

ORIGINAL ARTICLE

Enhancement of fungal DNA templates and PCR amplification yield by three types of nanoparticles

Fahad A. Al-Dhabaan¹, Heba Yousef², Tahsin Shoala^{3*}, Jumana Shaheen³,
Yousra El Sawi³, Tasneem Farag²

¹ Department of Biology, Science and Humanities College, Alquwayyah, Shaqra University, Saudi Arabia

² Plant Pathology Research Institute, Agricultural Research Centre (ARC), 12619, Giza, Egypt

³ College of Biotechnology, Misr University for Science and Technology, P.O. Box: 77, 6th of October City, Egypt

Vol. 58, No. 1: 66–72, 2018

DOI: 10.24425/119119

Received: August 21, 2017

Accepted: December 4, 2017

*Corresponding address:
tahsen.shoala@must.edu.eg

Abstract

Nanodiagnostic methods in plant pathology are used for enhancing detection and identification of different plant pathogens and toxigenic fungi. Improvement of the specificity and efficiency of the polymerase chain reaction (PCR) by using some nanoparticles is emerging as a new area of research. In the current research, silver, zinc, and gold nanoparticles were used to increase the yield of DNA for two plant pathogenic fungi including soil-borne fungus *Rhizoctonia solani* and toxigenic fungus *Alternaria alternata*. Gold nanoparticles combined with zinc and silver nanoparticles enhanced both DNA yield and PCR products compared to DNA extraction methods with ALB buffer, sodium dodecyl sulfate, ALBfree from proteinase K, ZnNPs and AgNPs. Also, by using ZnNPs and AgNPs the DNA yield was enhanced and the sensitivity of random amplified polymorphic DNA (RAPD) PCR products was increased. Application of nanomaterials in the PCR reaction could increase or decrease the PCR product according to the type of applied nanometal and the type of DNA template. Additions of AuNPs to PCR mix increased both sensitivity and specificity for PCR products of the tested fungi. Thus, the use of these highly stable, commercially available and inexpensive inorganic nano reagents open new opportunities for improving the specificity and sensitivity of PCR amplicon, which is the most important standard method in molecular plant pathology and mycotoxicology.

Key words: *Alternaria alternata*, DNA extraction, metal nanoparticles, nano-PCR, *Rhizoctonia solani*

Introduction

Nanobiotechnology is advancing rapidly in the life sciences and making great progress in biology. It is one of the most promising and influential areas of scientific research (Shen *et al.* 2005). Polymerase chain reaction (PCR) is a technique for *in vitro* amplification of nucleic acid with target sequence, and is one of the most important experimental assays in molecular biology (Trzewik *et al.* 2016).

The PCR process, which requires very little nucleic acid, can achieve higher detection sensitivity and larger amplification of specific sequences in less time than

morphological detection methods (Abd-Elsalam *et al.* 2007). Nano-PCR is a relatively new area of research in the field of biotechnology in which the idea of adding nanoparticles (NPs) into PCR for enhancing its efficiency and specificity has attracted several researchers (Yuce *et al.* 2014; Rehman *et al.* 2015). Recently, nanomaterials have received considerable attention and nanomaterials-based PCR is a new area in nanobiology that combines artificial nanomaterials and biomolecules for building and mimicking the DNA replication machinery *in vivo* (Li *et al.* 2005; Li *et al.* 2008; Narang *et al.* 2016).

