detected using transmission electron microscopy. The amplification of a phytoplasma-specific 1200-bp 16S recombinant DNA (rDNA) fragment confirmed that the plants were infected with a phytoplasma. To regenerate phyllody-free plantlets from meristem tips, Murashige and Skoog medium was utilized and phytoplasma indexing was performed using nested PCR (nPCR) on the mother plant and the *in vitro*-regenerated plantlets. In an effort to produce phyllody-free plants, explants from phyllody-positive *Sesamum* plants were cultured in Murashige and Skoog medium with varying concentrations of silver nanoparticles (Ag NPs). As the concentration of Ag NPs increased up to 300mg L<sup>-1</sup>, shoot regeneration was reduced. However, *in vitro*-regenerated plantlets at a concentration of 250mg L<sup>-1</sup> were tested negative for phyllody and proved to be phyllody free. The most effective concentration was found to be 250mg L<sup>-1</sup>, resulting in 100% phyllody-free plants and 100% of cultures showing shoot regeneration. Thus, treatment with Ag NPs is a promising technique for producing phyllody-free *Sesamum* plants.

**Keywords:** nPCR, Phyllody, *Sesamum*, Silver nanoparticles, Tissue culture.

### Introduction

Phytoplasma is a plant pathogen that causes several diseases in crops, including *Sesamum*. Tissue culture is a common method for producing disease-free plants, but it is often limited by contamination. In this study, silver nanoparticles (Ag NPs) were used to enhance the efficacy of tissue culture for producing phytoplasma-free *Sesamum* plants.

*Sesamum* production lags behind that of other significant oilseed crops despite its long history and high nutritional value due to its low seed yield, seed cracking, unpredictable growth pattern, and disease susceptibility (Ashri, 1998). *Sesamum* phyllody disease, characterized by stunted plants and floral parts transforming into leaf-like structures without capsules or seeds, poses a serious threat to *Sesamum* production. Reports

of *Sesamum* phyllody emerged in other countries, indicating the danger this disease poses to global *Sesamum* cultivation (Akhtar et al., 2009). Additionally, the disease has been identified in a number of Turkey's *Sesamum*-growing districts (Kersting, 1995; Sertkaya et al., 2007; Ikten et al., 2011).

The most economically significant species of the *Sesamum* genus is *Sesamum indicum* L., mainly because of its high oil content (50–60%) and high ratio of unsaturated fatty acids (Uzun et al., 2008). Sesamin and sesamol, two antioxidant lignins found in *Sesamum* oil, are particularly resistant to oxidative degradation (Yoshida & Takagi, 1997; Moazzami & Kamal-Eldin, 2006; Erbas et al., 2009). One of the most major diseases affecting *Sesamum* plants is phyllody disease, which results in great economic losses throughout the world (Sertkaya et al., 2007).

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Received 02/06/2023; Accepted 19/09/2023

DOI: 10.21608/ejbo.2023.212452.2340

Edited by: Prof. Dr. Neveen M. Khalil, Faculty of Science, Cairo University, Giza 12613, Egypt.

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Phytoplasmas are prokaryotic pathogens that live on plant and insect hosts and belong to the class Mollicutes, which also includes other infections with no cell walls. Plant diseases are brought on by phytoplasmas, which affect the phloem tissue and are transmitted from infected plants to healthy ones by insects that feed on sap (Seemuller et al., 1998; Lee et al., 2000; Bertaccini, 2007; Hogenhout et al., 2008). Multiple phytoplasma strains spread by diverse insect vectors have been linked to the phytoplasma disease known as *Sesamum* phyllody, which contains flower parts that resemble leaves. Previous investigations showed that the *Sesamum* phyllody phytoplasmas and their insect vectors showed diversification despite the disease signs being the same everywhere (*Orosius orientalis* (Esmailzadeh-Hosseini et al., 2007; Sertkaya et al., 2007; Akhtar et al., 2009), *Circulifer haematoceps* (Salehi and Izadpanah, 1992; Kersting, 1993), and *Neolaliturus haematoceps* are among the leafhoppers).

Several phytoplasma diseases, such as *cassava witches' broom* (CaWB), *sugarcane grassy shoot* (SCGS), *sugarcane white leaf* (SCWL), *sugarcane yellow leaf* (SCYL), *rice orange leaf* (ROL), *maize bushy stunt* (MBS), *Sesamum phyllodia* (SP), and *cinnamon witches' broom* (CinWB) were detected in Vietnam (Hoat et al., 2023).

Phytoplasmas infect hundreds of plant species, causing numerous diseases with a wide range of symptoms. Common phytoplasma-induced symptoms include yellowing, decline, witches' broom, leaf curl, abnormally long internodes, floral virescence and distortion, shoot multiplication, sterility, and phyllody. Among these, phyllody—the transformation of floral parts into leafy structures—is the most prominent and noticeable symptom of phytoplasma infection (Bertaccini et al., 2005; Al-Zadjali et al., 2007; Kamińska et al., 2011; Salehi et al., 2011; Win et al., 2011).

Phytoplasmas are poorly understood since they are difficult to be isolated *in vitro* (Šeruga et al., 2003), and the additional techniques that were frequently employed in the past are ineffective for identifying and differentiating phytoplasmas (Lee et al., 1998; Hodgetts et al., 2007; Santos-Cervantes et al., 2008). Recently for the detection, characterisation, and phylogeny of phytoplasmas, DNA-based molecular approaches have been widely used (Lee et al.,

1993; Bhat et al., 2006). For molecular detection, identification and phylogenetic analysis of phytoplasmas, universal phytoplasma-specific primers have been used extensively (Seemuller et al., 1998; Lee et al., 2000; Marccone et al., 2000). This is because the 16S rRNA gene is highly conserved among phytoplasma groups, present in two copies, and easy to amplify. The phytoplasmas infecting *Sesamum* plants have been found and characterized in a number of molecular investigations using the 16S rRNA gene. *Sesamum* plants all over the world are infected with various phytoplasma strains that belong to various 16Sr groups, according to PCR-RFLP investigations. For instance, research has revealed that India's *Sesamum* plants are infected with a phytoplasma from the 16SrI group (Khan et al., 2007), 16SrI-B (Myanmar) (Win et al., 2010), 16SrII (Iran) (Esmailzadeh-Hosseini et al., 2007), 16SrII-D (Sertkaya et al., 2007). The ultrastructural alterations generated by phytoplasma infection are thought to be the result of sugar and starch accumulation, which impedes sieve tube performance and phloem flow. The quantity of phytoplasma units in different phloem cells varied, and this has been due to the severity of the symptoms, according to Kesumawati et al. (2006) and El-Banna et al. (2015).

According to El-Beltagi & Mohamed (2013), plants have evolved a variety of defense systems. Enzymes have a critical role in pathogenesis, infection, and defense. Peroxidase was a vital enzyme that regulated plant growth and development. This process includes the development of cell walls and their eventual lignification, as well as the protection of tissue against injury and infection by harmful bacteria.

Polyphenols are well-known antibacterial chemicals that also operate as signal molecules in the host plant to activate plant defense genes, ROS scavengers, and singlet oxygen quenchers (Nicholson & Hammerschmidt, 1992; Reveles-Torres et al., 2018). The presence of high amounts of polyphenols in plants following phytoplasma infection demonstrates the host's reaction, and their increased accumulation may be related to the host's defense mechanisms (Junqueira et al., 2004).

