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Protocols

Localized surface plasmon resonance biosensing of *tomato yellow leaf curl virus*

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ABSTRACT

Current techniques for plant virus detection, such as RT- PCR and ELISA, require multistep procedures and rely on sophisticated equipment. Due to the global spread of plant viruses, the development of simpler, faster and cheaper assay methods is inevitable. Gold nanoparticles (AuNPs) had raised much interest during recent years due to their novel optical properties or diagnostic purposes. The localized surface plasmon resonance (LSPR[1]) of AuNPs had been used in the development of novel colorimetric nano-biosensing systems. The frequency and intensity of the LSPR peak generally depend on the shape, size and the surrounding medium of the AuNPs. In this study, unmodified AuNPs had been used to detect the unamplified *Tomato yellow leaf curl virus* (TYLCV) genome in infected plants. A specific DNA probe complementary to the coat protein region of virus genome was designed. The extracted total DNA of uninfected and infected plants was mixed with hybridization buffer and the designed probe. The mixture was denatured, annealed and then cooled to room temperature and was followed by AuNPs addition. The color changes in the samples indicating the presence of target virus infections were assessed visually after the addition of salt and confirmed by UV–Vis spectroscopy. The results showed that this strategy allowed for fast and sensitive detection of TYLCV genome and eliminated the need for PCR amplification and detection equipment.

1. Introduction

The continuing worldwide spread of plant viruses has created an urgent need to develop sensitive, specific and cost-effective detection techniques. Current techniques, such as PCR[2], RT-PCR[3] and ELISA[4], mostly require multistep procedures that are time-consuming and require trained personnel and expensive equipment (Azzazy et al., 2012; Shawky et al., 2010). Recently, AuNPs had been used to develop new diagnostic assays that could be used even by non-specialized personnel and would allow specific and sensitive detection of various analytes without the need for high-cost dedicated equipment (Liandris et al., 2009; Vaseghi et al., 2013; Yarbakht and Nikkhah, 2016). AuNPs exhibit a unique phenomenon known as Localized Surface Plasmon Resonance (LSPR) which is responsible for their intense red color (Azzazy and Mansour, 2009; Baptista et al., 2008). The frequency and intensity of the LSPR peak depend on the shape, size and the surrounding medium of the AuNPs. The addition of salt to a solution containing negatively charged AuNPs leads to the aggregation of the particles, resulting in a change in solution color from bright red to blue and a reduction of the intensity and broadness of the LSPR peak. (Abbasian et al., 2014; Li and Rothberg, 2004a). Colorimetric assays for direct detection of short sequences of DNA had been developed based on the fact that adsorption of single-stranded DNA (probe) on AuNPs increases the negative charge of the particles and consequently, increases the repulsion between them and thus preventing their aggregation upon salt addition (Li and Rothberg, 2004a, 2004b). Upon

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[1] LSPR: localized surface plasmon resonance.
[2] PCR: polymerase chain reaction.
[3] RT-PCR: Reverse transcription polymerase chain reaction.
[4] ELISA: enzyme-linked immunosorbent assay.

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hybridization of the single-stranded DNA with its complementary strand, the resulting double stranded DNA does not efficiently adsorb on the surface of AuNPs and as a result salt aggregation of AuNPs occurs. Shawky et al. (2010) showed that in the colorimetric assay of unamplified hepatitis C virus (HCV) RNA from clinical specimens, four factors were important. These factors included the concentration of NaCl, size of the AuNPs, the assay temperature and the probe sequence. The length of the probe and the percentage of adenine bases influence the adsorption efficiency of probe on AuNPs (Abbasian et al., 2014; Nelson and Rothberg, 2011). Optimization of these factors in AuNPs-based colorimetric assay is essential to prevent false positive and negative results.