Various types of nanomaterials have been used as PCR enhancers such as gold nanoparticles (AuNPs) (Li *et al.* 2008; Kambli and Kelkar-Mane 2016), quantum dots (Wang *et al.* 2009; Liang *et al.* 2010), magnetic NPs (Alghuthaymi *et al.* 2016), and carbon-based materials such as carbon-coated silica (Park *et al.* 2015). AuNPs could be used as a cheap, highly stable and efficient substance to simultaneously improve the specificity and efficiency of PCR reactions without changing the thermal cyclers (Vanzha *et al.* 2016). By adjusting the PCR conditions (decreasing DNA denaturation time, reducing annealing temperature), high-specific PCR amplification products can still be efficiently generated even with low concentrations (10 ng) of DNA template (Lin *et al.* 2013). Enhancement of the efficiency of PCR by using magnesium oxide (MgO) nanoparticles which are highly stable, shows good dispensability and are less toxic than other metallic oxide nanoparticles (Narang *et al.* 2016). Furthermore, the isolation of PCR products becomes very convenient because ssDNA (single stranded DNA) and dsDNA (double stranded DNA) amplified by PCR based on AuNPs are bound to the AuNPs surface. Therefore, the study of the effects of AuNPs on PCR yield has great prospects in molecular nucleic acid probes, material science and isolation of PCR products. The present research focused on the effect of three types of nanoparticles on enhancement of fungal DNA templates and increase of PCR amplification yield when used at a specific concentration.

Materials and Methods

Fungal growth on duplex media

Fungal isolates used in the current study including, soil-borne fungus *Rhizoctonia solani* and toxigenic fungus *Alternaria alternata* were obtained from the Cotton Diseases Department, Plant Pathology Research Institute, Agricultural Research Centre, Giza, Egypt. Petri dishes were used instead of liquid shake cultures to grow the different fungal species for DNA isolation. Disposable Petri dishes (4 cm) were filled with 1.8 ml of solid medium potato dextrose agar (PDA), and 1.4 ml of peptone yeast glucose (PYG) liquid medium was added. The selected fungal isolates were cultured by inoculating a small mycelial disk from a stock onto the prepared Petri dishes that were subsequently incubated for 2–3 days at 28°C. Mycelium was lifted from the medium using sterilized inoculating loops and transferred into sterile 1.5 ml microfuge tubes. For some fungal species, the mycelium mats were pelleted by centrifugation for 15 min at 4,000 rpm in a deep wells wing-bucket rotor (Microcentrifuge 5804 R; Eppendorf). The mycelium pellet was washed with 600 μ l

TE (Tris-EDTA) buffer and centrifuged again for 5 min at 4,000 rpm. Finally, the TE buffer was decanted. Fungal DNA was isolated according to the method of Moslem *et al.* (2010).

Synthesis of zinc nanoparticles (ZnNPs)

Zinc nanoparticles were prepared by conventional precipitation with NaOH as the precipitation agent. 0.4 mg NaOH was added dropwise continually to aqueous 5.75 mg zinc sulphate solution at the molar ratio 1 : 2 with continuous stirring. The resulting slurry was continuously stirred for 24 h. The obtained precipitate was filtered and washed thoroughly with distilled H₂O. The washing procedure was repeated several times and then the collected residue was dried in an oven at 100°C for 12 h and grinded to a fine powder.

Synthesis of silver oxide nanoparticles (AgNPs)

AgNPs were synthesized according to Pal *et al.* (2007). AgNPs were obtained after reducing AgNO₃ with NaBH₄ in a water suspension using citrate as a stabilizing agent. Briefly, 5 ml of 10 mM AgNO₃ was mixed with 45 ml of ultrapure water at 45°C and rapidly heated to boiling. Subsequently, 1 ml of 1% sodium citrate dihydrate and 300 μ l of 3 mM NaBH₄ were injected dropwise under vigorous stirring, and the resulting solution was boiled for 60 min. After cooling under ambient conditions, the solution was filtered through a polycarbonate membrane (0.22 μ m). The final mixture was centrifuged at 8,000 rpm, precipitated, and lyophilized. The prepared AgNPs were further stabilized using selected surfactants (SDS, SDBS, TX-100, and Tween 80) at a final concentration of 1% (w/w), and the pure and modified dispersed AgNPs were then serially diluted.

