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Silver and Magnetic Iron Oxide Nanoparticles-Assisted PCR for the Phytopathogenic Bacteria *Ralstonia solanacearum*

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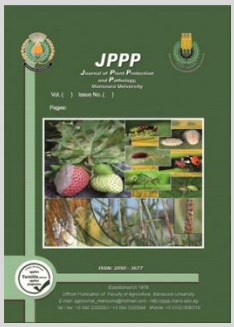

ABSTRACT

Nanotechnology became an integrated science in different fields. Integration between nanoscience and molecular diagnosis could enhance the early diagnosis of different pathogens. Enhancement of different molecular techniques by using nanomaterials increases the specificity, sensitivity and application of these techniques in biology. Polymerase chain reaction (PCR) is considered as one of the main basic molecular techniques that could be enhanced by using nanomaterials. Silver (Ag) and magnetic iron oxide (Fe_2O_3) nanoparticles were chemically synthesized and characterized by using transmission electron microscope (TEM) according to standard protocol. The sizes of Ag and magnetic Fe_2O_3 nanoparticles were ~15-20 and ~12-23 nm diameters respectively. DNA template of *Ralstonia solanacearum* was extracted by using Ag and magnetic Fe_2O_3 nanoparticles separately and in mixture of both nanomaterials in addition to the control. Extraction of DNA template by using combined nanomaterials increased the DNA yield followed by significant augmentation of the PCR efficacy for small concentration of bacterial DNA template. Inspections of the mechanism of such PCR augmentation suggested an indication of quick transfer of heat in the presence of Ag and magnetic Fe_2O_3 nanoparticles. Silver and magnetic Fe_2O_3 nanoparticles-supported PCR could be applicable for reducing the total PCR cycles and enhancing the augmentation of DNA amplicons from a diversity of samples, as well as GC-rich patterns (such as the bacterial genome of *R. solanacearum)* that are frequently observed to yield disappointing results.

keywords: Silver, Magnetic, Iron Oxide, Nanoparticles, PCR, *Ralstonia solanacearum*

INTRODUCTION

Ralstonia solanacearum is the causative agent of potato brown rot (bacterial wilt) disease (Smith, 1896), that causes economic losses in tropical, subtropical and warm temperate regions (Popoola *et al*., 2015 and Fegan and Prior, 2005). This bacterium is a soil borne pathogen causing an extreme devastation of many economic crops such as potato, tomato, banana and tobacco (Karim and Hossain, 2018 and Wan *et al*., 2009). Conventionally, *R. solanacearum* strains were separated into five races consistent with variances in the natural host range (Buddenhagen *et al.*, 1962) and six biovars according to biochemical properties (Hayward, 1991). In Egypt, the most common strain of *R. solanacearum* is race 3 biovar 2, which cause severe damage of the potato crop production (Anon a, 2014 and Janse *et al*., 2004).

Isolation, identification and detection of *R. solanacearum* based through different ways, such as using semi selective medium (triphenyl tetrazolium chloride (TTC) medium, Kelman, 1954), pathogenicity test on tomato seedling, biochemical and physiological tests, immunofluorescent antibody stain (IF); which may gave false positive reaction refer to cross reacting bacteria (Janse, 1988), and the polymerase chain reaction (PCR) (Mullis *et al*., 1983); which is the most sensitive and rapid method to detect pathogens from the plants samples. Screening of *R. solanacearum* from soil and tuber is quite difficult where; PCR is affected by inhibitors, contamination and experimental conditions (Yamamoto, 2002). PCR technique is used to produce millions copies of specific sequence of DNA. Also, it is the most accurate and specific detection technique for many organisms and it became necessary to find new applicable ways to increase the sensitivities of PCR detection methods (King *et al.*, 2014).

Nanotechnology is one of the most important technologies in the current Era which integrated rapidly with many scientific fields such as chemistry, biology, physics, engineering technology and agriculture. Change of macromaterials into nano size particles (1-100 nm) grant new different materials with different physical characteristics according to the nanosize and behave differently from the original size (Dubchak *et al*., 2010 and Khan and Fatima, 2014). Nanomaterial could be applied in plant disease management and enhancing the diagnostic techniques for phytopathogens (Boonham *et al*., 2008). The nanoparticles increase sensitivity and specificity for identifying single nucleotide alteration in bacteria and viruses (Lopez *et al*., 2009), for example, fluorescence silica nanoparticles have been applied to detect the causal of bacterial spot disease *Xanthomonas axonopodis pv. Vesicatoria* (Yao *et al*., 2009). Nano-PCR is the integration between PCR technique and nanotechnology, in which the notion of using nanomaterials into polymerase chain reaction (PCR) for increasing its efficiency and specificity (Pan *et al*., 2011). Metallic

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nanoparticles attracted researchers to enhance the extraction method of DNA yield and accordingly PCR product will be increased and other techniques could be applied for detecting many organisms. Silver nanoparticles were the main target of researchers because of their distinctive possessions (e.g., size and shape related optical, nanomicrobial activities, and electrical features). Silver nanoparticles could be synthesized using many methods such as laser ablation, gamma irradiation, electron irradiation, chemical reduction, photochemical methods, microwave processing, and biological synthetic methods (Wei *et al*., 2015).