Wang et al used unmodified **gold** nanoparticles as probes for Colorimetric detection of RT-PCR amplified Cucumber green mottle mosaic virus (Wang et al., 2017). In another study, colorimetric detection of PCR amplified DNA of *Tomato leaf curl New Delhi virus* using **gold** nanoparticle conjugated bifunctional oligo probe has been reported (Dharanivasan et al., 2016). In both of these studies PCR amplification of the virus genome is nessessary. In another report, AuNPs functionalized with single-stranded oligonucleotides have been served to detect *Ralstonia solanacearum* genomic DNA without the need for amplification of the target DNA (Khaledian et al., 2017). Surface plasmon resonance (SPR) technique has been used to directly detect an intact form of insect pathogen: the baculovirus, Autographa californica multiple nuclear polyhedrosis virus (Baac et al., 2006).

Tomato yellow leaf curl virus (TYLCV) is one of the most destructive viruses of tomato that causes serious economic losses to tomato crops in tropical, subtropical and warm temperate regions of the world (Azizi et al., 2008; Moriones and Navas-Castillo, 2000). Symptoms of disease include severe stunting of plant growth, erect shoots, miss shaped leaflets, inward and upward leaf curling and subsequently, developing leaves are severely chlorotic and show curling of the leaflets (Azizi et al., 2011).

This virus is a small circular single-stranded DNA virus that belongs to the *Geminiviridae* family and *Begomovirus* genus (Azizi et al., 2011). This report describes the application of unmodified AuNPs as a tool for the rapid detection of unamplified genomic DNA of *Tomato yellow leaf curl virus* (TYLCV) in infected plants (Fig. 1). This method would be an easy, fast and promising way to detect infected plants.

2. Materials and methods

2.1. Plant and viruses

Tomato plants (*Solanum lycopersicum* L.) showing TYLCV like symptoms such as leaf curling, yellowing and stunting have been collected from a field in Hormozgan province, southern Iran. The isolate was named TYLCV-Ir2 and its nucleotide sequence was registered in NCBI under the accession NO. EU085423 (Azizi et al., 2011).

2.2. DNA extraction and PCR reaction

Total DNA was extracted from chlorotic and curly leaves of infected plant according to Dellaporta et al. (1983) with slight modifications (Azizi et al., 2011). The extracted DNA was diluted and used as a template for PCR and colorimetric assays. PCR reaction was performed by using TYLCV- specific primers (TYLCV-F: 5′-CGCCCGTCTCGAAGG TTC-3′ and TYLCV-R: 5′- GCCATATACAATAACAAGGC -3′) (Macrogen, South Korea) that were designed according to the conserved sequences of TYLCV-Sar and TYLCV-Isr (Pico et al., 1999). The reaction was carried out in a total volume of 25 µl containing 2.5 µl of 10x PCR buffer, 200 ng of extracted plant DNA, 1.25 unit *Taq* DNA polymerase (Fermentas), 1 µl of each of the primers (10 µM each), 1 µl of $MgCl_2$ (50 mM), 0.25 µl of dNTPs (20 mM each) and deionized H_2O up to the final volume of 25 µl. The mixture was incubated in a Thermal Cycler (BIO-RAD, PTC1148) at 94 °C for 4 min for the first denaturation step followed by 27 cycles at 94 °C for 30 s 54 °C for 30 s, and 72 °C for 45 s and the final extension was done at 72 °C for 10 min. The amplified PCR products were analyzed by 1% agarose gel electrophoresis.

2.3. Synthesis and characterization of AuNPs

AuNPs were synthesized by the citrate reduction method (Storhoff et al., 1998; Shawky et al., 2010). Briefly, the reflux system was cleaned by aqua regia and then rinsed with Milli-Q water.

An aqueous solution of tetrachloroauric acid ($HAuCl_4$) solution (250 µM, 50 mL) was boiled while stirring and when it was brought to reflu × 2 ml of 1% trisodium citrate (38.8 mM) was added quickly. This resulted in consequent changes in solution color from transparent light yellow to gray, black, purple and finally cherry red, which indicated the formation of AuNPs. Afterwards, the solution was refluxed for an additional 15 min and then allowed to cool to room temperature while stirring. This solution was stored at 4 °C for further use. It should be mentioned that, all the glassware and the reflux system used in the experiments were cleaned by aqua regia and then rinsed thoroughly with Milli-Q water.