Lysis buffer used for DNA isolation

Alkaline lysis buffer (ALB) and three additional lysis buffers, derived from ALB by excluding one or more of its components were used. The ALB buffer containing: i) 20 mM Tris-HCl, pH 8.0, 5 mM methylenediaminetetraacetic acid (EDTA), 400 mM NaCl, 0.3% sodium dodecyl sulfate (SDS), and 200 μ g \cdot ml⁻¹ Proteinase K (Alshahni *et al.* 2009); ii) ALB-Zn (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 400 mM NaCl, 0.3% SDS, 200 μ g \cdot ml⁻¹ Proteinase K and 0.2/ml ZnNPs; iii) ALB-Ag (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 400 mM NaCl, 0.3% SDS, 200 μ g \cdot ml⁻¹ Proteinase K and 0.2/ml AgNPs. DNA templates were prepared by suspending portions of fungal cultures in 25 μ l of deionized water, and aliquots of 5 μ l of suspensions were added to 70 μ l of each lysis buffer in microtubes and mixed slowly.

Then, they were incubated at 65°C for 1 h followed by 5 min at 100°C. The resulting lysates were used as DNA template sources.

Random polymorphic DNA (RAPD)-PCR analysis

RAPD-PCR analysis was performed using 10-mer primers (MWG, Germany). RAPD analysis was performed in 25- μ l reaction volumes containing PCR buffer (Promega, Mannheim, Germany), 0.2 mmol \cdot l⁻¹ dNTPs, 0.5 mmol \cdot l⁻¹ primer (5'-TGCCGAGCTG-3'), 4.0 mmol \cdot l⁻¹ MgCl₂, 1.25 units of Taq Polymerase (Promega, Mannheim, Germany) and 10–20 ng genomic DNA. PCR reactions were carried out in a T-Gradient thermal cycler (Biometra, Germany) using the following profile: 94°C for 1 min, 36°C for 1 min and 72°C for 1 min for 30 cycles, and a final extension at 72°C for 5 min. Following amplification, the samples were separated by electrophoresis in 1.4% agarose gel, stained with 0.5 μ g \cdot ml⁻¹ of ethidium bromide and viewed under ultra-violet light. A 100 bp ladder (Promega, Mannheim, Germany) was used as a molecular mass marker (Abd-Elsalam *et al.* 2007).

Internal transcribed spacer (ITS)-PCR conditions

PCR amplifications were carried out in PCR reactions in a total volume of 50 μ l, containing 20 ng genomic DNA, 1X PCR buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100), 0.2 mM of each of dNTPs, 0.2 unit of Taq DNA polymerase (Roche) and 10 pmol of ITS1/ITS4. The sequences of the ITS1 and ITS4 primers were 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3' (White *et al.* 1990) for *R. solani*. *Alternaria alternata*-specific primers Aalt-For (5'-GTGCCTTCCCCAAGGTCTCCG-3') and Aalt-Rev (5'-CGGAAACGAGGTGGTTCAGGTC-3') were used to detect 184-bp PCR product (Kordalewska *et al.* 2015). PCR amplification was carried out according to the following PCR parameters: an initial step of 2 min at 94°C, 40 cycles of 60 s at 94°C, 1.5 min at 52°C and 2 min at 72°C, and a final step of 7 min at 72°C. ITS-PCR was carried out with gold nanoparticles (0.2 nM), in order to check the efficiency of gold nanoparticles to improve PCR products.

Gel electrophoresis

Electrophoresis of the amplified PCR products was performed in 1.5% agarose gels (low melting) for 1.5 h at 7.0 V \cdot cm⁻². PCR products were stained with 0.5 g \cdot ml⁻¹ of ethidium bromide and visualized with 305 nm ultra-violet light.

Results

Enhanced DNA templates and RAPD amplification from *Alternaria alternata* using ZnNPs and AgNPs

RAPD-PCR amplification of genomic DNA was conducted for isolate of *A. alternata* using 10-mer random primer. Figure 1 shows different RAPD patterns in all the different DNA extraction methods of *A. alternata*. Lanes 2 and 3 show a limited number of RAPD bands (>3 bands). Most of the observed bands were around 500 bp, 1,000 bp and above 1,500 bp. Lane 4 shows two bands ranging from 500 bp to 1,000 bp. Lanes 5 and 6 show different patterns of RAPD-PCR bands (>4 bands) with the following sizes: 600 bp, 700 bp, 1,200 bp and above 1,500 bp. On the other hand, lane 7, *A. alternata* DNA template extracted by ALB + AgNPs shows new distinctive patterns of RAPD-PCR bands (>6 bands) with high polymorphisms. We found that there was an improvement of the DNA yield and PCR quality when the extraction was performed using nanoparticles.

