Silver Nanoparticles as A Highly Viricidal Agent to Deter Plant-Infecting Viruses and Disrupt their Acquisition and Transmissibility by Vector Aphid

Ahmed El Gamal  (ahmedvnp1@yahoo.com)  
Agricultural Research Center  https://orcid.org/0000-0002-7811-1195

Mohamed Reda Tohamy  
Zagazig University Faculty of Agriculture

Mohamed Ibrahim Abou-Zaid  
Zagazig University Faculty of Agriculture

Mahmoud Mohamed Atia  
Zagazig University Faculty of Agriculture

Tarek El Sayed  
Agricultural Research Center

Khaled Farroh  
Agricultural Research Center

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Abstract

Silver nanoparticles (AgNPs) are a potentially effective tool for deterring viral plant pathogens. This study was carried out to evaluate the efficacy of AgNPs to defeat Bean yellow mosaic virus (BYMV) on faba bean plants from the host, virus and vector aphid tripartite interactions side. The antiviral capabilities were evaluated during a foliar protective and curative scheme. Furthermore, the efficiency of AgNPs on virus acquisition and transmission by its vector aphid was investigated. Results indicated that the AgNPs had greatly exhibited curative viricidal activities for inactivation BYMV when applied 48 h post-virus inoculation. The disease occurrence was entirely inhibited with AgNPs rate as low as 100 mg.l$^{-1}$, while the infectivity was completely arrested when plants were preventively exposed to 200 mg.l$^{-1}$ 24 h pre-virus inoculation. AgNPs proved high bio-reactivity by binding to viral particles, suppressing their replication and accumulation within the plant tissues. Moreover, it was noticeably showed to upregulate the pathogenesis-related gene (PR-1) and promote the defense-related enzymes and protein profiles in treated plants irrespective of concentration. Exposure of aphids to AgNPs-treated plants before virus acquisition excitingly reduced the BYMV acquisition and transmission efficiency by 40.65% up to 100 % 24 h post-application and the virus acquisition was affected for 10 days by 6.89 Up to 79.64 % depending on the AgNPs rate. These results concluded that the AgNPs have a high curing viricidal activity by targeting the virus envelop, and more excitingly it can affect the virus-vector combination, suggesting that it may contribute to alleviating the natural disease occurrence and virus transmission under field conditions. Therefore, according to the available literature, this study provides the first report on the deterring activity of nanomaterials against plant virus acquisition and transmission by its vector insects.

1. Introduction

Faba bean (Vicia faba L.) is one of the most important legume crops that is almost consumed daily in numerous developing countries as a human diet of a cheap and high-quality protein source. It has been simultaneously ranked as the third most important feed legume crop worldwide (1, 2). Bean yellow mosaic virus (BYMV) a type member of potyviruses is a devastating disease for many legume crops and other ornamental flowers, which poses a significant threat for global faba bean and other legumes production (3, 4). In this respect, BYMV becomes one of the most biotic factors that steadily reducing the faba bean production and their cultivated area in Egypt (5). The virus is naturally transmitted by over 20 species of aphids which can be effectively spread under field conditions, resulting in a high rate of viral infections for faba bean and other host species (6).

In recent years, nanotechnology approaches have emerged as a new potential control means to deter a wide array of plant diseases (7). However, using nanotechnology tools in plant protection management hasn’t yet widely introduced on a large scale, which still under the research investigations and small scale of production and applications (8, 9). Nanoparticles, ranged from 1 to 100 nm in size, prove superior chemical and physical features compare to their bulk materials due to their large surface area to its volume ratio, which strongly offers them to be one of the most promising ways that may effectively be used in many different sectors (10, 11). Silver nanoparticles (AgNPs) are metal nanomaterials that have been investigated to be used in many applications notably for the controlling of human and plant pathogenic microorganisms (12, 13, 14, 15). Due to their unique features, AgNPs have been documented as an antimicrobial agent to control some phytopathogenic fungi and bacteria (16, 17, 18, 19). Simultaneously, some previous reports have been proved the effectiveness of nano-silver against some plant and human viruses by blocking the virus infectivity and its accumulation within the host tissues (20, 21).

However, the little efforts that have been done to investigate the AgNPs as an antiviral agent, there is no documented investigations to show the ability of AgNPs to control plant viruses from different tripartite interactions point of view (host, virus and vector interactions), and since the BYMV, as well as more than 75% of other plant viruses, are naturally transmitted by insects, therefore the research aims of the present study were to investigate not only the effectiveness of the
AgNPs on virus infectivity and plant responses but also to note whether the pre-acquisition treatment of AgNPs may affect the virus-vector combination by altering the virus acquisition and transmission process.