Nanotechnology plays an important role in agriculture, especially in the production of disease-resistant crops, increasing crop quality,

producing high yields, and enhancing resistance to biotic and abiotic stresses (Sompura et al., 2023). For biological therapies, the usage of generated Ag NPs has a high antibacterial efficiency (Saqib et al., 2022). Agriculture, water resources, safety and security, environment, engineering, biology, energy conversion, and many other fields could be affected by nanotechnology (Hoang et al., 2022). However, a few factors, such as their composition, surface charge, physical and chemical characteristics, size, concentration, and susceptibility of the plant species, affect how the generated nanomaterials affect the treated plants. According to Elsahhar et al. (2022), potato plants that had been infected with Potato Virus Y were completely cleared from the affected plants when the concentration of chitosan nanoparticles was increased from 100 to 250mg L<sup>-1</sup>. After 48 hours of virus inoculation, therapy with Ag NPs can show curative antiviral activity that renders the virus inactive. Aphids were less effective in acquiring and dispersing Bean Yellow Mosaic Virus (BYMV) when they were exposed to plants treated with Ag NPs before the virus was acquired (El Gamal et al., 2021). The three objectives of the current study were to, first, identify and characterize the phytoplasma strains and vectors linked to *Sesamum* phyllody in Egypt; second, assess the impact of silver nanoparticles on phytoplasma; and third, cultivate *Sesamum* plants free of phytoplasma using tissue culture and Ag NPs.

### Materials and Methods

#### *Sesamum* samples source

In this study, samples were collected from *Sesamum* fields in Shibin El Qanater City, Al Qalyubia Governorate, between June and September of 2022. Phyllody symptoms on *Sesamum* plants have been visually detected by foliar and floral symptoms (Fig. 1). The plant samples have been collected and maintained at -80°C for molecular analysis.

*PCR amplification of the 16Sr RNA and the SecY gene, as well as DNA extraction*

#### *Plant material collection*

Leaf samples from symptomatic and asymptomatic *Sesamum* plants have been collected from Al Qalyubia Governorate. The samples were kept in the greenhouse conditions and used for further studies.

#### *PCR amplification and DNA extraction*

Dellaporta extraction was used to extract DNA from fresh *Sesamum* plants with phyllody, witches broom symptoms (Dellaporta et al., 1983). Degenerate and specific PCR primers amplified extracted DNA. High-quality Milli-Q water adjusted primer concentrations to 10 mM. The first PCR reaction used degenerate PCR primers P1/P7 (Table 1) followed by nested (specific) R16F2n/R16R2. 250ng (10µL) extracted DNA, 10 mM dNTPs, 0.1µM primers, 1.25mM MgCl<sub>2</sub>, and Taq polymerase (Thermo Scientific) were in the 50µL PCR reaction mixture. In a Bio-Rad DNA Thermal Cycler (PTC100), viral DNA was amplified by 35 cycles of melting, annealing, and DNA extension conditions: 1 cycle of 3min at 94°C, 30 cycles of 1min, 2min at 53°C and 55°C for nested primer, 2min at 72°C, and final extension for 10min at 72°C. The reaction mixtures were then withdrawn at 4°C. After amplification, the DNA was electrophoresed at 120 volts on a 0.5 TBE-buffer of 1% agarose gel for an hour. The gel was stained with ethidium bromide (0.5µg/mL) and photographed with a gel documentation system (Bio-Rad, GelDoc XR). PCR markers were the Vivantis VC 100 bp Plus and 1kb Plus DNA ladders.

#### *Phyllody transmission*

The main methods of phytoplasma transmission from plant to plant are through the vegetative propagation of infected plant material or through graft inoculation.

TABLE 1. The PCR primers used for identifying phytoplasma on *Sesamum* plants

Primers	Sequencing	Expected size (bp)	Reference
P1	5' AAGAGTTGATCCTGGATT-3	1800	Sinclair et al. (2001)
P7	5' -CGTCCTTCATCGGCTCTT-3'		
R16F2n	5'-GAAACGACTGCTAAGACTTGG-3'	1200	Wang & Hiruki (2001)
R16R2	5'-TGACGGGCGGTGTGTACAAACCCC-3'		

#### *Transmission through seeds*

*Sesamum* test plant seeds were seeded in pots set up in insect-free cages for transmission trials using *Orosius orientalis*. Twelve healthy *Sesamum* plants were put into each of the three cages (totaling 36 plants). In each cage aside from the control one, 50 of *O. orientalis* adults were let to feed continuously on *Sesamum* plants with the phyllody infection. No insects were released onto the 12 healthy *Sesamum* plants in one cage, which was utilized as a control. Each cage's plants were continuously observed for the emergence of symptoms, and leaf samples were repeatedly taken for molecular testing. After the first phyllody symptoms on the plants appeared, the bug samples were also collected for study.

#### *Graft transmission*

Graft transmission tests were also run to see if healthy *Sesamum* plants might spread the phyllody phytoplasma to afflicted plants. The infected *Sesamum* plants were grafted onto a total of ten healthy *Sesamum* plants. A section of a *Sesamum* branch that was 10 centimeters long and had typical phyllody was grafted onto the stem of a *Sesamum* plant that was healthy after it was cut from an infected plant (Akhtar et al., 2009). Within the controlled environment of a greenhouse, grafted plants were monitored for the development of any symptoms while the graft unions were covered with parafilm. Utilizing molecular analysis, it was further proven that grafted plants were affected by the phytoplasma. The study applied five periwinkle and *Hibiscus syriacus* plants. Graft transmission was done using the side grafting technique. *Sesamum* infected plants' disease scions were trimmed into a "V" form from both sides. On the periwinkle stock plants' healthy slanting cut, the scions were put. High-density polythene strip was used to tie around the grafted area. For the development of symptoms, the inoculated plants were housed in an insect-proof glasshouse. The number of plants exhibiting symptoms and the length of time it took for signs to develop were noted. The grafted periwinkle plant exhibiting phyllody signs was also subjected to PCR testing. Two sets of primers, P1/P7 (Table 1) and R16F2n/R16R2, were used in direct and nested PCR to analyze the DNA from healthy and phyllody-symptomatic periwinkle plants.

#### *Insect vector transmission*

Using a hand-held suction device, samples

of insects were collected in the morning. The remainder of each insect sample was caged-reared with *Sesamum* plants in a greenhouse environment while a portion of each insect sample was maintained at 20°C for DNA extraction and phytoplasma testing by PCR. At least ten samples of each type of insects were used in the *Sesamum* phyllody phytoplasma PCR analysis. It was determined to stop breeding insect species that gave positive PCR results for phytoplasma. However, when the phyllody in the leafhopper samples was detected by DNA methods, *Orosius orientalis* species was continuously raised in cages.

#### *Transmission electron microscopy (TEM)*

To detect ultrastructural abnormalities, healthy and diseased *Sesamum* plant leaves were examined using transmission electron microscopy (TEM). Sliced into separate vials, 2x2mm portions of leaf samples were fixed for an hour in 2.5% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.4). The tissues were then rinsed three times in sodium phosphate buffer for a total of 30 minutes each after the fixative solution was withdrawn. After washing, osmium tetroxide ( $\text{OsO}_4$ ) in a 1% solution was applied before removing the buffer. After that, the tube was kept at 4°C for an additional 15min. The samples were dehydrated in a sequence of five concentrations of ethanol prior to each phase's 15min exposure to the 100% concentration. The procedure with 100% ethanol was carried out two times. Once every 15min, fresh epoxy resin is dripped copiously into the sample tube until an overnight resin saturation rate of 75% is achieved. Before being inserted into flat BEEM capsule molds, samples were immersed in 100% resin for at least a day.

The resin was then incubated overnight at 60°C in an oven. Leica model EM-UC6 ultramicrotome was used to segment samples into 60nm-thick (90-100nm) sections that were placed on copper grids (400 mesh). 2% uranyl acetate and 10% lead citrate were used to stain ultra-thin sections twice; the sections were then given lead citrate to dry for five minutes after 10min of thorough drying. Images were taken using a CCD camera type AMT and obtained using a JEOL (JEM-1400 TEM, Japan) transmission electron microscope while stained slices were being viewed by Optronics camera with side mounts layout and 1632x1632 pixel resolution.