UV/Vis spectroscopy of AuNPs was performed using Alpha-1860S/1900S spectrophotometer (LAXCO, Inc,). The hydrodynamic radius and size distribution of the nanoparticles were measured using Zetasizer Nano ZS instrument (Malvern Instruments, UK).

2.4. Determination of the salt concentration threshold for the induction of AuNPs aggregation

The threshold of the salt concentration that would induce aggregation of nanoparticles was determined by addition of different amounts of phosphate buffer saline (phosphate buffer 100 mM and NaCl 1 M) in the range of 20–40 mM to 100 µl of colloidal AuNPs and color modification was observed at different time points. The changes in LSPR peak of the salt-treated AuNP solutions were analyzed by UV/Vis spectroscopy.

2.5. Probe design and optimization of probe concentration

The reverse primer (20-mer) (pico et al., 1999) which contains 45% adenine bases and has a high level of specificity to all TYLCV genotypes available was used as the probe.

The probe concentration was optimized by addition of different amount of probe (5 pmol/µL) to 96 µl of colloidal AuNPs. The mixture incubated at room temperature for 5 min and then 4 µl of phosphate buffer saline (phosphate buffer 100 mM and NaCl 1 M) was added and the color changes were observed. The changes in corresponding LSPR peak intensities were followed by UV/Vis spectroscopy.

2.6. Detection of viral genomic DNA using AuNPs with the AuNPs colorimetric assay

The colorimetric assay was performed as follows: 4 µl of deionized water, 4 µl of phosphate buffer saline, 1 µl of probe (5 pM) and 1 µl of extracted plant DNA (200 ng/µl) were mixed together. The mixture was heated at 95 °C in a thermocycler (BIO-RAD) for 3 min and then cooled to 54 °C for 30 s and then cooled to room temperature for 10 min. Then 90 µl of colloidal AuNPs was added to the mixture and the color was observed after 15 min. The final concentrations of NaCl, probe and plant DNA were 40 mM, 0.05 pM and 2 ng/µl respectively.

2.7. Specificity and sensitivity of the AuNPs colorimetric assay

The specificity of the method was assessed by applying it to detect the total DNA in Beet curly top virus (BCTV-Svr) infected beet leaves. To assess the sensitivity of the assay, serial dilutions of the extracted DNA from TYLCV-infected tomato (0.75–200 ng/µl) were prepared and