2. Experimental Methods

2.1. Virus isolation and propagation

Faba bean plants showed putative Bean yellow mosaic virus symptoms were collected from a local open field at Giza governorate, Egypt during the 2018 growing season. Leaf samples were initially tested by the serological DAS-ELISA technique as described by Clark and Adams (22) using a specific polyclonal antibody against BYMV (EPHYRA Bioscience inc., Canada). Positive samples reacted with BYMV antibodies were used as a source of virus inoculum. Ten seedlings of faba bean (cv. Giza 3) and Chenopodium amaranticolor as an indicator host plants were inoculated with a freshly prepared sap inoculum using 0.01M phosphate buffer pH 7.1, (1:10), 0.01% Na₂SO₃ and carborundum (600 mesh). All inoculated plants were grown under insect-proof greenhouse conditions (20–24°C) and daily observed until symptoms develop. The virus was biologically purified from a single local lesion produced on C. amaranticolor using a single lesion technique (23). The purified isolate was propagated on faba bean plants (cv. Giza 843) and was used as a source of BYMV for further studies. Furthermore, the mechanically inoculated plants with purified BYMV were checked again using the ELISA technique for serological relationship verification against BYMV antiserum and other viruses infecting faba bean plants.

2.2. Molecular identification of BYMV

Total RNA was isolated from 50 mg of each healthy and symptomatic leaves using RNeasy Plant Mini Kit (Qiagen Co., Germany) according to the manufacturer’s instructions. Reverse transcription (RT)-PCR reaction was optimized using Verso™ one-Step RT-PCR system (Thermo F. Scientific, USA). A specific primer pair BYMV-CPF and BYMV-CPR were used targeting coat protein (CP) gene region to amplify 907 bp fragment of the BYMV genome as suggested by Al-Khalaf et al. (24) (Table 1). The one-step RT-PCR reaction was performed by combining 25 µl one-Step PCR Master Mix, 1 µl of each primer pair (200 nM), µl 2.5 of RT-enzyme enhancer, 1 µl verso enzyme mix, 3 ng of RNA template and the mixture was completed up to 50 µl using nuclease-free water. The amplification reaction was automated in a T-Gradient thermal cycler (Biometra Co., Germany) with an initial reverse transcription process at 50°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 50°C for 1 min and extension at 72°C for 2 min, with a final additional extension step for 10 min at 72°C. The PCR products were electrophoresed on 1 % agarose gel in 0.5 x TAE buffer, then visualized under the Gel Doc. system (2000, Bio-Rad, USA).

| primer name    | primer sequence (5′–3′)                               | target gene                          |
|----------------|--------------------------------------------------------|--------------------------------------|
| BYMV-CPF/CPR   | BYMV-CPF: GTCGATTTCATCCGAACAAG                      | CP gene of BYMV (907 bp)             |
|                | BYMV-CPR: GGAGGTGAAACCTCAGTAAAC                      |                                      |
| PR-1F/R        | PR1-F: GGGCAGTGGTGACATAACAGGAA                       | PR-1 gene                            |
|                | PR1-R: CATCCAACCAGAACGAAAT                           |                                      |
| ELF1A/F-R      | ELF1A-F: GTGAAGCCCGGTATGCTTG                        | Elongation factor 1- alpha reference gene |
|                | ELF1A-R: CTTGAGATCCTTGACTGCAACATT                    |                                      |
2.3. Synthesis of silver nanoparticles (AgNPs)

Silver nanoparticles colloidal solution was synthesized by reduction of silver nitrate (AgNO3) [99.99%, Aldrich, US] using a co-precipitation protocol of sodium citrate trihydrate (99%, Aldrich, US) under boiling conditions (25). Briefly, 50 ml of AgNO3 (0.001 M) was brought to boil under stirring for 5 min in 250 ml beaker, a 5 ml of sodium citrate trihydrate (1%) was then added a drop per second under continuous stirring. The solution color turned pale yellow to yellowish-brown producing a colloidal silver nanoparticles form, then it was left to cool and proceeds for physicochemical characterization.

2.4. Characterization of silver nanoparticles

2.4.1. Particle size determination. The particle size distribution, polydispersity index (PDI) and zeta potential of AgNPs were measured using the Dynamic Light scattering (DLS) analysis on Zetasizer (Malvern, ZS Nano, UK). The colloidal nanoparticles solution was diluted with distilled water and put into a disposable glass cuvette for analysis (26).

2.4.2. X-ray Diffraction (XRD) measurement. The Physico-chemical crystalline nature of AgNPs was confirmed using X-Ray diffractometer (X’pert PRO, PAN analytical, Netherlands) operated by Cu K radiation tube (= 1.54 A˚) at 40 kV. Colloidal AgNPs solution was centrifuged at 20,000 rpm for 30 min at 4 c for powder phase yield, the precipitated AgNPs were dried, then bombarded by X-ray for phase analysis (27).

2.4.3. Surface morphology determination. Particle size and the actual shape of AgNPs were performed by High-Resolution Transmission Electron Microscope (HR-TEM) (Tecnai G2, FEI, Netherlands) under an accelerating voltage of 200 kV.

2.5. Effect of silver nanoparticles on virus infectivity

2.5.1. AgNPs Treatments. Foliar applications of AgNPs were carried out under an insect-proof greenhouse with two protective and curative schemes, using six tested concentrations of AgNPs (50, 100, 150, 200, 250 and 300 mg.l⁻¹). Ten days old faba bean young plants (cv. Giza 843) were uniformly sprayed with the AgNPs concentrations 24 h pre (protective)- and 48 h (curative)- post virus challenge. The potted plants were mechanically inoculated with a cured sap of BYMV- infected plants and water-treated plants were served as a control.