Enhanced DNA templates and RAPD amplification from *Rhizoctonia solani* using ZnNPs and AgNPs

Figure 2 shows the qualities of extracted DNA which were evaluated with the RAPD-PCR technique. All samples were amplified by using 10-mer random primer. Figure 2 shows different RAPD-PCR patterns in all the different DNA extraction methods of *R. solani*. Lanes 2 and 3 show exactly the same number of RAPD-PCR bands (~7 bands) ranging from 500 bp to 800 bp and above 1000 bp. In lane 4, the DNA template which was extracted by ALBfree from Proteinase K shows only two bands ranging in size from 500 bp to 800 bp. Lanes 5 and 6 show different patterns of RAPD-PCR bands (>4 bands) ranging in size from 700 bp, 900 bp to above 1,000 bp. On the other hand, in lane 7 *R. solani* DNA template extracted by ALB + AgNPs shows new distinctive patterns of RAPD-PCR bands (>6 bands) with high polymorphisms. Most of the amplified fragments were around the following sizes: 600 bp, 900 bp, 950 bp, 1,000 bp and above 1,000 bp.

Enhanced DNA templates and specific PCR amplification from *Alternaria alternata* using ZnNPs and AgNPs

Figure 3 shows typical specific PCR results of DNA from *A. alternata* fungus by different extraction methods. *Alternaria alternata* specific primers were used to detect 184-bp PCR product. Lanes 2 and 7 show

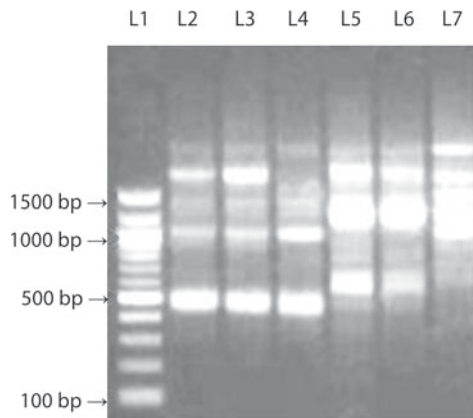


Fig. 1. Gel electrophoresis of RAPD-PCR amplification for *Alternaria alternata*. Lane 1 – 100 bp ladder; lane 2 – *A. alternata* DNA template extracted by ALB; lane 3 – *A. alternata* DNA template extracted by ALB + ZnNPs; lane 4 – *A. alternata* DNA template extracted by ALBfree from Proteinase K; lane 5 – *A. alternata* DNA template extracted by ALBfree from Proteinase K + ZnNPs; lane 6 – *A. alternata* DNA template extracted by ALBfree from SDS; lane 7 – *A. alternata* DNA template extracted by ALB + AgNPs

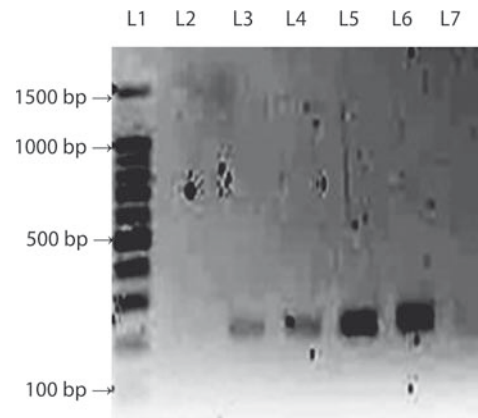


Fig. 2. Gel electrophoresis of RAPD-PCR amplification for *Rhizoctonia solani*. Lane 1 – 100 bp ladder; lane 2 – *R. solani* DNA template extracted by ALB; lane 3 – *R. solani* DNA template extracted by ALB + ZnNPs; lane 4 – *R. solani* DNA template extracted by ALBfree from Proteinase K; lane 5 – *R. solani* DNA template extracted by ALBfree from Proteinase K + ZnNPs; lane 6 – *R. solani* DNA template extracted by ALBfree from SDS; lane 7 – *R. solani* DNA template extracted by ALB + AgNPs