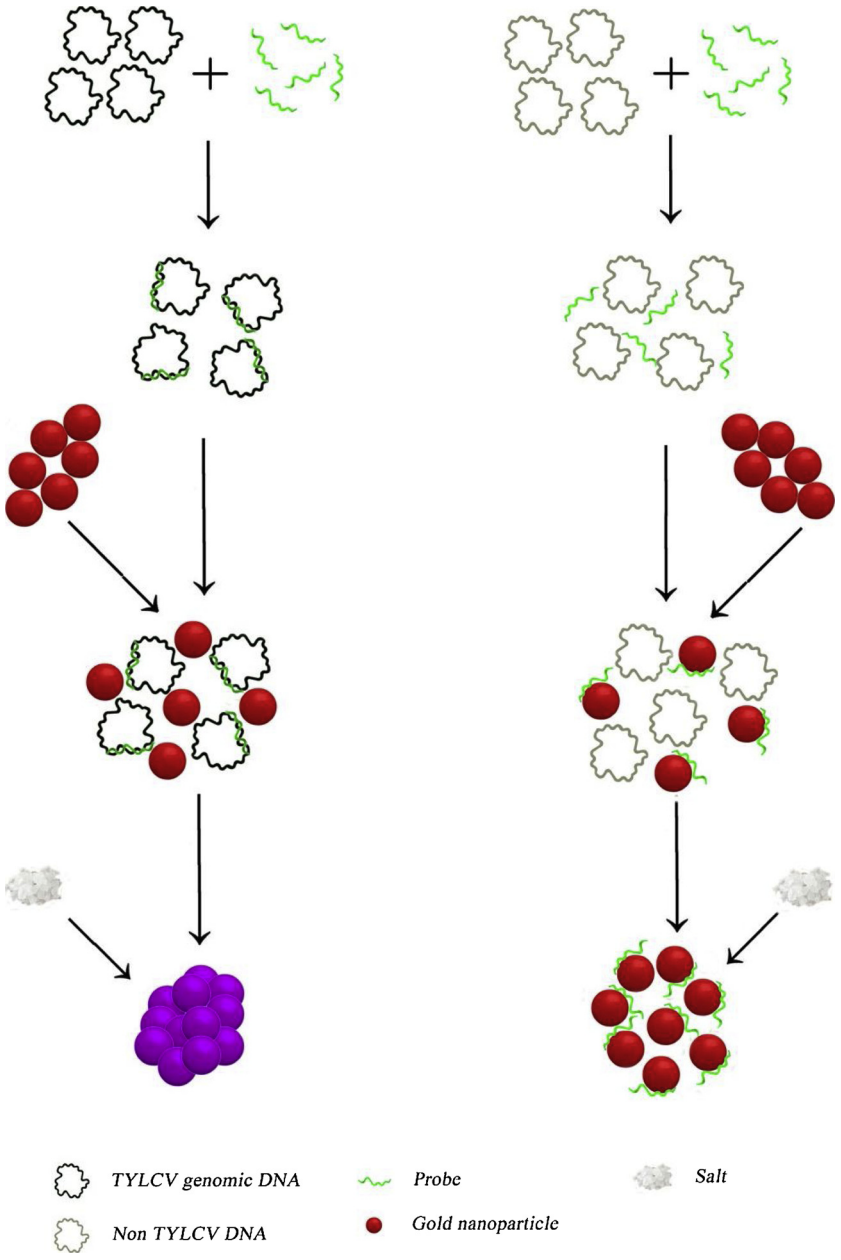

Fig. 1. Schematic representation of TYLCV colorimetric detection by using gold nanoparticles.

assayed using the AuNPs colorimetric assay and the PCR method as described earlier.

3. Results

3.1. PCR detection of TYLCV infected plants

PCR amplification of a 670 bp fragment was performed using extracted total DNA of all suspicious plant samples as template (Pico et al., 1999). As it can be seen in Fig. 2, for the samples 3 and 5 that were suggested to be infected, the 670 bp fragment was amplified but the infection of other morphologically suspected plants was not confirmed.

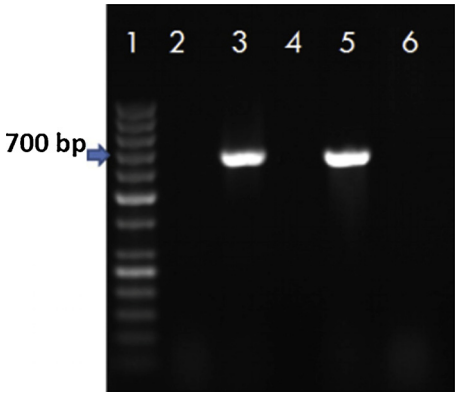

Fig. 2. Detection of TYLCV DNA by PCR. The numbers refer to 1: DNA ladder, 2: uninfected plant, 3-5: plants that showed symptoms of viral infection 6: PCR negative control (without template).

3.2. Characterization of the synthesized AuNPs

The plasmonic peak of AuNPs was recorded by UV–Vis spectroscopy. The absorbance spectrum of the prepared AuNPs displayed a

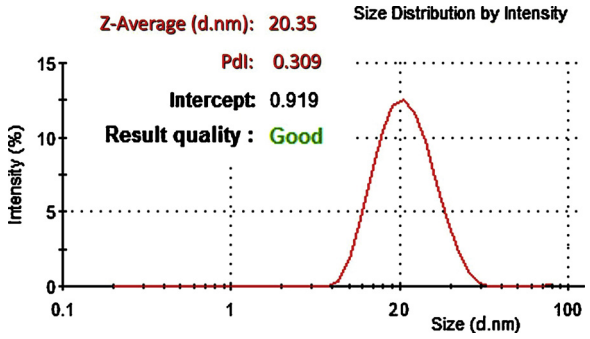

Fig. 3. Dynamic light scattering analysis of synthesized AuNPs. The mean hydrodynamic diameter of the synthesized AuNP is 20.39 nm and the polydispersity index (PDI) represents narrow size distribution of the AuNPs.

single peak in the visible region with λ_{max} at 520 nm. Based on dynamic light scattering (DLS) results the average diameter and polydispersity index of AuNPs were 20 nm and 0.3 respectively (Fig. 3).