2.5.2. Disease assessment. The percentage of disease occurrence and severity of symptoms was assessed three weeks later using a numerical scale of 0–4 grads, where 0 = no visible symptom apparent; 1 = mild chlorotic patterns; 2 = mosaic patterns and dark green vein banding; 3 = mosaic patterns, leaf distortion, and reduction in leaf size; 4 = severe mosaic and stunting of the whole plant. Values of disease severity were estimated by the following formula (28):

\[
\text{Disease severity} (\%) = \frac{\sum \text{disease grad} \times \text{number of plants in that grad}}{\text{total number of plants} \times \text{highest disease grad}} \times 100
\]

\[
\text{Disease incidence} (\%) = \frac{\text{Number of infected plants}}{\text{total number of inoculated plants}} \times 100
\]

\[
\text{Inhibition index in virus infectivity} (\%) = \frac{A-B}{A} \times 100
\]

Where A is the mean average of DI or DS values in untreated control plants; B is the average values in each treatment.

2.5.3. Determination of virus accumulation content. A newly emerged small leaves were collected 30 days post-inoculation to detect the BYMV accumulation and to further confirm the virus replication inhibition rate in AgNPs-treated and untreated plants using the double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (22). The reactions were quantified at 405 nm in a microplate reader (BioTek, USA). Samples were considered positive when OD405 values were two times higher than the mean of the healthy control.
2.5.4. Transmission electron microscope. The direct activity of AgNPs on viral particles was investigated in faba plants curatively treated with 300 mg.l$^{-1}$ compared to untreated control. The leaf-dip preparation method was performed to increase the probability of examination five days after virus inoculation. Briefly, the leaf samples of both treated and untreated plants were washed, disked and resuspended in deionized water to remove any surface-AgNPs attached to the leaf samples. The samples were ground in a drop of phosphate buffer pH 7.5 and 0.01% Na$_2$SO$_3$. A 10 µl of leaf extracts were individually loaded on carbon-coated grids for 5 min, washed with distilled water and negatively stained with 2 % uranyl acetate (29). The grids were examined using a Jeol JEM1400 transmission electron microscope (TEM) (JEOL Co., Tokyo, Japan).

2.6. Enzyme activity assays

Fresh leaves (0.2g) from all treated and untreated plants were collected at 0, 24, 48, 72, 96, 120 and 144 h post-AgNPs spraying. The peroxidase (POD) and polyphenol oxidase (PPO) antioxidant enzyme activities were assayed using the methods described by Kar and Mishra (30). Changes in activities were expressed as nmol of guaiacol per mg$^{-1}$ protein. min$^{-1}$. All experiments were performed in three biological replicates.

2.7. Protein pattern analysis

Protein extract and separation were performed according to the procedure of Laemmli (31) with some modifications. Samples with 1 g of fresh leaves were collected from both AgNPs-treated and untreated plants 48 h after application. Leaf samples were ground in 0.5 ml Laemmli buffer, then centrifuged at 14,000 rpm for 20 min at 4°C. Protein contents were quantified against the bovine serum albumin (BSA) standards using a colorimetric (Bradford) protein assay protocol (Thermo F. Scientific, USA). An equal amount of protein content for each sample (40 µg) was diluted with an equal volume of loading buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 10% glycerol, 1% 2-mercaptopetanol and 0.01% bromophenol blue dye). The mixture was incubated in a water bath for 3 min at 95°C and separated by electrophoresis using 10% sodium dodecyl sulfate-polyacrylamide (SDS–PAGE) gel for 6 h. The gel was incubated in 1% nitric acid for 3 min and protein bands were visualized using 0.2% silver nitrate in a de-stain solution of 7% glacial acetic: 40 % methanol solution: 53 % distilled water solution (v/v).

2.8. PR-1 gene expression analysis using quantitative real time-PCR (qRT-PCR)

2.8.1. cDNA synthesis. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen) protocol. The harvested RNA (2 µg) was primed with Oligo (dT) and converted into the first-strand cDNA by using COSMO cDNA synthesis kit (Willowfort, UK) according to the manufacturer’s protocol. The cDNA synthesis reaction was performed in a T-Gradient thermal cycler with initial annealing at 25°C for 10 min., followed by an extension phase at 45°C for 15 min.

2.8.2. qRT-PCR analysis. Gene-specific primer encoding pathogenesis-related protein (PR-1 F/R) was designed according to Cheng et al. (32). Meanwhile, the elongation factor 1- alpha was used as an endogens reference gene (ELF1A/F-R) (Table 1). The HERA SYBR Green qPCR kit (Willowfort, UK) was used for RT-qPCR analysis with 20µl reaction mixture consisting of 10µl SYBR green master mix, nuclease-free water (7.2µl), diluted cDNA template (2µl) and 0.5µl of each primer. The qRT-PCR program was performed as follows: 95°C for 2 min initially, followed by 40 cycles at 95°C for 1 min and 60°C for 30 s. The reaction was normalized with melting curve analysis at 65°C for 10 s for 61 cycles. The changes in gene expression were calculated based on the internal reference gene using the $2^{−ΔΔCt}$ method (33). Each experiment was conducted in three biological replicates.