Iron oxide nanomaterials are distinctive metal oxide nanoparticles, with exceptional magnetic possessions, high adsorbing features, and higher nanoactivities. Iron oxide-based magnetic nanoparticles (MNPs) have been widely studied as valuable diagnostic platform for bacteria as a result of their magnetic properties (Wang *et al*., 2018). According to surface modifications, MNPs linked with dissimilar metals allowing the improvement of innumerable bacterial detection protocols, as well as colorimetric, luminous, and surface-improved Raman identifications (Yuan *et al*., 2018).

The main aim of the current research is enhancing DNA yield and improving PCR product specificity and efficacy of *R. solanacearum* using silver and magnetic iron oxide nanoparticles.

MATERIALS AND METHODS

Isolation and Identification of *R. solanacearum*:

Infested potato tubers with *R. solanacearum* obtained from Al- Kanater Alkhiria, Kaliobiya governorate were cleaned up in running tap water, superficial disinfected using 70% ethanol then flaming (OEPP/EPPO 1990). Cores of 5-10 mm diameter and 5 mm length, containing main vascular and cortical tissues were macerated in sterile water (vascular tissues full with bacterial oozing). The resulting suspension was streaked onto plates of the triphenyle tetrazolium chloride (TTC) medium (Kelman, 1954). The inoculated plates were incubated at 28±1 °C for 48hrs. Colonies; typical for *R. solanacearum* virulent form, were picked and propagated on King's B agar (KBA) medium for 48 hrs at 28±1 °C. The isolates were identified using the morphological, physiological and biochemical tests according to the methods of Lelliot and stead (1987). Tomato seedlings (cultivar Beto 86) were used as a tested plant to detect the pathogen from collected isolates. The bioassay was made by inoculating tomato plants (two weeks old) grown in pots under greenhouse conditions, by the stem puncture technique (Janse, 1988). Injection was made at the leaf axis by a needle laden with the bacterial growth (10^7 cfu/ml) of the pathogen. Control treatments were prepared by the same method by using sterile water instead of bacteria. The inoculated plants were incubated under humid conditions at 28-30°C.

Immunofluorescent Antibody Staining (IFAS)

The obtained isolates of *R. solanacearum* were grown on (KBA) medium, and typical colonies of *R. Solanacearum* were serologically examined using IFAS to confirm the possible *R. solanacearum* identity. Typical bacterial colonies were used to prepare a suspension containing ca. 10^6 cells per ml from the culture and reference. A measured standard volume (20 µl for 6 mm window diameter) was pipetted on five successive window of a 10-window test slide. Slides were air dried at room temperature (or at 40°C) and lightly heat-fixed by flaming. All slide wells were loaded with 25 µl of the antiserum (anti *R. solanacearum* polyclonal) in 4 dilutions (1:800, 1:1600, 1:3200 and 1:6400). Slides were incubated for 30 min at room temperature in a wet condition, washed with tween buffer and 0.01 M phosphate buffer (PB) incubated with 25 µl of anti-rabbit Nordic SW/AR fluorescein isothiocyanate conjugated in a 100 fold dilution for 30 min in a wet condition chamber. Slides were washed again with tween buffer and 0.01 M PB, and excess wetness was removed carefully using filter paper. One droplet of 0.1µ of 0.01 M phosphate buffered glycerine (pH 7.6) was loaded to separate well and the slides were topped with long cover glasses. Slides were observed using a microscope (tube factor 1.25) with an epifluerescent light source and suitable filters with fluorescin isothyocyanate (FITC), using a 100X oil immersion lens and an l0X eyepiece. At least 20 microscope spots per window were examined for the absence or presence of typical morphologically fluorescing bacterial cells (Janse, 1988).

Nanoparticles synthesis and characterization

Chemical synthesis of silver nanoparticles:

Silver nanoparticles (Ag NPs) were chemically synthesized using organic and inorganic reducing agents. Silver nitrate ($AgNO_3$), sodium citrate ($Na_3C_6H_5O_7$) and double distilled water were used in the chemical reduction of Ag NO_3 according to the method described by Suriati *et al*. (2014).