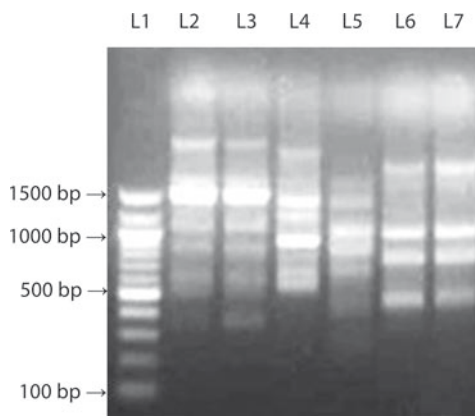


Fig. 3. Gel electrophoresis of *Alternaria alternata*-specific PCR amplification. Lane 1 – 100 bp ladder; lane 2 – *A. alternata* DNA template extracted by ALB; lane 3 – *A. alternata* DNA template extracted by ALB + ZnNPs; Lane 4 – *A. alternata* DNA template extracted by ALBfree from Proteinase K; lane 5 – *A. alternata* DNA template extracted by ALBfree from Proteinase K + ZnNPs; Lane 6 – *A. alternata* DNA template extracted by ALBfree from SDS; lane 7 – *A. alternata* DNA template extracted by ALB + AgNPs

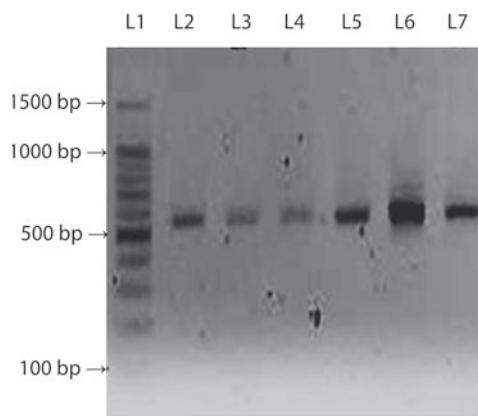


Fig. 4. Gel electrophoresis of ITS-PCR amplification for *Rhizoctonia solani*. Lane 1 – 100 bp ladder; lane 2 – *R. solani* DNA template extracted by ALB; lane 3 – *R. solani* DNA template extracted by ALB + ZnNPs; Lane 4 – *R. solani* DNA template extracted by ALBfree from Proteinase K; lane 5 – *R. solani* DNA template extracted by ALBfree from Proteinase K + ZnNPs; lane 6 – *R. solani* DNA template extracted by ALBfree from SDS; lane 7 – *R. solani* DNA template extracted by ALB + AgNPs

no PCR amplification, while lanes 3 and 4 show faint bands. Lanes 5 and 6 show very strong and distinctive bands as a result of a very good yield of *A. alternata*-specific PCR product.

Enhanced DNA templates and ITS-PCR amplification from *Rhizoctonia solani* using ZnNPs and AgNPs

Figure 4 shows typical ITS-PCR results with a template of DNA from *R. solani* fungus by different extraction methods. *Rhizoctonia solani* specific primers, ITS1

and ITS4 primers were used to detect 184-bp PCR product. Lanes 3 and 4 show faint bands of PCR product. Lane 2 shown a good yield of PCR product from *R. solani* DNA extracted by ALB. Lanes 5 and 7 show very sharp and distinctive bands as a result of a very good yield of PCR product from extracted DNA of *R. solani* by ALBfree from Proteinase K + ZnNPs and ALB + AgNPs, respectively. Lane 6 shows very intensive and distinctive band on agarose gel as a result of increasing the PCR product to the highest level on the extracted DNA of *R. solani* by ALBfree from SDS.