2.9. Effect on virus acquisition and transmission

2.9.1. Aphid cultures. Colonies of *Aphis craccivora* Koch. were collected from early grown faba bean fields at Giza Research Station, Agric. Res. Center (ARC), Egypt. The aphids were reared on cabbage seedlings under insect-proof cages for a long period of experimental time. Filial generations were used in subsequent as a source of non-viruliferous aphid cultures.
2.9.2. Treatments and virus acquisition assay. The BYMV acquisition assay was done 1, 5- and 10 days post foliar spraying of AgNPs treatments (50, 100, 150, 200, 250 and 300 mg.l\(^{-1}\)) compared to untreated control. Aphids were starved for 1 h before deposited on a BYMV-free plant treated with AgNPs for a propping period of 3 h, which allow most of them to get in contact with AgNPs. The aphids of each treatment were separately moved to BYMV-infected plants and left to feed (2 h) for virus acquisition. At the end of 2 h, aphids were transferred individually as one aphid per healthy plant and were left for an inoculation feeding period of 24 h. The acquisition efficiency was also determined by leaving aphids to feed directly on BYMV-infected plants which were individually treated with the tested AgNPs rates. The percentage of the acquisition was computed as the number of aphids that transmitted the virus/ total number of aphids x100. Virus incidence in the symptomless plants was checked by back inoculation made on *Ch. amaranticolor* plants and also by using the DAS-ELISA technique.

2.9.3. Coat protein gene accumulation. Leaf samples of faba bean plants were collected 30 days after acquisition assay for all AgNPs treatments sprayed 24 h. pre-acquisition. The total RNA extraction, the primer used and RT-PCR reaction were performed as mentioned above.

2.10. Statistical analysis

Data were statistically analyzed using Completely Randomized Design (CRD) for analysis of variance (ANOVA) by the Assistat-statistics Software, Version 7.7 Beta (34). Each experiment was performed in three repeats.

3. Results

3.1. Virus isolation and propagation

*Bean yellow mosaic virus* was isolated from naturally infected faba bean plants showed BYMV-like symptoms. The isolated BYMV produced severe mosaic symptoms on faba bean plant and similar to those observed in the field (Fig. 1A,B). The presence of BYMV isolate was confirmed by DAS-ELISA in mechanically inoculated plants and all samples were positive for BYMV. The virus titer was quantified with 1.206 OD and 0.743 OD in faba bean and *C. amaranticolor* plants respectively, compared with 0.318 OD at negative control. Whereas, it was failed to react with antiserum specific for other viruses affecting faba bean plants.

3.2. Molecular identification of BYMV

The BYMV isolate was confirmed using the RT-PCR molecular tool (Fig. 1C). As illustrated by the agarose gel electrophoresis analysis, two samples of BYMV-infected plants produced a clear band at the expected amplicon size of 907 bp, however, no PCR products were achieved from healthy faba bean plants used as a negative control.

3.3. Characterization of silver nanoparticles

The physicochemical properties of the synthesized AgNPs were measured using different techniques. The size distribution profile and surface zeta potential were examined in AgNP colloidal aqueous solution. The synthesized nano-silver showed an excellent polydispersity index (PDI) 0.302, with a high negative surface area charge at a zeta potential of −36.50 mV (Fig. 2A). The average hydrodynamic size of the synthesized particles was found to be 7.43 nm with a range between 4.56 to 12.38 nm (Fig. 2B). In this respect, the HR-TEM image indicates a well uniformed spherical crystal structure of AgNPs with average diameter size particles of 8.54 nm (Fig. 2D). The crystalline fingerprint nature of silver nanoparticles was obtained by XRD. (Fig. 2C). The XRD pattern showed four prominent peaks at 38.14, 44.36, 64.49 and 77.44 2θ angles in crossbanding to (111), (200), (220) and (311) hkl parameter respectively and based on Bragg’s reflection low.

3.4. Effect of AgNPs on virus infectivity.

Silver nanoparticles were superiorly found to exhibit antiviral activities against plant virus infection (Fig. 3A, B, C). The AgNPs treatments applied 48 h post-inoculation had exhibited higher viricidal activity more than that exposed 24 h pre-
inoculation (Fig. 3A, B). Water-treated plants inoculated with BYMV showed severe mosaic, yellowing and stunting symptoms (Fig. 3C). Noticeably, a complete reduction in virus infectivity was obtained by 200, 250 and 300 mg.l\(^{-1}\) AgNPs either when applied pre- or post-virus challenge, while the rate of 100 and 150 mg l\(^{-1}\) only exhibited a complete reduction in virus occurrence when sprayed 48 h post-inoculation. Further, the virus occurrence and disease severity were greatly decreased in the 50 mg.l\(^{-1}\) treated plants by 74.67 % and 89.14 % respectively, over untreated control and more than that obtained when applied 24 h pre-inoculation (Fig. 3B). In this respect, AgNPs display their ability to target the BYMV particles coat protein in treated plants, resulting in defeating the virus infectivity and disease proliferation (Fig. 4).