### *Determination of enzymes activity*

#### *Preparation of enzymes extract*

Fresh plant tissues were ground in a cold mortar and pestle and homogenized in cold sodium phosphate buffer (100mM, pH 7) containing 1% (w/v) polyvinylpyrrolidone (PVP) and 0.1mM EDTA. 4mL of extraction buffer was used for every gram of plant tissue throughout the extraction process. The homogenate was centrifuged at 4500 rpm and 4°C for 15min. In the supernatant, polyphenol oxidase (PPO) and peroxidase (POD) activities were assessed according to El-Beltagi et al. (2012).

#### *Peroxidase activity (POD)*

Fresh plant tissues were broken up using a cold mortar and pestle, and then they were homogenized in a cold sodium phosphate buffer (100mM, pH 7) that contained 1% (w/v) polyvinylpyrrolidone (PVP) and 0.1mM EDTA. During the entirety of the extraction process, 4 milliliters of extraction buffer was utilized for each gram of plant tissue that was being processed. The homogenate was centrifuged for 15min at a temperature of 4 degrees Celsius and 4500 revolutions per minute. It was determined whether the polyphenol oxidase (PPO) and peroxidase (POD) activities were present in the supernatant according to El-Beltagi et al. (2012).

#### *Polyphenol oxidase activity (PPO)*

The reaction mixture contained 100mL of crude enzyme, 600mL of catechol, and 2.3mL of phosphate buffer (0.1M, pH 6.5). The absorbance at 420nm was measured using a spectrophotometer both immediately and one minute later. One unit of PPO activity was defined as the amount of enzyme that caused a rise in absorbance of 0.001 per minute at 420nm (Oktay et al., 1995).

### *Photosynthetic pigments assessment*

The amounts of photosynthetic pigments including chlorophyll a, chlorophyll b, and carotenoids were measured in about 1gm of *Sesamum* leaves 25 days after infection by phytoplasma (Murdock et al., 2008; Phanjom et al., 2012). To balance the acidity of the sap and prevent chlorophyll from turning into pheophytin, fresh leaf samples were crushed in a mortar with 10mL of 90% acetone, 3g of fine sand, and 2g of calcium carbonate. The acetone extract was filtered through a fine glass filter (G4) in the center, and the residue was rinsed with small amounts of 90% acetone until it became pigment free. Each filtrate was made up to 50mL volume with 85% acetone.

Using Lichtenthaler's method (1987), pigment concentrations in mg g<sup>-1</sup> fresh weight (FW) were determined.

### *Preparation of free hand sections for Diene's stain*

Dienes' staining is a quick and simple preliminary technique for assessing plant samples quickly in order to find phytoplasmas in tissues of infected *Sesamum* plant. Both healthy and diseased *Sesamum* plants' stems were collected for sampling. The free hand cross sections made from these samples were then stained with Diene's stain for 5 minutes in accordance with the procedure described in the publications by Deeley et al. (1979), Musetti & Favali (2004) and Musetti et al. (2013). After staining, the slices were immediately examined under a light microscope after being rinsed with distilled water (LEICA ICC50 HD).

### *Silver nanoparticles synthesis*

Silver nitrate (AgNO<sub>3</sub>, Sigma-Aldrich, USA) was reduced to a colloidal solution of Ag NPs using the co-precipitation procedure with Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub> (molecular grade, Sigma-Aldrich) (Van Dong et al., 2012). 50mL of AgNO<sub>3</sub> (0.001M) were added to a 250mL flask and heated for five minutes while being agitated. Five minutes later, 5mL of sodium citrate trihydrate (1%) were added, one drop at a time, while stirring. The colloidal solution containing Ag NPs was allowed to cool at room temperature until it attained a pale-yellow to yellowish brown tint before starting the physicochemical characterization. Colloidal Ag NPs were created at that stage.

### *Silver nanoparticles characterization*

*Determination of particle size:* Using a Zetasizer Nano ZS instrument, dynamic light scattering (DLS) analysis was used to assess the particle size distribution and zeta potential of Ag NPs (Malvern, UK). In order to conduct the analysis, the colloidal nanoparticle solution of Ag NPs was diluted in distilled water (Murdock et al., 2008).

*The X-Ray diffraction measurement:* A 1.54-mm Cu K radiation tube was used in an X-ray diffractometer (X'pert PRO, Panalytical, The Netherlands) that ran at 40kV used for measurement of the physico-chemical crystalline nature of the Ag NPs. The colloidal solution of Ag NPs has been centrifuged at 25,000 × g for 30min at 4°C till the powder phase yield forms; then the precipitated Ag NPs have been dried and bombarded using X-rays for phase analysis (Phanjom et al., 2012).

*Determination of surface morphology:* Using a high-resolution transmission electron microscope (HRTEM), the Ag NPs' size and shape were evaluated (Tecnai G2, FEI, The Netherlands). Ag NPs were ultrasonically treated for 10min. The particles were then dropped onto a copper grid covered in a 10-nm-thick carbon layer. The photos were captured at 200kV of voltage.

#### *In vitro cultures initiation*

Explants of *Sesamum* (*Sesamum indicum* L.), previously analyzed using RT-PCR techniques, were obtained from *Sesamum* plants originating from phytoplasma-infected plants and used to start *in vitro* propagation materials (Fig. 1).

*Sesamum* explants were cleaned in distilled water before being surface sterilized with 0.2% mercury chloride (HgCl<sub>2</sub>) for a few minutes, 10% sodium hypochlorite for ten minutes, and 70% ethanol. Prior to culture in Murashige & Skoog (MS) medium (1962) supplemented with 6g L<sup>-1</sup> agar, 30g L<sup>-1</sup> sucrose, and 4.4g MS medium, the pH of the explants was adjusted to pH 5.7. The media were then autoclaved at 121°C for 20min and pressure cooked at 1kg cm<sup>2</sup>. The prepared medium has been incubated for 3 days at the media incubation room before the cultivation to avoid any contamination could be found in the medium. Then the sterilized explants were cultivated in the prepared medium inside laminar air flow hood under controlled conditions. Following cultivation, cultures were incubated for 16h each day in a growth environment with 1 white fluorescent light, temperature of 27°C, and 70% relative humidity. The regenerated shoots were subcultured in a 250mL jar for full multiplication after 4 weeks of cultivation. The produced shoots were taken out of the jars and separated into segments with two to three nodes. The nodal segments were then arranged vertically on new multiplication media, which contains the same components as in the previous phase. The regenerating *Sesamum* shoots were subcultured three times (4 weeks each).

#### *Ag NPs treatments for phytoplasma eradication*

In order to evaluate the response to shoot regeneration, shoot proliferation, plantlet growth, and phytoplasma eradication, the regenerated shoot (3cm in length) was cultured on MS culture media supplemented with different concentrations (100, 150, 200, 250, and 300mg L<sup>-1</sup>) of Ag NPs.

Filtered, sterile silver treatments were added to the autoclaved medium. The same conditions that were outlined for initiation cultures were used to grow the cultures.

#### *In vitro rooting induction stage*

After being subcultured for 4 weeks, the shoots that grew on medium containing antiviral Ag NPs were removed from the culture jars and separated into individual shoots. These were then cultivated on a rooting medium free of Ag NPs, which was supplemented with 4.4g MS, 30 g L<sup>-1</sup> sucrose, 6g L<sup>-1</sup> agar, and 0.1mg L<sup>-1</sup> 3- $\alpha$ -naphthalene acetic acid (NAA) at pH 5.7, with a density of 10 shoots per culture jar. Following cultivation, the cultures were exposed to optimal light conditions (1-10 klx) for 16 hours daily and maintained at a temperature of 27°C with 70% relative humidity under 40 mol m<sup>-2</sup> s<sup>-1</sup> cool white fluorescent light.