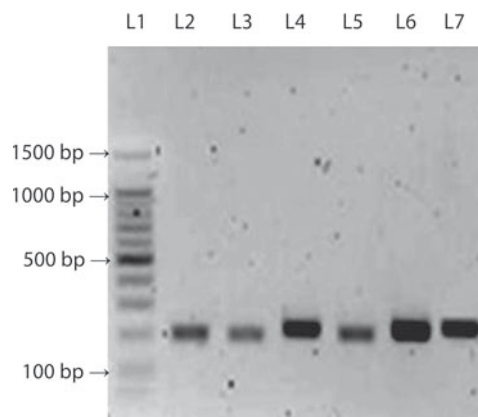


Fig. 5. Gel electrophoresis of *Alternaria alternata*-specific PCR amplification in the presence of gold nanoparticles. Lane 1 – 100 bp ladder; lane 2 – *A. alternata* DNA template extracted by ALB; lane 3 – *A. alternata* DNA template extracted by ALB + ZnNPs; lane 4 – *A. alternata* DNA template extracted by ALBfree from Proteinase K; lane 5 – *A. alternata* DNA template extracted by ALBfree from Proteinase K + ZnNPs; lane 6 – *A. alternata* DNA template extracted by ALBfree from SDS; lane 7 – *A. alternata* DNA template extracted by ALB + AgNPs. The results of PCR performed in the presence of gold nanoparticles (0.2 nM) mixed with PCR components

Enhanced DNA templates and specific PCR amplification from *Alternaria alternata* using ZnNPs, AgNPs and AuNPs

Figure 5 shows typical specific PCR results with DNA from *A. alternata* fungus by different extraction methods. Lanes 2, 3 and 5 show faint bands as a result of a low yield of PCR product from extracted *A. alternata* DNA by ALB, ALB + ZnNPs and ALBfree from Proteinase K + ZnNPs, respectively, in the presence of gold nanoparticles. Lanes 4, 6 and 7 show very intensive, sharp and clear bands as result of increasing the yield of PCR amplification to the highest level from extracted *A. alternata* DNA by ALBfree from Proteinase K, ALBfree from SDS and ALB + AgNPs, respectively, in the presence of gold nanoparticles.

Enhanced DNA templates and ITS-PCR amplification from *Rhizoctonia solani* using ZnNPs, AgNPs and AuNPs

Figure 6 shows typical ITS-PCR results with a template of DNA from *R. solani* fungus by different extraction methods. Lane 2 shown faint bands as a result of a low yield of PCR amplification from extracted *R. solani* DNA template by ALB in the presence of gold nanoparticles. Lanes 3 4, 5, 6 and 7 show very sharp, clear and intensive bands as a result of increasing the PCR amplification of extracted *R. solani* DNA template by ALB, ALB + ZnNPs, ALBfree from Proteinase K, Proteinase K + ZnNPs, ALBfree from SDS and ALB + AgNPs, respectively, in the presence of gold nanoparticles.

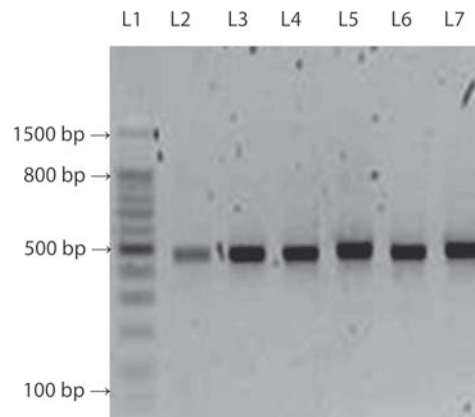


Fig. 6. Gel electrophoresis of ITS-PCR amplification for *Rhizoctonia solani* in the presence of gold nanoparticles. Lane 1 – 100 bp ladder; lane 2 – *R. solani* DNA template extracted by ALB; lane 3 – *R. solani* DNA template extracted by ALB + ZnNPs; lane 4 – *R. solani* DNA template extracted by ALBfree from Proteinase K; lane 5 – *R. solani* DNA template extracted by ALBfree from Proteinase K + ZnNPs; lane 6 – *R. solani* DNA template extracted by ALBfree from SDS; lane 7 – *R. solani* DNA template extracted by ALB + AgNPs. The results of PCR performed in the presence of gold nanoparticles (0.2 nM) mixed with ITS-PCR components

Discussion

Modifications of PCR techniques aim to normally enhance and increase both specificity and efficiency of the amplified products plus minimizing the costs for high-throughput applications. At the moment, diverse forms of nanoparticles, for example, gold, silver, or zinc, play important roles in detection, identification and management of some pathogens (Elechiguerra *et al.* 2005; Mocan *et al.* 2017).