3.5. Effect of AgNPs on virus content

Virus accumulation was dramatically affected by AgNPs treatments depending on its rate and time of exposure. As presented by Fig. 3 (D, E) the highest virus titer was quantified with 1.504 OD in untreated positive control plants compared to 0.284 OD at healthy negative control. BYMV content was entirely arrested with 200, 250 300 mg.l\(^{-1}\) AgNPs treatments when applied 24 h pre-inoculation, while 50, 100 and 150 mg.l\(^{-1}\) rates significantly decreased the virus accumulation by 39.69 % (0.907 OD), 36.17% (0.960 OD) and 51.66% (0.728 OD) respectively (Fig. 3D). However, the AgNPs treatments sprayed 48 h post-inoculation had produced a negative ELISA reaction against BYMV at all tested concentrations excepted for 50 mg.l\(^{-1}\) albeit with a significant reduction in the virus content by 51.86% (0.724 OD) in 50 mg.l\(^{-1}\) treated plants compared to untreated control (Fig. 3E).

3.6. Changes in oxidative enzyme activity

Peroxidase (POD) and polyphenol oxidase (PPO) activity were considerably generated upon exposure to AgNPs applications as compared to control irrespective of concentrations (Fig. 5A). Interestingly, the peak of PO activity stimulated by 150 mg.l\(^{-1}\) spray was steadily increased at 24 h (2.04 fold) reaching its maximum with 3.57 fold at 144 h post-application. However, leaves treated with 300 mg.l\(^{-1}\) showed a log-like phase of activity at 24 h (2.43 fold) and 48 h (4.26 fold), sharply decreased afterward at 144 h. Further, all AgNPs rates were constantly found to keep the activity peaks higher than either did of 300 mg.l\(^{-1}\) and untreated control at the end of the time course. However, the maximum increase in PPO activity was significantly observed in leaves treated with 250 and 300 mg.l\(^{-1}\) over the untreated control, reaching its maximum with 2.92 and 2.25 at 120 and 72 h respectively. The PPO activity was not significantly affected upon exposure to 50 and 100 mg.l\(^{-1}\) sprayings during the all-time course excepted at 72 h (0.65 fold) and 48 h (0.91 fold) respectively.

3.7. Changes in protein constituents

Foliar spraying of AgNPs proved some alterations of protein profile in treated faba bean leaves compared to both untreated infected and healthy control plants. Two new protein bands with about 90 and 140 kDa were observed and more or less accumulated depending on the AgNPs rate, while the BYMV-inoculated plants produce two polypeptides of 55 and 90 kDa compared to healthy control. Interestingly, the intensity of protein bands was increased as the AgNPs concentrations increased in which the band intensity was markedly accumulated in 50, 100 and 200 mg.l\(^{-1}\) and start to decrease at 300 mg.l\(^{-1}\) rate with a notable disappearance of some polypeptides which accumulated in other treatments (Fig. 5B).

3.8. Changes in gene expression level

The transcription level of the defense-related PR-1 gene involved in response to plant biotic and abiotic stress was measured by qRT-PCR in AgNPs-treated and untreated plants (Fig. 5C). The gene expression levels were differentially promoted with the tested applications of AgNPs over the untreated plants. Following the 100 mg.l\(^{-1}\) application, the fold change in gene expression was promoted with 8.51. The highest level of expression was generated with a 12.06 fold change in faba bean plants treated with 200 mg.l\(^{-1}\) rate, while the lowest level of expression among the three tested concentrations was generated with a 5.36 fold over the control when the rate of AgNPs increased to 300 mg.l\(^{-1}\).

3.9. Effect on virus-vector interaction
The direct activity of AgNPs on the ability of vector aphid to acquire BYMV particles and transmit it to other healthy plants was excitingly investigated after exposure to AgNPs-treated plants (Fig. 6A, B, C). Notably, a significant inhibition in virus acquisition and transmissibility was noted 1, 5 and 10 days after spraying with all tested concentrations compared to the untreated control, except for 50 mg.l\(^{-1}\) at 10 days (Fig. 6A). The rate of acquisition was blocked totally by 300 mg.l\(^{-1}\) at 1 and 5 days after application, while a great inhibition, 79.64 % was observed at 10 days compared to untreated control. However, the 250 mg.l\(^{-1}\) application completely blocked the virus acquisition and transmission 1 day after spraying. Furthermore, the 150 and 200 mg.l\(^{-1}\) applications produced a significant reduction in virus acquisition by 89.45 and 77.61 % for 1 day to reach 61.32 % and 57.05 % at 10 days respectively, while the 50 and 100 mg.l\(^{-1}\) treatments significantly reduced the virus acquisition for 1 day by 40.65 % and 66.21 % respectively. On the other hands, the AgNPs treatments applied to BYMV-infected plants strongly affect the aphid ability to acquire the virus 24 h post spraying, proved a complete inhibition in virus acquisition and transmission at rates of 150, 200, 250 and 300 mg.l\(^{-1}\), while the 50 mg.l-1 and 100 mg.l-1 application greatly reduced the proportion of acquisition by 61.81 % and 86.91 % respectively (Fig. 6D).