#### *Hardening and acclimatization*

After 4 weeks of transplanting *Sesamum* plantlets with healthy root systems, they were gently removed from the culture jar and washed with tap water to discard any remaining medium that might prevent roots from absorbing nutrients from the substrates of the acclimatization culture. Next, the rooted plantlets were treated with a 2% liquid fungicide (Topsin M-70) by dipping the root base for 10min to prevent pathogenic infection. Then the plantlets transferred to plastic pots 10 x 15 cm<sup>2</sup> containing a mixture of peat moss and perlite in the ratio of 3: 1. The *Sesamum* plantlets were covered by transparent polythene plastic bags with small holes for air circulation with temperature at 25 $\pm$ 2°C and 1000 lux illumination and for keeping high humidity conditions of 95% around the plants. Following a period of 10 days, the plantlets were gradually exposed to natural atmospheric conditions for 30 to 40 minutes each day. Another 10 days later, the plantlets were fully exposed to natural atmospheric conditions and transplanted into bigger pots filled with natural soil. Once the plantlets were adequately hardened, they were transferred to a greenhouse and subjected to RT-PCR to determine the presence or absence of phytoplasma at 30 days of age.

#### *Phytoplasma detection after Ag NPs treatments*

After 30 days of acclimatization, leaves were collected from *Sesamum* plants that underwent the *in vitro* therapy and controls for checking the presence/absence of phytoplasma using RT-PCR.

### Statistical analysis

This experiment included 5 Ag NPs concentrations, equivalent to 4 treatments. The experiment was planned in a purely random manner. Each treatment had 10 replicates (10 x 5 = 50 replicates), and each replicate had 10 jars, with each jar containing 10 plants (50 +10 = 60 replicates).

The impact of antiviral Ag NPs on the percentage of shoot regeneration was analyzed using a one-way analysis of variance (ANOVA). Mean separation was performed by the High-Range Statistical Domain (HSD) via Tukey's test, which compares the means of each treatment to the means of all other treatments, to identify any differences between the means (Meyers & Grossen, 1974). The results were analyzed using ANOVA in SAS (SAS Institute, 2006).

## Results

### Samples source and symptomatology

*Sesamum* infected and healthy samples were collected from *Sesamum* fields in Shibin Elqanater City, Al Qalyubia Governorate, Egypt. The collected samples have showed symptoms of phytoplasmas like phyllody, yellowing, small leaves, short internodes, proliferation, and green leaf like floral (Fig. 1). All collected samples were

subjected directly to molecular detection using nPCR.

### Molecular detection

Phytoplasma detection was done using universal phytoplasma PCR primer P1/P7. Nested primer R16F2n/R16R2 was used to confirm the infection with a product size range about 1200 bp for all infected samples, while no results were shown in healthy plants (Fig. 2).

### Transmission studies

#### Transmission through seeds

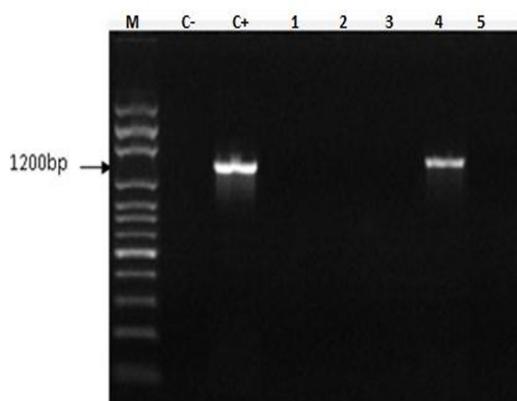
Seed transmission of phytoplasma disease through *Sesamum* seeds has not succeeded in greenhouse experiment while disease transmission through dodder was achieved in only 22% of the tested plants.

#### Graft transmission

Graft transmission from infected plants to healthy plants through grafting has successfully transmitted the phytoplasma that causes symptoms like phyllody disease. The causative agent was transmitted to 5 healthy plants of periwinkle and *Hibiscus syriacus*, producing disease symptoms after 25-35 days from the inoculation in all the grafted plants (100% transmission), whereas the results were confirmed by PCR.



Fig. 1. (A) Healthy plants, (B) Floral proliferation, (C & D) Proliferation of auxiliary shoots, (E & F) Empty *Sesamum* pods associated with phytoplasma



**Fig. 2.** Agarose gel electrophoresis of PCR amplification products [M: 100 bp DNA ladder, lane C-: negative control, lane C+: positive control, lanes 1, 2, and 3: healthy *Sesamum* samples, lane 4: infected sample, and lane 5: negative samples]

#### *Insect vector transmission*

The tested plants showed symptoms after 4 weeks from inoculation. The results indicated that leafhopper (*Orosius orientalis*) species was capable to transmit the phytoplasma diseases from the infected plants to the healthy plants. These results were confirmed by PCR.

#### *Transmission electron microscopy*

Analysis of ultrathin sections of healthy and infected *Sesamum* leaves using electron microscopy revealed that the sieve elements (SEs) in infected phloem tissue contained phytoplasma bodies. In contrast, no phytoplasma units were found in healthy plants. The phytoplasma bodies, which were spherical or pleomorphic in shape and ranged in size from 100 to 600nm, were surrounded by a cell membrane but lacked a cell wall. Floating individuals or groups of phytoplasma were observed linked to the plasma membrane, and thin strands of phloem protein structures were also present. The results suggest that phytoplasma bodies are present in the SEs of infected phloem tissue, while healthy plants do not contain these units. Additionally, the SEs displayed a variety of organizational forms of protein filaments (Fig. 3 A, B, C, and D).

#### *Enzymes activity*

The activities of peroxidase (POD) and polyphenols oxidase (PPO) were significantly increased in infected *Sesamum* plants and healthy plants. The activity of the antioxidant enzymes was higher in the affected plants than the control

as shown in Table 2 and Figs. 4 & 5. The activity of POD in the infected *Sesamum* was higher than that in the healthy plants. The activity of PPO in the infected *Sesamum* was higher than that in the healthy *Sesamum* plants.

#### *Photosynthetic pigments determination*

Data represented in Table 3 indicated that the infected *Sesamum* plants showed significant reduction in Chl a, Chl. b, and carotenoid contents compared with the healthy plants (Fig. 6).

#### *Examination of free hand sections stained with Diene's stain*

By using Diene's stain, infected and healthy *Sesamum* stems were identified. The diseased phloem area was always being stained dark blue while the phloem of sections prepared from healthy plants remained unstained as shown in Fig. 7.