In the present research we tested the effect of ZnONPs, AgNPs, AuNPs and combinations of (AgNPs + AuNPs) and (ZnONPs + AuNPs) nanoparticles by using different extraction methods for the DNA template for two plant pathogenic fungi. AgNPs uniqueness is based on their constant physical properties, which are strictly dependent on their sizes, which vary from 1 to 100 nm. Moreover, the fascinating range of their properties is due to the large surface area that may be adjusted or functionalised for different biological applications (Hoshino *et al.* 2007). These modifications in the physical properties can lead to higher water solubility or targeting specific biomolecule sites within biomolecules including proteins and nucleic acids (McCarthy *et al.* 2010). AgNPs can also bind to phosphorus containing DNA to enhance electrical conductivity and chemical stability, which has resulted in the quick amplification of the DNA strands (Rai *et al.* 2009).

The present study showed an increase in the extracted DNA template of *A. alternata* by (ALB + AgNPs)

and impacted the RAPD-PCR results as new distinctive patterns of RAPD-PCR bands (>6 bands) with high polymorphisms. ZnO nanoparticles increased PCR efficiency and specificity as well as decreased non-specific products (Cui *et al.* 2004). ZnO nanoparticles could improve the efficacy and specificity of PCR and RAPD-PCR reactions by improving the specificity of the primers or increasing thermal conductivity of the fluid. ZnO nanoparticles may be useful for enhancing amplification of DNA. Our nanoparticle PCR strategy allows reliable PCR amplification even at lower annealing temperatures, obviating time-consuming "touchdown" PCR (Fig. 4).

Gold nanoparticles have shown significant enhancement of PCR sensitivity and specificity. AuNPs can increase the specificity of PCR by enhancing the thermal conductivity of the PCR solution and facilitating the competitive binding of the correct primer to the template under the same heating conditions, rather than by increasing DNA polymerase accuracy through selective binding to ssDNA (Li *et al.* 2005). AuNPs promoted their dissociation from the template and reduced the inhibitory effect, similar to increasing the annealing temperature (Wan and Yeow 2009; Yao *et al.* 2012). AuNPs inhibit the amplification of longer DNA molecules and favour the amplifications of the shorter one, rather than enhancing the efficiency of PCR (Binh *et al.* 2008). For this reason Au nanoparticles possess some limitations for their use in this technique.

In conclusion, we have developed a highly selective nano-PCR strategy by employing three types of nanoparticles. We have demonstrated that in the presence of appropriate concentrations of gold nanoparticles, PCR amplification can be optimized with respect to both yields and specificity. Gold nanoparticles combined with zinc and silver nanoparticles enhanced both DNA yield and PCR products compared to DNA extraction methods with ALB buffer, sodium dodecyl sulfate, ALB free from Proteinase K, ZnNPs and AgNPs. Also, different combinations of two nanoparticles could enhance the PCR products and specificity for specific fungus but not for others. Combinations of nanoparticles could be applied by using one nanomaterial for DNA extraction and the other one in the PCR reaction or *vice versa*. Our study confirmed that application of nanomaterials in the DNA extraction from different fungi could increase or decrease the yield of DNA. So, application of nanomaterials in the DNA extraction could vary from one fungus to another. Furthermore, application of nanomaterials in the PCR reaction could increase or decrease the PCR product according to the type of applied nanomaterial. Non-specific amplification problems can be overcome in the presence of NPs. Application of AuNPs increase both sensitivity and specificity of the PCR product for different fungi, which is of great significance for molecular diagnosis of fungal plant pathogens and toxigenic fungi.

Acknowledgements

This work was partially funded by the Science and Technology Development Fund, Egypt (STDF)-South Africa (NRF) Scientific Cooperation Grant, Grant ID. 27837 to Kamel Abd-Elsalam.

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