3.3. Optimization of salt and probe concentrations

To find the salt concentration that could induce the aggregation of AuNPs, PBS buffer containing 1 M NaCl in the concentration range of 20–40 mM was added to AuNPs colloidal solution. Fig. 4 depicts the color changes of the AuNPs solutions 15 and 45 min after addition of increasing amount of salt. It was found that 40 mM of NaCl was enough to induce aggregation and the color change of the of AuNPs colloidal solution in 15 min. The UV–Vis spectra of the samples after addition of 40 mM of NaCl at different time points have been presented in Fig. 5. In this study, at final salt concentration of 40 mM, the optimal probe concentration was found to be 0.05 µM (data not shown). Although high concentrations of probe are effective in stabilizing nanoparticles, in the presence of the target sequence higher probe concentrations would prevent aggregation leading to false negative results. On the other hand, in the absence of the target sequence, lower probe concentration will not be sufficient to prevent aggregation leading to false positive results (Shawky et al., 2010).

3.4. Colorimetric detection of viral genome

In order to assess the method performance regarding the detection of viral genome, the optimized conditions were applied to the DNA isolated from infected and uninfected plants. The color change of the

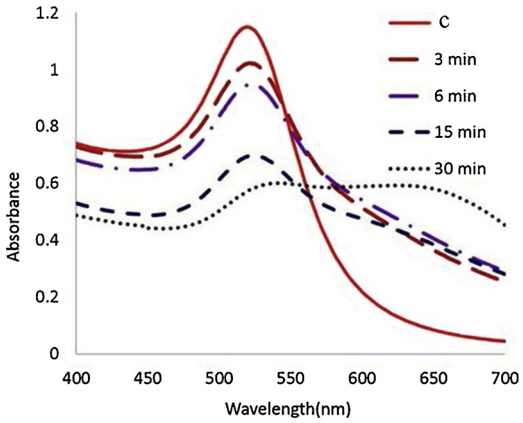

Fig. 5. UV–Vis spectra of the AuNPs colloidal solution measured at different time points after addition of 40 mM of NaCl. During 30 min after addition of salt, significant decrease of LSPR peak due to gradual aggregation of AuNPs is observed.

reactions was inspected after the addition of salt, which can be clearly seen in Fig. 6a. After addition of salt/ buffer solution to samples 1 (AuNPs) and 2 (AuNPs + total DNA), the aggregation of nanoparticles was observed as the color of the samples turned to gray and the characteristic peak of the nanoparticles at 520 nm was completely destroyed (Fig. 6b). These observations confirm that the presence of total DNA could not prevent the aggregation of AuNPs. In sample 3 (AuNPs + probe) the color of the AuNPs remained red by salt addition and the λ_{max} was shifted to 534 nm indicating the resistance of AuNPs against salt induced aggregation. In the sample 4 (AuNPs + probe + TYLCV genome) the color changed and the maximum absorbance of the nanoparticles shifted toward longer wavelengths (636 nm) indicating the nanoparticle aggregation. In this case due to the hybridization of probe with target sequence, free probes are not available to stabilize the AuNPs so the nanoparticles were aggregated upon salt addition. The results show that the unmodified AuNPs were able to detect the viral genomic DNA extracted from the infected plant. In the case of uninfected plant sample (sample 5) the extracted DNA did not hybridize to probes and hence the color remained red with λ_{max} at 528 nm.