#### *Silver nanoparticles characterization*

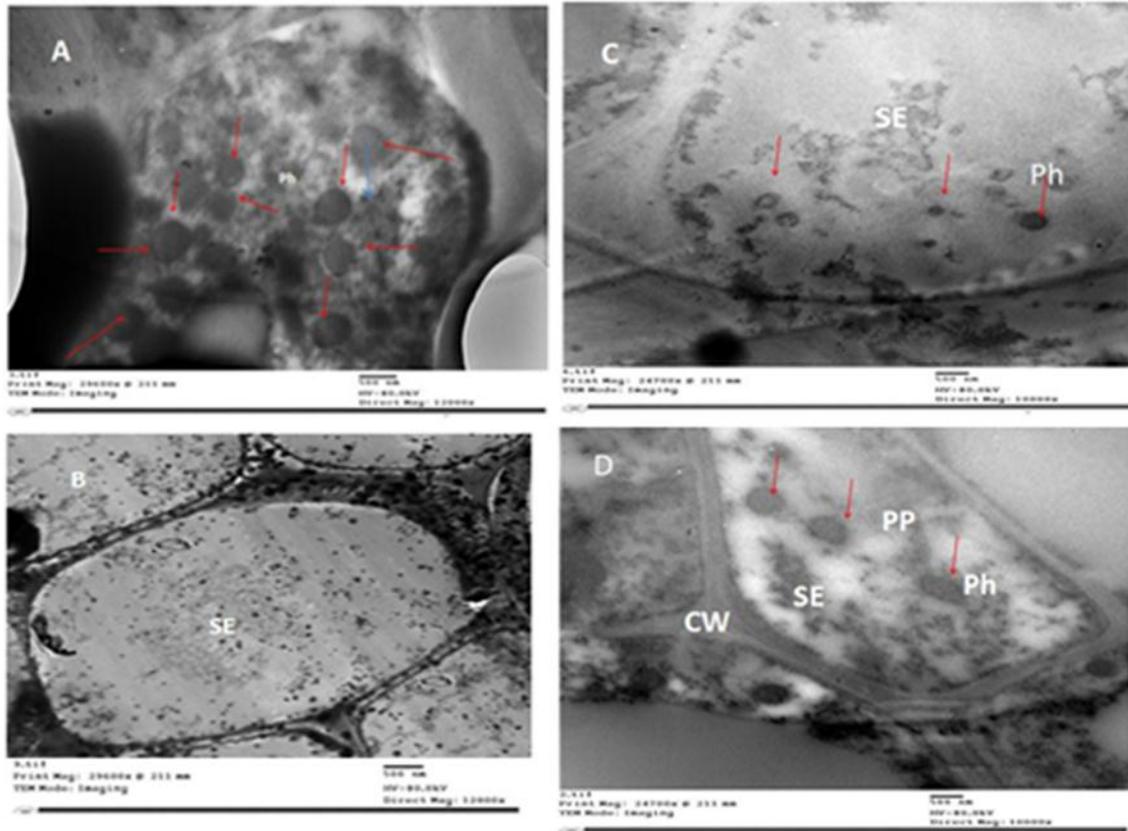
The synthesized Ag NPs physicochemical properties have been measured using different techniques. The surface zeta potential and size distribution profile have been tested in aqueous solution. The synthesized silver nanoparticles showed a high negative surface area charge at a zeta potential of -36.50mV (Fig. 8), while the average hydrodynamic size of the synthesized particles was 8.0nm. The HR-TEM image showed a uniform spherical crystal structure of Ag NPs (Fig. 9).

#### *Ag NPs treatments for phytoplasma eradication*

Ag NPs had been found to reveal antiviral activity against phytoplasma infection on *Sesamum* plants. A complete block of phytoplasma infectivity was found at 250mg L<sup>-1</sup> and 300mg L<sup>-1</sup> while 200mg L<sup>-1</sup> Ag NPs treatments resulted in significantly reducing phytoplasma accumulation infectivity compared with the control. On the contrary, the concentrations of 150mg L<sup>-1</sup> treatments resulted in no effect on accumulation of phytoplasma in infected plants. All results were confirmed by nested PCR (Fig. 10).

#### *Effect of AgNPs on phytoplasma accumulation*

Phytoplasma replication and accumulation inside the host tissues have been significantly affected in Ag NPs treated *Sesamum* plants. As shown in Fig. 11, phytoplasma replication has been completely arrested by Ag NPs concentrations of 250 and 300mg L<sup>-1</sup>.



**Fig. 3.** Electron microscopy analysis of ultrathin sections of healthy and infected *Sesamum* leaves revealed the following: (A) Spherical or pleomorphic phytoplasma units are shown in the sieve elements of infected phloem tissue, (B) No phytoplasma units were observed in the sieve elements of healthy plants, (C) Phytoplasma particles adhere to the plasma membranes of the sieve elements, (D) In the budding stage, simple or branched strands and thin networks of protein filaments (the red arrows) and phytoplasma are shown

**TABLE 2.** Effect of Ag NPs treatments on enzymes activity

Treatments	POD U/g FW	PPO U/g FW
Healthy	1.324	1.854
Infected	2.865	3.542
100 mg L <sup>-1</sup>	2.854	3.565
150	2.925	3.540
200	2.765	3.452
250	1.852	1.964
300	1.215	1.232

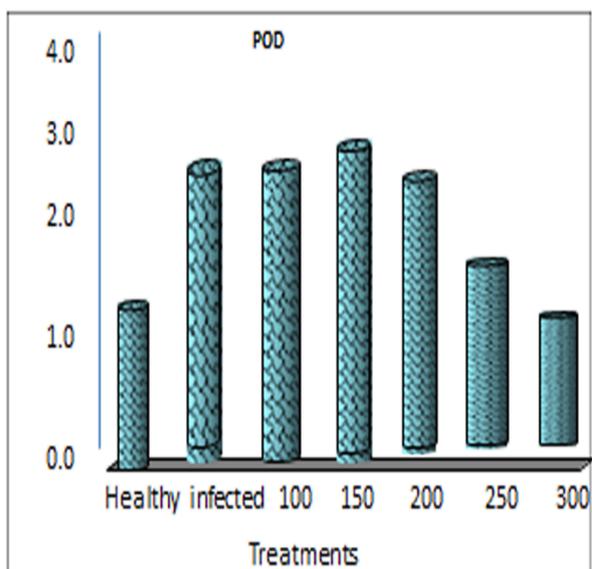


Fig. 4. The activity of the antioxidant of peroxidase (POD) enzyme

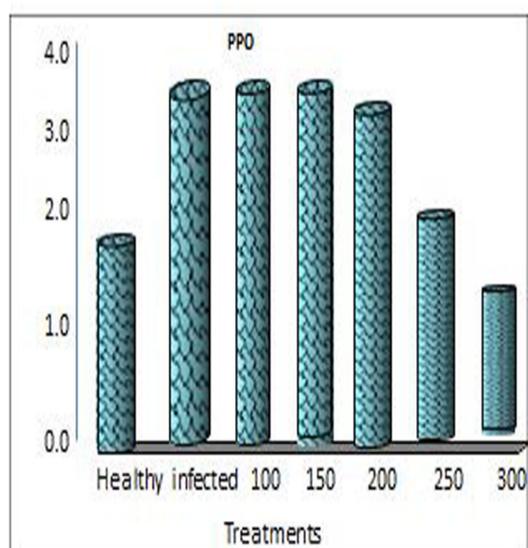


Fig. 5. The activity of the antioxidant of polyphenol oxidase (PPO) enzyme

TABLE 3. Effect of phytoplasma infection on photosynthetic pigments and carotenoids in *Sesamum* plants

Primary metabolites	Healthy plants (mg kg <sup>-1</sup> )	Infected plants (mg kg <sup>-1</sup> )
Chl. a	4.899	1.205
Chl. b	5.137	1.564
Total chl.	6.217	1.225
Carotenoids	3.125	1.297