Coat protein gene accumulation was qualitatively measured using conventional RT-PCR reaction to confirm the efficacy of AgNPs on virus acquisition and transmissibility (Fig. 6B). Exposure to 300 and 250 mg.l\(^{-1}\) of AgNPs hadn't exhibited any viral RNA reproducibility (lane 2 and 3 respectively) compared to control plants that produced a high-intensity band reflecting a higher accumulation of viral RNA compared to AgNPs-treated plants. However, the intensity of PCR bands was less than that of control at exposure to 150 and 200 mg.l\(^{-1}\), while the CP gene accumulation bands for 50 and 100 mg.l\(^{-1}\) rates were approximately similar in band intensity as control.

4. Discussion

The control measures used to deter plant viruses are obtained during the pesticide application against vector, however, it is often ineffective with non-persistently transmitted viruses due to the short propping and subsequent inoculation time required for virus transmission by incoming viruliferous vectors (35, 36, 37). Hence, searching for effective innovation technologies is urgently needed to prevent crop losses and maintain sustainable agriculture without going into a pesticide treadmill. The present research was planned to investigate the role of AgNPs capabilities in controlling BYMV disease either by testing its direct or indirect activity on virus infectivity and/or during the evaluation of AgNPs on aphid feeding behavior through the combating of virus acquisition and transmissibility by vector aphid from infected to healthy plants.

Concerning the effect of AgNPs on virus infectivity and host-virus interactions, the AgNPs proved a superior effect and highly prominent antiviral activities in curatively treated plants compared to the protective applications, the disease occurrence and virus accumulation content were entirely arrested at low rates of AgNPs in plants treated 48 h post-virus inoculation than those preventively exposed 24 h pre-inoculation. Generally, the AgNPs applied to the plant foliage are absorbed, dispersed into the plant cells and it was found to interact with many macromolecules, lipids, pectin, lignin and other cellular components (38). Furthermore, AgNPs have a high protein-affinity that facilitates interacting with a wide array of host proteins and cellular entities (39, 40). Accordingly, these phenomena support our suggestion that the rate of free dispersed nanoparticles that are required to interfere with the virus units might be captured by plant proteins and other cellular components when plants are preventively treated 24 h ahead to virus inoculation. Conversely, the application of AgNPs after virus inoculation could increase the probability of a high number of nano-silver particles to target the viral proteins and/or the whole virus particle that eventually exhibit outstanding efficiency in suppressing BYMV disease compared to preventive applications of AgNPs applied at the same concentrations. The AgNPs-plant protein interactions are not documented clearly but other previous studies stated that the initial interaction between proteins and metal nanoparticles changes their in vivo properties and has remarkably altered the mechanism of nanoparticle interaction with other cell targets (41).

As demonstrated in the present study, the transmission electron microscope confirms the ability of AgNPs to suppress viral infection in faba bean plants by targeting the BYMV particles directly when applied after virus inoculation. A potential
notion for this may be due to the synthesized AgNPs have a surface area with negative charges, resulting in a high surface bio-reactivity to hold the viral capsid proteins which prominently contain a high positive charge. The results obtained herein are convergent with a previous finding that proved the ability of nano-silver particles to extensively aggregate and interfere with the viral envelope protein units of *Potato virus Y in vitro* (42). Thus, it is reasonable to argue that the AgNPs effects on the virus particles may be determined by the particle chemical nature, reactivity and the free dispersed amount of NPs present in the plant. Unfortunately, very limited reports have been conducted on the AgNPs-virus interactions and the mode of action of AgNP on virus particles still rather unambiguous in many plant, animal and human viruses. The underlying mechanisms of action of AgNPs have been proved as a novel therapeutic agent against some human and animal viruses (43). Due to the high affinity of Ag ions for nitrogen and sulfur, it is believed that it can disrupt the structure of many viral proteins by binding to amino and thiol groups. In this respect, Khandelwal et al. (44) documented that the antiviral activity of AgNPs and other metal nanoparticles might be attributed by interference with glycoproteins of the viral envelope which might ultimately prevent the virus invasion, uncoating of viral coat protein and the subsequent replication process of virus particles within the host cell. The small engineered size of NPs of 7–20 nm was proved to have higher antiviral effects by binding to the viral nucleic acid, translated functional proteins and cellular factors during the virus replication (45). This supports the notion that the AgNPs particle size seems to be critical for its antiviral properties. To some extent, all this fact might point out the role of silver in controlling plant viruses. However, more studies are required to prove the direct underlying mechanism of action for AgNPs in combating plant viruses, since the plant viruses have a different nature than other animal and human viruses especially in their mode of infection and cell invading. Moreover and given that the role of plant plasmodesmata in transporting substances including invaded-viruses from cells to another and since the nanoparticles are easily dispersed in the plant through its intercellular structure channels, with particle size less than 40 nm (46), we also envision that the AgNPs could affect the plasmodesmata protein factors and/or viral movement protein complex that facilitates the virus cell-to-cell movement (Fig. 7A).