3.5. Determining the specificity and sensitivity of colorimetric assay methods

TYLCV and Beet curly top virus BCTV belong to the *Geminiviridae*

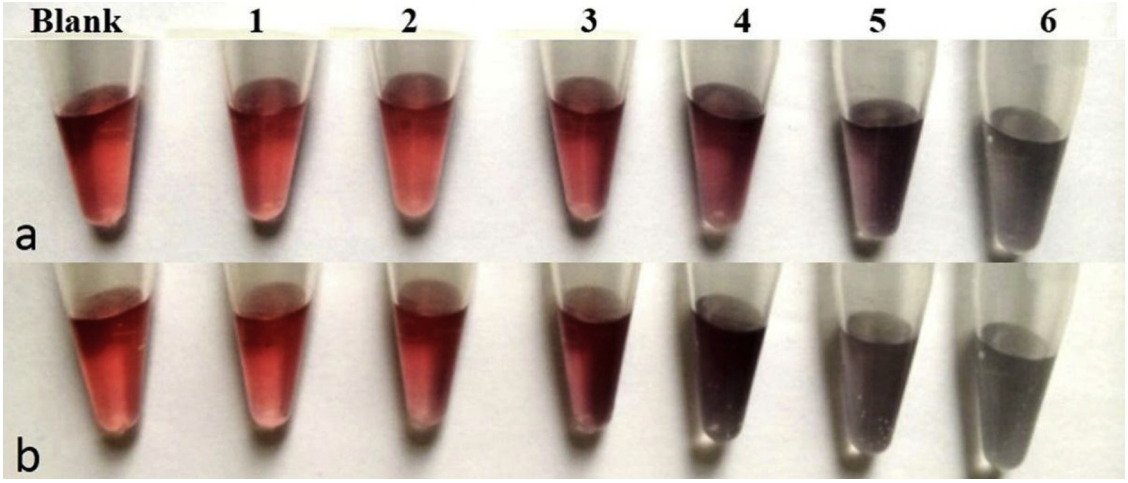

Fig. 4. Determination of salt concentration for induction of AuNPs aggregation. The effects of different concentrations of salt on the color of AuNPs after a: 15 min and b: 45 min. The numbers 1–6 refer to 20, 24, 28, 32, 36 and 40 mM of NaCl respectively.