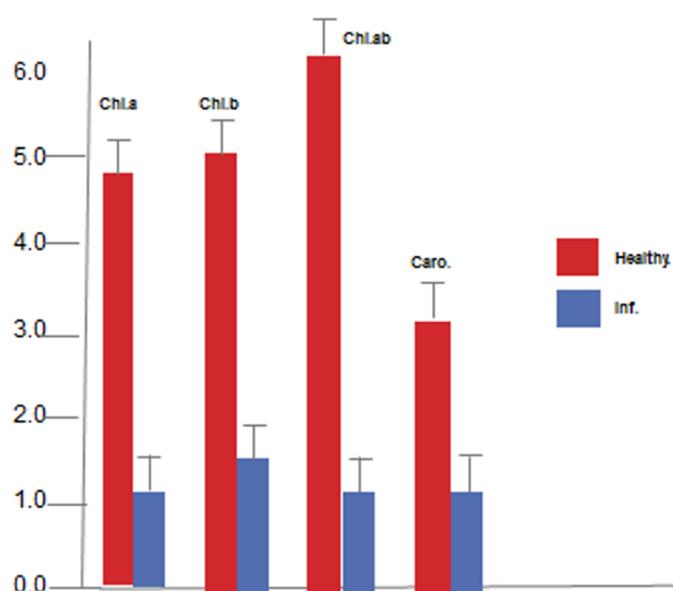


Fig. 6. Photosynthetic pigments and carotenoids in *Sesamum* plants

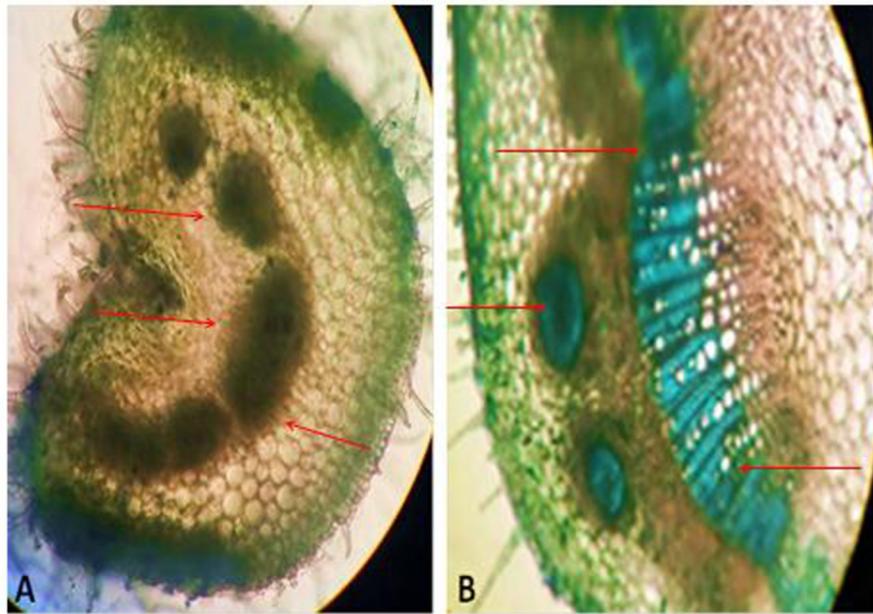


Fig. 7. Micrograph of transverse section of healthy and infected *Sesamum* plant stem stained with Diene's stain [(A) Cross section of healthy stem of *Sesamum* plant, (B) The blue areas in phloem cells indicating phytoplasma presence]

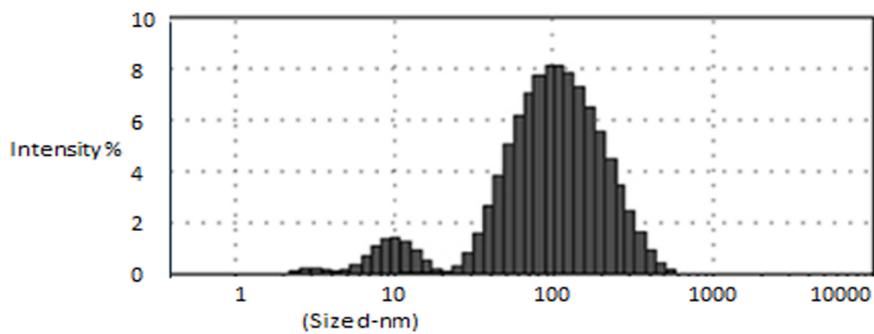


Fig. 8. Particle size distribution and zeta potential distribution of silver nanoparticles determined by dynamic light scattering

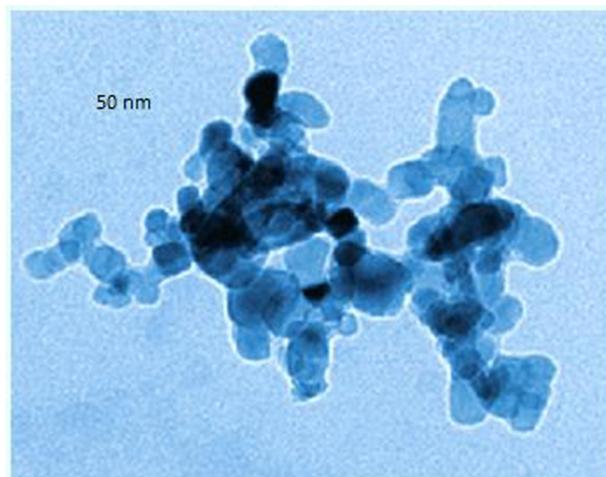


Fig. 9. Images of AgNPs with high resolution transmission electron microscopy (HR-TEM) and a selected area electron diffraction (SAED) pattern showing spherical crystal structure of AgNPs with 8.0nm average size

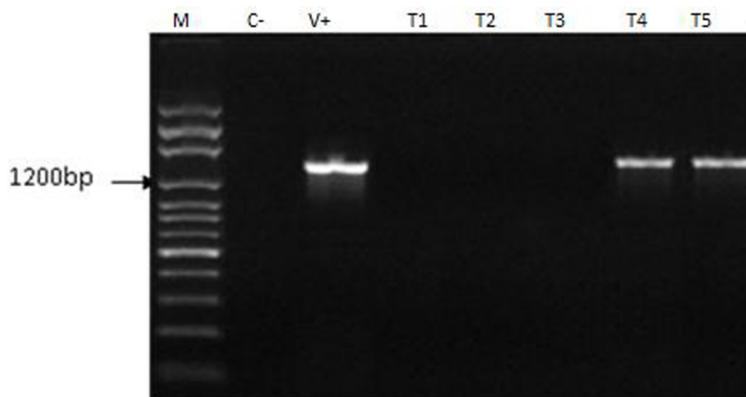


Fig. 10. PCR amplification products [M: 100bp DNA ladder, C-: negative control, V+: positive control, T1, T2 and T3: infected samples treated with Ag NPs at 300, 250, and 200mg L<sup>-1</sup>, and T4 and T5: infected samples treated with Ag NPs at 100mg L<sup>-1</sup> and 150mg L<sup>-1</sup>]