Chemical synthesis of magnetic iron oxide nanoparticles

Magnetic iron oxide nanoparticles (Fe NPs) were sensitized using organic and inorganic reducing agents. Ferric chloride ($FeCl_3$), Ferrous sulfate ($FeSO_4$), double distilled water, 25% ammonia and 70% alcohol were used in the procedure consistent with the process defined by Laurent *et al*. (2008).

Characterization of Nanoparticles:

Physical characterization was performed by using the TEM at Nanotechnology& Advanced Material Central Lab. (NAMCL), Agriculture Research Center (ARC), Cairo University, Egypt. The high-resolution transmission electron microscope (HR-TEM, Tecnai G20, FEI, Netherland) was applied for imaging, crystal construction exposure and elemental investigation (qualitative and semi-quantitative analysis of the samples). Two diverse approaches of imaging were applied; the bright field at electron accelerating voltage 200 kV using lanthanum hexaboride (LaB6) electron source gun and the diffraction pattern imaging. Eagle CCD camera with (4k*4k) image resolution was used to obtain and collect transmitted electron images. TEM Imaging & Analysis (TIA) software was used to spectrum acquisition and analysis of EDX peaks.

DNA extraction methods of *R. solanacearum*

DNA extraction method 1

Ralstonia solanacearum were grown on tetrazolium chloride agar medium at 30°C for 2 to 3 days. Cell suspension was prepared in distilled water and adjusted to an optical density of 0.1 unit at 650 nm using a spectrophotometer (Genova MK2 Life Science Analyser, Jenway Ltd, Essex, UK) to provide cell concentration of

approximately 10^8 cfu/ml. DNA isolated by heating 1 ml of the suspension for 6 min at 100º C to facilitate DNA release.

DNA extraction method 2

DNA of *R. solanacearum* was extracted according to the procedures of ZYMO REASEARCH Quick-gDNA™ extraction kit following.

[Extracted DNA mixed separately with each of 1mM Fe NPs, 40 mM Ag NPs or a combination of both (1 mM Fe NPs + 40 mM Ag NPs), and followed by PCR amplification].

PCR amplification condition

Master mix Dream Taq™ (Thermo Fisher Scientific™); with an expected size 750 bp (Poussier *et al*., 2000), was used for amplification of DNA. A region of the endoglucanase gene was amplified using the forward primer Endo-F (5̀ -ATGCATGCCGCTGGTCGCCGC-3′) and reverse primer Endo-R (5̀-GCGTTGCCCGGCACGAAC ACC-3′).

PCR amplification was performed in 50 µl reaction mixture as follows: initial denaturation at 95ºC for 3 min, followed by 35 cycles consisting of denaturation for 30 sec at 95 ºC, annealing for 30 sec at 55ºC, and extension for 1 min at 72ºC. A final extension step was carried out at 72ºC for 7 min.

Detection of PCR products

PCR amplification products were separated on 2% agarose in TBE buffer (10.8 g tris- base, 5.5 g boric acid, 0.74 g EDTA, and 1 L deionized water). PCR products (5 µl) were mixed with 1 µl of loading dye. Gels were run for 120 min at current 5 V/cm. Gels were then stained with ethidium bromide (0.005 g/L) and visualized under UV light. A known DNA ladder (hyperLadder™ 1kb, Thermo Fisher Scientific™) was loaded in the 1st and last lane to estimate the size of PCR products.

RESULTS AND DISCUSSION

Results

Isolation and identification of *R. solanacearum*.

Virulent colonies of *R. solanacearum* on TTC agar medium, which were white colored, irregular shape and fluidal texture with pink color in the midpoint, were designated and used in inoculation of tomato seedlings. The isolates were able to produce wilt after 3-7 days.

Morphological, physiological and biochemical characteristics indicated that all the selected isolates were gram negative, short rods, catalase and oxidase test positive.

Ralstonia solanacearum produced brown pigment on King's B medium (Fig. 1), reduced nitrate, unable to hydrolyze starch and liquefy gelatin. The isolates produced acid from maltose, lactose, glucose and fructose, but not from sorbitol, salicin, mannitol and D-arabinose (Table 1).

Fig. 1. Typical shape of the causal agent of potato brown rot, *Ralstonia solanacearum,* on King's B medium.

Table 1. Biovar and race determination of *R. solanacearum* isolate.