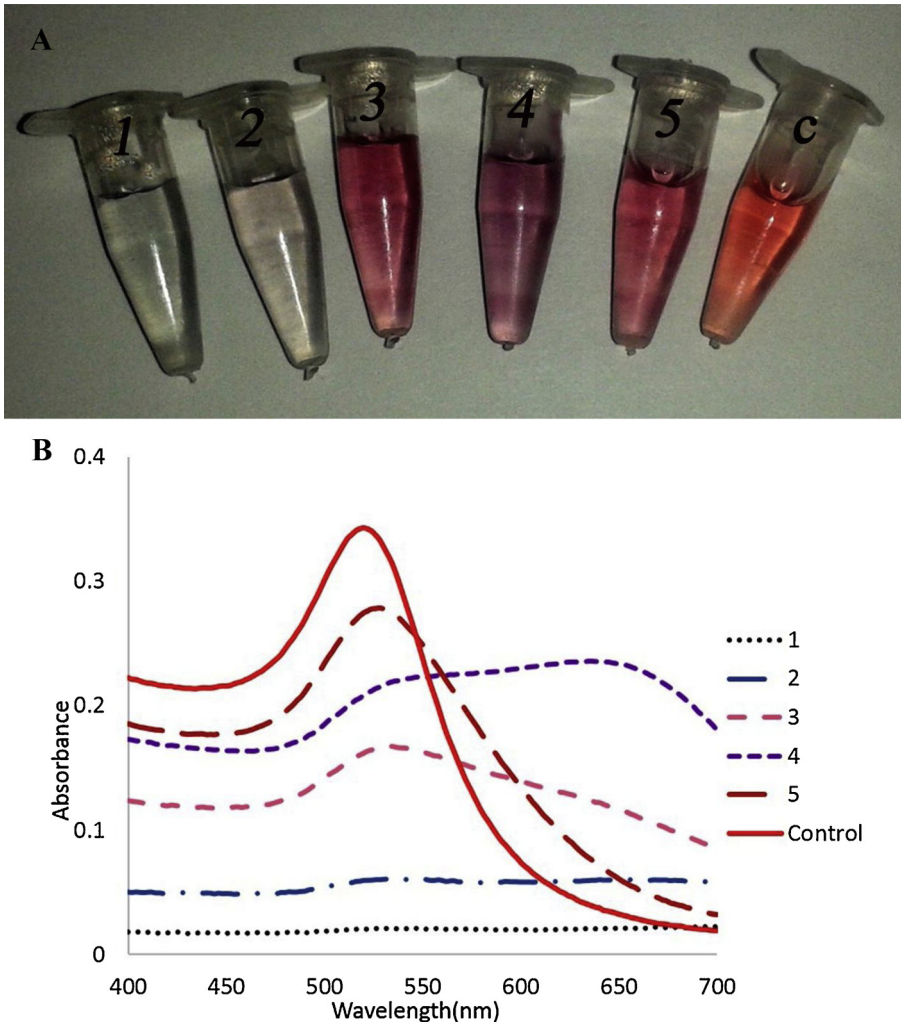

Fig. 6. a) Visual detection of TYLCV genome by the colorimetric assay. 1: AuNPs + salt/ buffer solution, 2: AuNPs + plant total DNA + salt/ buffer solution, 3: AuNPs + 1 µl of 5 pM/µl probe + salt/ buffer solution, 4: AuNPs + extracted total DNA from infected plant + 1 µl of 5 pM/µl probe + salt/ buffer solution, 5: AuNPs + extracted total DNA from uninfected plant + 1 µl of 5 pM/µl probe + salt/ buffer solution, C: AuNPs. b) The corresponding UV–Vis spectra of the samples.

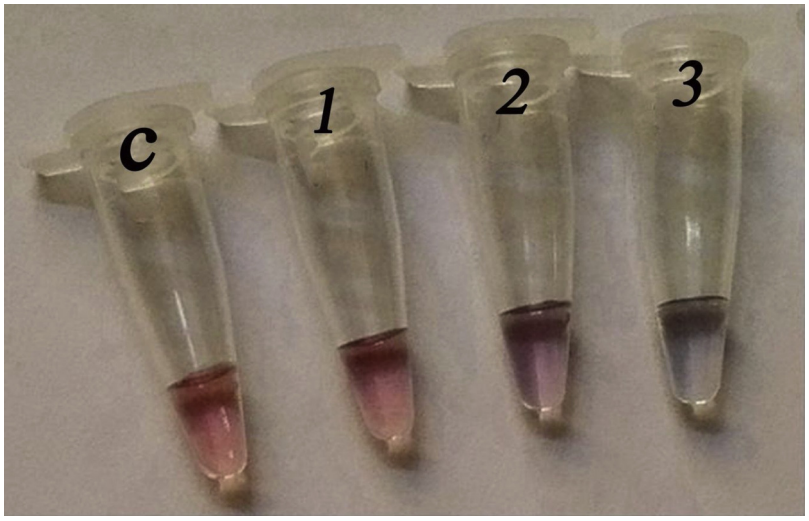

Fig. 7. Specificity of the developed colorimetric assay. C: AuNPs, 1: AuNPs + extracted total DNA from BCTV-Svr infected plant + 1 µl of 5 pM/µl probe + salt/ buffer solution, 2: AuNPs + extracted total DNA from TYLCV infected plant + 1 µl of 5 pM/µl probe + salt/ buffer solution, 3: AuNPs + salt/ buffer solution + healthy plant total DNA.

family; both the viruses contain a single-stranded DNA. Sequence alignment of probe and BCTV genome showed about 30% similarity. Although sugar beets are known to be the main host of BCTV, it also affects many plants including tomatoes (Chen et al., 2010). The colorimetric assay was performed for total DNA extracted from BCTV-Svr and TYLCV infected plants (Fig. 7). As can be seen, the color of sample 1 (AuNPs + probe + BCTV genome) did not change while the color of sample 2 was turned from red to purple, showing the presence