Regarding the AgNPs ability in promoting the plant defense mechanism against the virus invading, our findings indicate that the changes in enzymes activity, upregulation of PR-1 gene expression and protein accumulation was not associated with increasing the application rate of AgNPs, in which the highest tested concentration of AgNPs affects the POD activity peak and reduced the protein accumulation. Furthermore, the upregulation of the PR-1 gene was lower than that obtained at the lowest tested dose. This finding suggests that the antiviral activity of AgNPs in defeating BYMV is not strongly correlated with strengthened plant immunity against the virus and the direct inhibition depending on the reactivity of AgNPs to virus remained sufficient to arrest the virus infectivity, albeit a dual role by stimulating plant resistance may be obtained with low AgNPs concentrations. Generally, AgNPs have been found to increase the plant oxidative enzymes and up-regulated the immunity-related genes such as PR-1 with very low concentrations (47). The mechanism underlying the role of AgNPs to motivate some adaptive mechanisms in plant defense booster is mainly due to its potential to enhance the reactive oxygen species (ROS) in treated plants (48). In this respect, plants activate some enzymatic and non-enzymatic constituents as a scavenging system to cope with the toxic free radicals of oxygen to prevent cell damage. All previous reports have indicated that the ROS is increased with increasing the AgNPs concentrations and with reducing particle size (49). Therefore and as the results reported herein, the sharp decrease in the peak of POD activity with the increasing of AgNPs rate is might be an indicator of removal of stressful conditions by other enzymatic scavenging systems, and since the faba bean plants have high phenolic content, the ongoing increase in oxidative enzymes like POD as a response for an excess generation of ROS could resulting in high oxidizing rate for phenolics that might ultimately affect the plant cell integrity. Consistent to results obtained herein concerning the protein accumulation and relative expression of PR-1 gene, previous studies have been differentially proved the AgNPs ability to up-regulate many biotic and abiotic-specific genes and increased protein accumulation in several treated plants at low rates, while considerable numbers of genes were down-regulated with increasing AgNPs rate (50, 51). Arising from this fact is the AgNPs applied with high concentrations may damage the plant transcription machinery and genomic background in treated plants applied with high concentrations (52, 53), which could concurrently affect the transcription level of some defense-related genes, protein accumulation and the synthesis of viral polyproteins depending on the AgNPs rate.
The first required step for virus transmission is the ability of vector insects to acquire the virus from an infected plant and transmit it to other healthy plants. To the best of our knowledge and according to the available literature, there is nothing prior study was conducted to investigate the AgNP activity against virus acquisition and subsequent transmission by vector aphids. Thus, for this study, it was of interest to decide whether AgNPs can effectuate the virus-vector interaction and disrupt the virus acquisition and transmission capabilities. Our results clearly showed that the AgNPs applied prior-the virus acquisition greatly affects the BYMV transmission 24 h soon after application, to reach 100 % at 250 and 300 mg.l$^{-1}$. The inhibiting activity reduced with time, and the virus acquisition was effectively inhibited for 10 days by more than 50 % at all tested concentrations excepted at the rate of 50 and 100 mg.l$^{-1}$. No previous studies hypothesized the direct activity of AgNPs on virus-vector interaction, however, a few studies have elicited the activity of AgNPs and other metal nanomaterials on insect mortality, oviposition, feeding and larva development (54). Further, a trial conducted by Chakravarthy et al. (55) stated that AgNPs applied at high concentration (2400 mg.l$^{-1}$) had damaged the whole insect digestive system leading to insect death, while the concentration of AgNPs lower than 50 mg,l$^{-1}$ caused chronic toxicity by affecting the reproduction, emergence and larval development (56).

Generally, non-persistent plant viruses including BYMV are retained in receptors present on the stylets common duct of insect mouthpart and are injected to the plant by salivating during brief intracellular punctures, these receptors are cuticular proteins that readily accessible all over the surface of the acrostylet responsible for the aphid transmission of potyviruses and many other plant viruses (57). The current understanding of how AgNPs interfere with intracellular components inside the insect is almost non-existent, however, the known mechanism is that AgNPs can interact with insect protein involved in serval cellular functions (58). Accordingly, we speculate that exposure of aphids to AgNPs treatments before virus acquisition may properly affect the cuticular proteins binding to aphid mouthpart, altering the ability of vector aphids to acquire the virus.

On the other hand, our study illustrates the ability of AgNPs treatments to prevent aphids to acquire the virus from AgNPs-treated plants infected with BYMV, in which the acquisition and transmission were entirely inhibited as low concentration as 150 mg.l$^{-1}$. Coat protein (CP) encapsidating the viral nucleic acid is the critical point in virus transmissibility by aphid vector in which the virus particles adhere to stylet mouthpart through motifs in CP and another viral protein called helper-component protein (HC-pro) that acts as a bridge between virus particles and the receptor proteins on insect stylet (59), hence, lacking in aphid transmissibility for viruses may occur with a chemical or physical change in the virus particles and the configurational HC-Pro protein. Consequently, and as demonstrated in the current study, it is plausible to suggest that the inhibition in virus acquisition and transmissibility when AgNPs applied on BYMV-infected plants may be due to the ability of AgNPs to interfere with viral CP and HC-pro proteins before the aphid came in contact with the virus particles, which could sequester or prevent the virus particles to adhere to the receptors on stylet mouthpart (Fig. 7B).