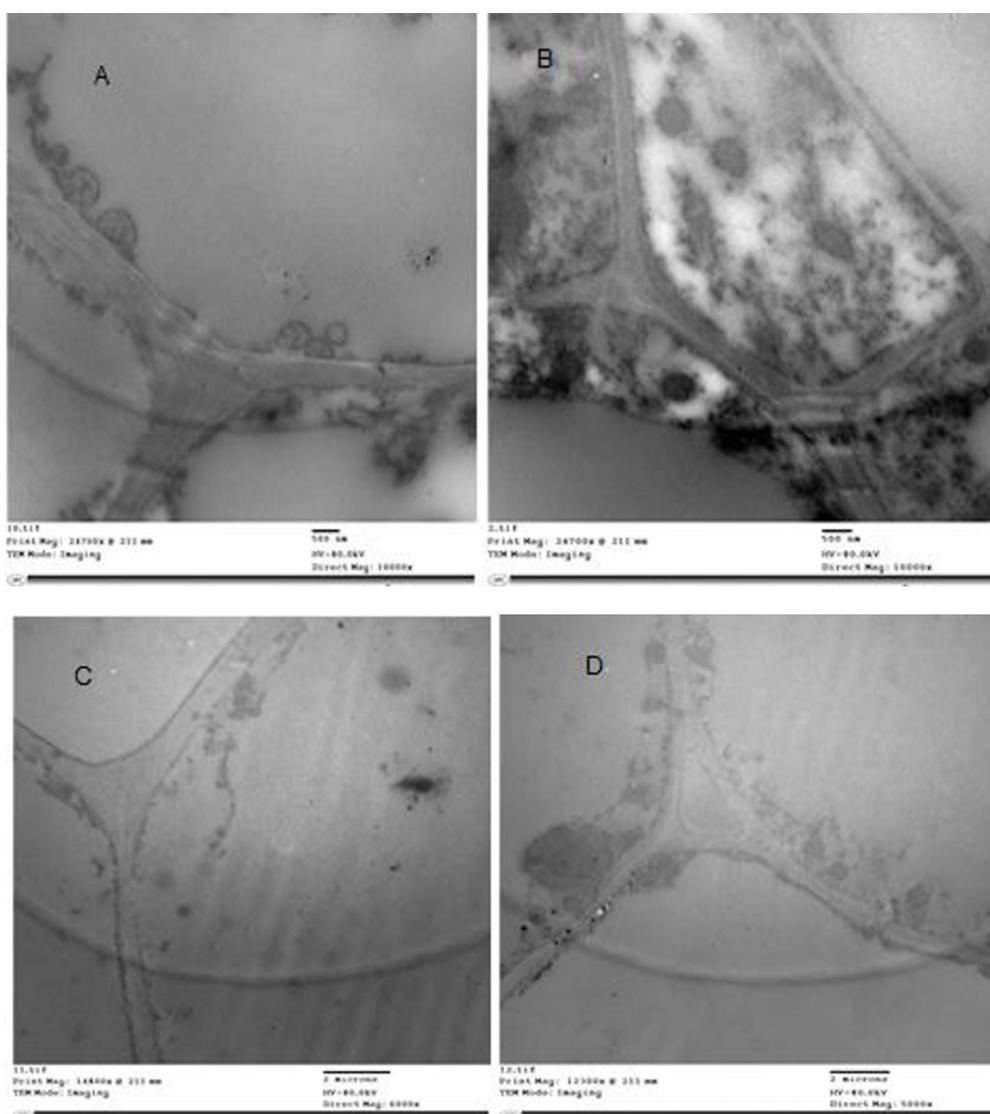


Fig. 11. Leaf dip preparations transmission electron micrographs of phytoplasma showing the direct effect of Ag NPs on phytoplasma particles in treated *Sesamum* plants in vitro [(A) Healthy control, (B) Infected control, (C) Ag NPs' treated plants at 250mg L<sup>-1</sup>, (D) Ag NPs' treated plants at 300mg L<sup>-1</sup>]

The outcomes showed that Ag NPs were successful in removing phytoplasma from infected *Sesamum* plants under *in vitro* circumstances (Fig. 11). Ag NPs at concentrations of 100mg L<sup>-1</sup> and 200mg L<sup>-1</sup> promoted shoot regeneration (4.69 and 6.11%, respectively). Additionally, the regeneration response and quantity of phytoplasma-free plants produced 100% phytoplasma-free plants with 46.80% of cultures exhibiting shoot regeneration at 200 mg L<sup>-1</sup>, 38.78% phytoplasma-free plants with 30.50% cultures exhibiting shoot regeneration at 100mg

L<sup>-1</sup> Ag NPs, and so forth. The explant regeneration rate was highest at 250mg L<sup>-1</sup>.

*Effect of Used Nanoparticles (Ag NPs) on Vegetative Growth and Parameters of Phyllody.*

Before the application of Ag NPs, phytoplasma reduced the growth parameters. The plant leaf area, shoot length, and chlorophyll content decreased in the infected plants compared with the healthy plants. Data in Table 4 and Fig. 12 (A, B, and C) illustrated that Ag NPs significantly increased the plant growth compared with untreated plants.

TABLE 4. Effect of NPs on vegetative growth and parameters

Treatment	Conc. (mg L <sup>-1</sup> )	Growth parameters		
		Shoot length	Leaf area	Chlorophyll content
+ve	-	28.33 e	11.75 b	22.85 c
-C	-	39.41 bc	16.47 a	44.55 a
Ag NPs	100	28.62 e	11.78 b	22.75 c
	150	29.63 e	11.96 b	22.83 c
	200	38.59 a	15.24 ab	35.80 b
	250	39.37 a	16.57 a	41.87 ab
	300	39.82 a	16.57 a	45.99 a
LSD at 0.05		6.4	2.3	6.01

+ve: positive control      -c: negative control

Conc.: concentration

Values with the same letter have no significant difference.

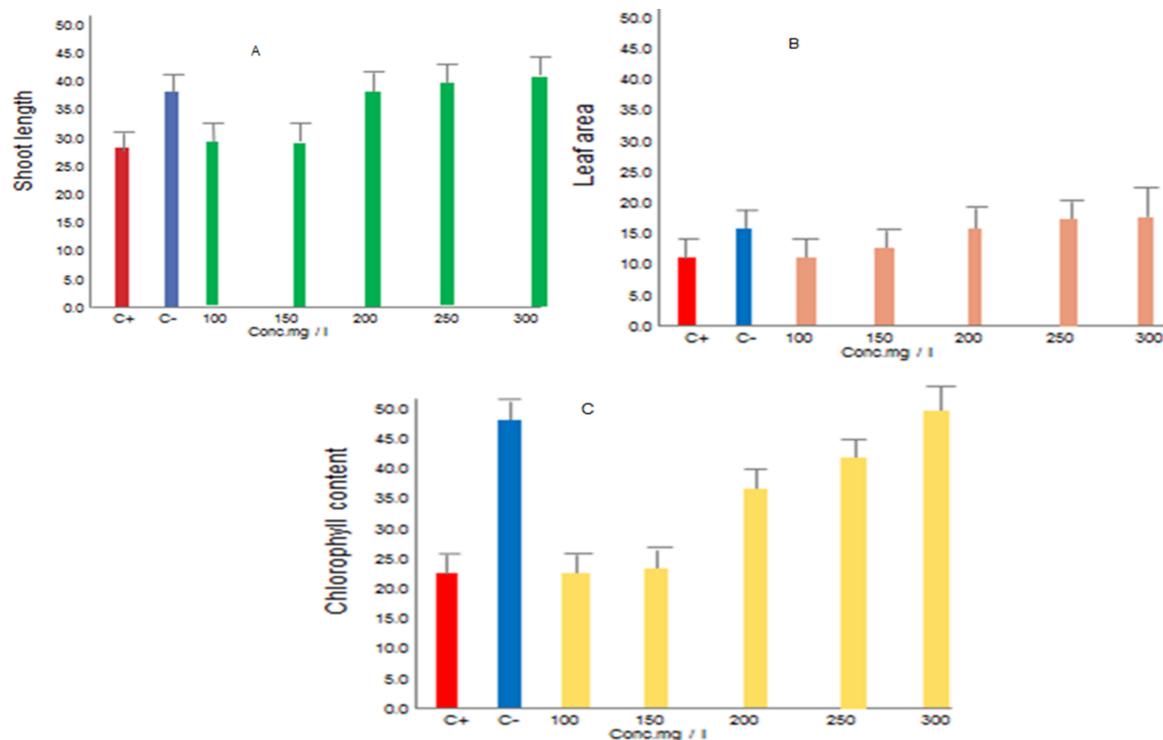


Fig. 12. Vegetative parameters of *Sesamum* plants treated with Ag NPs compared with untreated plants (C+: infected control and C-: healthy control) [(A) Shoot length (cm), (B) Leaf area (cm<sup>2</sup>), (C) Chlorophyll content]