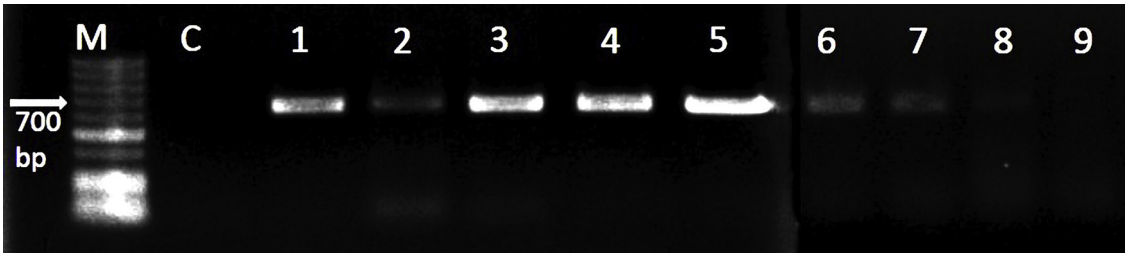

Fig. 8. TYLCVdetection of the PCR assay. M: DNA ladder (50 bp), C: Healthy plant 1-9: different amounts of extracted DNA from TYLCV-infected plants (200, 100, 50, 25, 10, 5, 2.5, 1.5 and 0.75 ng respectively).

Table 1
Comparison of the limits of detection of DNA extracted from TYLCV infected plants by PCR and the developed colorimetric assay. In colorimetric assay "+" indicates the color change that can be detected by eye and in PCR method "+" indicates the observation of the amplified DNA on the gel.

sample	TYLCV infected Plant DNA (ng/ μL)	PCR	Colorimetric assay
1	200	+	+
2	100	+	+
3	50	+	+
4	25	+	+
5	10	+	+
6	5	+	+
7	2.5	+	
8	1.5	+	
9	0.75		

of TYLCV genomic DNA in sample 2. Although, the infection of samples 1 by BCTV was proven by PCR, the suggested nanobiosensor which was specifically designed for detection of TYLCV could not detect the BCTV genome and the color of AuNPs remained red. In the next step, the sensitivity of the assay was compared to conventional PCR. The PCR has been very successful in TYLCV genome detection and was able to amplify the 670bp fragment from 1.5 ng of the extracted DNA from infected plants (Fig. 8). The developed nanobiosensor is able to detect the presence of TYLCV genome in 5 ng of the extracted DNA from infected plants without the need for amplification (Table 1).

4. Discussion

AuNPs- based colorimetric biosensing assays have attracted considerable attention for diagnostic applications due to their simplicity and versatility (Liandris et al., 2009; Vaseghi et al., 2013; Zhao et al. 2008). Unmodified AuNPs can significantly contribute to the development of simpler, faster, and cheaper assays for nucleic acid detection (Azzazy et al., 2012; Shawky et al., 2010). Nevertheless, excepting very few cases, this potential platform has not been exploited for detection of plant pathogens. Herein, we developed a LSPR- based colorimetric nanobiosensor which is able to detect unamplified TYLCV genome in the context of plant total DNA. This biosensing approach is able to discriminate between TYLCV and BCTV that belongs to the *Geminiviridae* family and have several Common features including: small circular single-stranded DNA and similarity in the probe hybridizing region (Heydarnejad et al., 2007; padidam et al., 1995; Rochester et al., 1994). By using the probes that hybridize to the conserved regions of the genome among the members of the family, all the members of the genus or family can be detected simultaneously. In addition, the genomes of the most plant viruses are RNA that are not directly detectable by PCR and require experimentations, the developed colorimetric assay is theoretically eligible for detection of RNA viruses.

5. Conclusions

Overall, The results showed that use of AuNPs allows for fast and sensitive detection of TYLCV genome and eliminates the need for PCR amplification and detection instruments and specialized personnel.

Conflict of interest

The authors declare that they have no conflict of interest.

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