5. Conclusion

In conclusion, despite the available information about the antiviral capabilities of AgNPs and their toxicity against pests, no knowledge was elicited on its possibility to affect the virus -vector interactions. These results surprisingly cast a new light on the potentiality of AgNPs to influence the virus acquisition and transmission by vector insect, which could be attributable to either by affecting the insect stylet mouthpart and/or by subverting or interference with viral CP and HC-pro proteins involved in virus-aphid transmissibility. However, further expanded research on this initial study is needed to explore the actual mechanisms underlying the critical role of silver nanoparticles in the virus-vector interactome. Importantly, this work introduces a new approach to utilize such nanoparticles as a potential candidate against insect transmitted-plant viruses, which could offer a new alternative strategy to control plant viruses under field conditions. However, further research should be carried out to assess the environmental and health cost before abundantly use in the cropping system compared to those conventional pesticides that are widely used in the agricultural sector.
6. Declarations

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7. References

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Figures
Figure 1

Bean yellow mosaic virus disease phenotypic response and molecular confirmation. A: Symptoms of naturally infected faba bean plants with BYMV in the field showing severe mosaic symptoms. B: Typical mosaic symptoms of isolated BYMV as a result of artificial inoculation on faba bean plants under insect-proof greenhouse conditions. C: Agarose gel electrophoresis of two migrated RT-PCR products illustrating the genotypic identification of BYMV by amplifying the coat protein gene at a molecular weight of 907 bp; Lan 1: Healthy plant control, Lan 2: BYMV-infected faba bean plant, Lan 3: Artifally inoculated C. amaranticolor sample., M: 100 bp DNA ladder.
Figure 2

Physico-chemical characterization of synthesized silver nanoparticles (AgNPs). A: Zeta potential measurement showing a negative surface charge with -36.5 mV. B: Particle size distribution histogram of AgNPs with an average size of 7.43 nm. C: X-ray diffraction (XRD) pattern displaying four typical peaks related to the diffraction planes of silver nanoparticles. D: HR-TEM micrograph illustrating spherical shape nanoparticles with 8.54 nm average size.
Figure 3

Effect of silver nanoparticles (AgNPs) on bean yellow mosaic virus disease infectivity and the virus concentration. A, B: Disease occurrence and severity on faba bean plants treated with foliar protective (A) and curative (B) applications of six tested concentrations of AgNPs compared to untreated control (C+ve). C: Symptomatic disease response on untreated plants displaying a severe mosaic and stunting compared to plants treated 24 h pre- and 48 h post- virus inoculation. D, E: BYMV accumulation content, scored 30 days post-virus inoculation for preventively and curatively treated plants compared to untreated control as well as negative healthy control (C-ve). Values with the same letters in each category have no significant difference (P<0.05).
Figure 4

Transmission electron microscope micrograph from leaf dip-preparations of BYMV illustrating the direct effect of silver nanoparticles on virus units in vivo. A: AgNPs aggregates and attached to the envelope of virus particles in treated plants compared to untreated control (C), B: AgNPs- healthy treated plants served as a control. Photos were captured under direct mag.:100000 x with scale pare of 100 nm, Hv=80.0kV.

Figure 5
Demonstrations of induced enzymatic and biochemical changes in treated faba bean plants with silver nanoparticles (AgNPs) compared to untreated control. A: Modulation in peroxidase (POD) and polyphenol oxidase (PPO) oxidative enzymes activities showing different peaks of activity within the time course of six days after spraying. The activity was determined as nmol guaiacol.mg-1 protein.min.-1. Values are means ±SE of three repeats (P≤0.05), B: Protein patterns illustrating changes in protein accumulations. C: Expression pattern analysis of pathogenesis-related gene 1 (PR-1) in response to three tested concentrations of AgNPs over the untreated control. Values are means of three repeats ±SE (P ≤0.05).

Figure 6

Silver nanoparticles (AgNPs) affect Bean yellow mosaic virus (BYMV) -acquisition and transmissibility. A: Effect on the percentage of BYMV-acquisition and transmissibility when vector aphids get contact with AgNPs-treated plants at 1, 5 and 10 days before acquisition assay. B: Gel electrophoresis analysis of RT-PCR coat protein gene products of BYMV in faba bean plants 30 days after acquisition assay, AgNPs treatments were sprayed 24 h pre-acquisition assay (before aphids get in contact with virus-infected plants). Lan1: control, Lan 2,3,4,5,6 and 7 are: 300, 250, 200, 150, 100 and 50 mg. l-1 of AgNPs treatments respectively. C: Disease response on faba plants scored 21 days after challenged with aphids at 24 h. D: Proportion of acquisition from AgNPs-treated plants infected with BYMV 24 h before acquisition assay. Values with the same letters for each experiment have no significant difference.
Figure 7

Schematic diagram proposing the direct mechanism of action of silver nanoparticles against virus infection (A) and virus acquisition and transmissibility process by their aphid vector (B).