## Discussion

This study demonstrates that silver nanoparticles can be used to effectively reduce phytoplasma infection in *Sesamum* tissue cultures, leading to the production of phytoplasma-free plants. This method has significant potential for improving crop yields and reducing the spread of plant diseases. The phyllody, yellowing, witches' broom, virescence, and stunting symptoms that were observed were the same as those that had been previously described by Pal & Pushkarnath (1935), Choopanya (1973), and Salehi & Izadpanah (1992). The results of this investigation revealed a number of indicators, including phyllody, yellowing, seed capsule breakage, seed germination in capsules, and presence of black exudates. Salehi & Izadpanah (1992) hypothesized that the Iranian phyllody agent infection with sugar beet curly top virus may be responsible for the development of black exudates on leaves. Foliar yellowing is brought on by a *Sesamum* phyllody agent and a *Spiroplasma citri* infection, according to Başpınar et al. (1993). Tamimi et al. (1989) reported some pleomorphic entities in curious *Sesamum* plants using TEM. Currently, only the symptoms are used to define this condition as a phytoplasma disease (Akhtar et al., 2009). With the help of specific primers R16R2/R16F2n, nested PCR was successfully used to confirm the presence of phytoplasma in *Sesamum*-infected plants. This technique has been widely used to detect all phytoplasma strains, while healthy plants reacted negatively (Smart et al., 1996). Even with low titer in the infected plants, nested PCR using various universal primers has the potential to improve the diagnosis of unknown phytoplasma (Marzachi, 2004). Phyllody disease is caused by a 16SrII-D phytoplasma, according to molecular diagnostics, a positive reaction to Diane's stain, and other factors. The presence of pleomorphic bodies in the sieve elements (based on TEM) and recovery in response to treatment with Ag NPs in conjunction with a tissue culture technique are among the ultrastructural changes brought on by phytoplasma infection. The accumulation of sugars and starch, which impairs sieve tube functionality and disrupts phloem transport, may also be responsible for these changes. Diane's staining of the infected samples from our investigation showed uniformly dispersed areas in the phloem region that were very similar to those mentioned by Salehi & Izadpanah (1992) in Iran. Pleomorphic entities (phytoplasma structures) were revealed by TEM studies, as previously described by Salehi

& Izadpanah (1992), Credi (1994), Samad et al. (2002), and Ajayakumar et al. (2007). Phytoplasma units were found in distinct phloem cells at varying quantities, and this has been linked to the severity of symptoms in accordance with Kesumawati et al. (2006) and El-Banna et al. (2015). The phytoplasma amplified a 1250-bp 16S rRNA fragment to demonstrate that it belonged to the 16SrII-D group and was associated with *Sesamum* phyllody. The outcomes of this investigation demonstrated that grafting, dodder, and the leafhopper vector were effective ways to successfully spread the phyllody disease from infected *Sesamum* plants to healthy ones (*O. albicinctus*). These findings confirmed earlier studies which showed that *O. albicinctus* was disseminating phytoplasma in India. *Circulifer haematoceps* has also been implicated in the transmission of phyllody from infected *Sesamum* to *Catharanthus roseus* L. in Turkey (Kolte, 1985; Kersting, 1993; Srinivasulu & Narayanasamy, 1995); and Iran (Esmailzadeh-Hosseini et al., 2007). Therefore, the antioxidant enzyme activity on the phytoplasma-infected *Sesamum* plants was examined, and substantial differences between the infected and healthy plants were found. According to Table 2, it was found that the activity of antioxidant enzymes in the infected *Sesamum* plants was higher than that of antioxidant enzymes in the healthy controls. These findings are consistent with those of earlier studies that found a relationship between an increase in antioxidant enzyme activity and pathogen resistance in plants (Kobeasy et al., 2011; Kesba & El-Beltagi, 2012). In addition to serving as signal molecules to activate plant defense genes, reactive oxygen species (ROS) scavengers, and singlet oxygen quenchers in the host plant, polyphenols are well-known antibacterial substances (Reveles-Torres et al., 2018; Nicholson & Hammerschmidt, 1992). After phytoplasma infection, plants' high levels of polyphenols represent the host's response, and their higher accumulation may be due to the host's defense systems (Junqueira et al., 2004).

According to earlier studies, the peroxidase and polyphenol oxidase activities in the infected *Sesamum* plants were higher than those in the healthy plants. This finding suggests that the infected plants had increased defenses against infection. According to studies by El-Beltagi et al. (2012) and Sowmya et al. (2011), the activity of peroxidase and polyphenol oxidase increased in infected plants in comparison to healthy plants. In this study, we assessed the antiviral activity of Ag

NPs against phytoplasma infectivity to determine their ability to control phytoplasma diseases. These results were in agreement with Sompura et al. (2023). Infection with phytoplasma and its accumulation in the treated plants were both reduced by Ag NPs. *Sesamum* plants cultivated *in vitro* on culture media supplemented with Ag NPs at a concentration dose of (250mg L<sup>-1</sup>) significantly suppressed phytoplasma infection. According to our findings and PCR indexing, all *Sesamum* plants treated with Ag NPs were completely clear of phytoplasma. These results are in agreement with Elshahar et al. (2022). The same outcomes have been observed with Ag NPs, which were successful in protecting *Faba* bean plants from the BYMV disease (El Gamal et al., 2021).

### Conclusions

A novel technique for eradicating phytoplasma from infected *Sesamum* plants has been developed in the current study, which involves the use of meristem tip culture combined with nanoparticle treatments. The study's results indicate that an ideal concentration of Ag NPs can encourage shoot regeneration in plant tissue cultures. This method shows promise for producing *Sesamum* plants and seeds that are free of phytoplasma and could have practical applications. Further studies are needed to identify the effect of Ag NPs on the behavior of the leafhopper insects as the main method of the spreading and transmission phytoplasma.

*Competing interests* The authors report no conflicts of interest regarding this work.

*Authors' contributions:* Conceptualization, T.E.A.; methodology, H. F.E and A.E.E.; software, S.M. S. and K. I.S. writing—original draft preparation, T.E.A.; writing—review and editing. All authors have read and agreed to the published version of the manuscript.

*Ethics approval:* Not applicable

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## استخدام بعض مركبات الفضة النانو متريية في زراعة الأنسجة لإنتاج نباتات سمس خالية من الفيتوبلازما

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يعتبر الفيتوبلازما في السمسم مرض خطير. وأعراضه الأكثر شيوعا هي تشوه الأزهار، والأصفرار، وتشقق كبسولات البذور، وتكوين الإفرازات الداكنة على أوراق النبات. أظهرت صبغة دينيز بواسطة المجهر الضوئي مناطق زرقاء بمنطقة اللحاء للنباتات المصابة. وأشار الفحص الجزيئي لجزء من الحمض النووي الريبوزي المميز للفيتوبلازما إلى أن نباتات السمسم مصابة بالفيتوبلازما. تم استخدام المجهر الإلكتروني النافذ للكشف عن الفيتوبلازما بعناصر اللحاء في النباتات المصابة. كما تم استخدام وسط غذائي (موراشيغ وسكوج) لتجديد النباتات من القمة المرستيمية. وتم استخدام تحليل nPCR المتداخل لفحص الفيتوبلازما بالنبات الأم والنباتات الصغيرة المجددة بالمعمل. تمت زراعة نباتات السمسم المستأصلة إيجابياً الفلودي (الفيتوبلازما) على نفس الوسط الغذائي وإضافة عدة تركيزات (100، 150، 200، 250، 300 ملجم/لتر) من جزيئات الفضة النانوية (Ag-NPs). انخفضت البراعم الجديدة مع زيادة تركيز (Ag-NPs) حتى 300 ملجم/لتر. بتركيز 250 ملجم/لتر وكانت النباتات المجددة بالمعمل خالية من الفيتوبلازما وأظهرت نتائج سلبية في PCR-n. عند 150 ملجم/لتر نتج 18.3% نباتات خالية من الفيتوبلازما وأظهر 20.10% تجددا بالبراعم. أنتج مستوى 200 ملجم/لتر 29.5% نباتات خالية من الفلودي بينما أظهرت 36.8% من الزراعات داخل المعمل تجددا بالبراعم. كان مستوى 250 ملجم/لتر الأكثر فعالية وأنتج 100.0% نباتات خالية من الفلوديات وأظهرت 100.0% من الزراعات تجددا بالبراعم. نستنتج من ذلك أن المعالجة باستخدام Ag-NPs أداة فعالة قادرة على إنتاج نباتات خالية من الفلودي.