Code	Race	Biovar	Maltose	Lactose	Glucose	Fructose	Sorbitol	Salicin	Mannitol	D-Arabinose
Tested Isolate	3	2	+/+	+/+	+/+	+/+	–/–	–/–	–/–	–/–
Negative	---	---	-----	-----	----	----	-----	-----	----	-----

The isolated cells showed short rod shaped morphology, stained evenly as bright green fluorescent by using immunofluorescent antibody staining, IFAS (Fig. 2).

The results of identification indicated that the isolate belongs to race 3 biovar 2 of *R. solanacearum*.

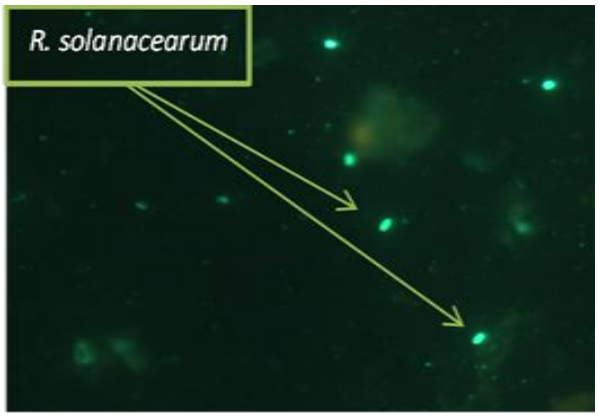

Fig. 2. Typical morphological shape of potato bacterial wilt disease, R*alstonia solanacearum* under immunofluorescent microscope (IF).

Physical characterization of iron oxide magnetite (Fe NPs) and silver (Ag NPs) nanoparticles.

Analysis of magnetic Fe_2O_3 nanoparticles by TEM revealed that the mean diameter size of 16 nm (Fig. 3). The particle size distribution proofed that most of the nanoparticles size within the range of average diameter (15 ± 8 nm). TEM Analysis of the silver nanoparticles (Ag NPs) showed that average diameter of 15 nm (Fig. 4). The particle size distribution confirmed that most of silver nanoparticles are within the range of mean diameter (14 ± 8 nm).

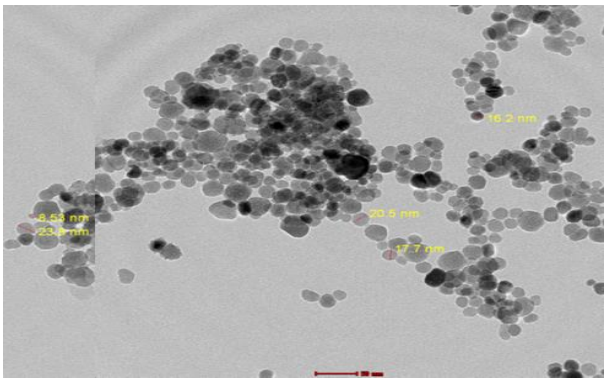

Fig. 3. Transmission electron microscope (TEM) image of magnetic iron oxide nanoparticles (Fe NPs).

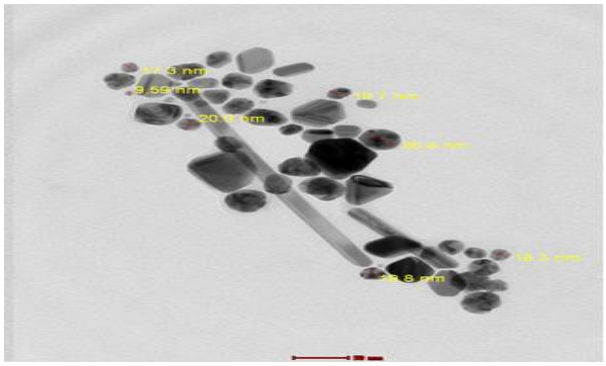

Fig. 4. Transmission electron microscope (TEM) image of silver nanoparticles (Ag NPs).

Figure (5) shows PCR profile of *R. solanacearum* endoglucanase gene (750 bp) hat obtained by different extraction methods with and without Fe NPs and Ag NPs. Lanes 3, 4, 6, 7 and 8 showed very intensive and sharp bands as an outcome of enhancing the PCR product to the highe level as a result of DNA extraction by (physical method + 40 mM Ag NPs), (physical method + a combination of both Fe and Ag nanoparticles), (ZYMO kit+ 1 mM Fe NPs), (ZYMO kit + 40 mM Ag NPs) and (ZYMO kit + a combination of both nanoparticles), respectively. While, lanes 1, 2 and 5 showed normal, and low intensive bands compared to the other treatments as a result of low PCR amplification.

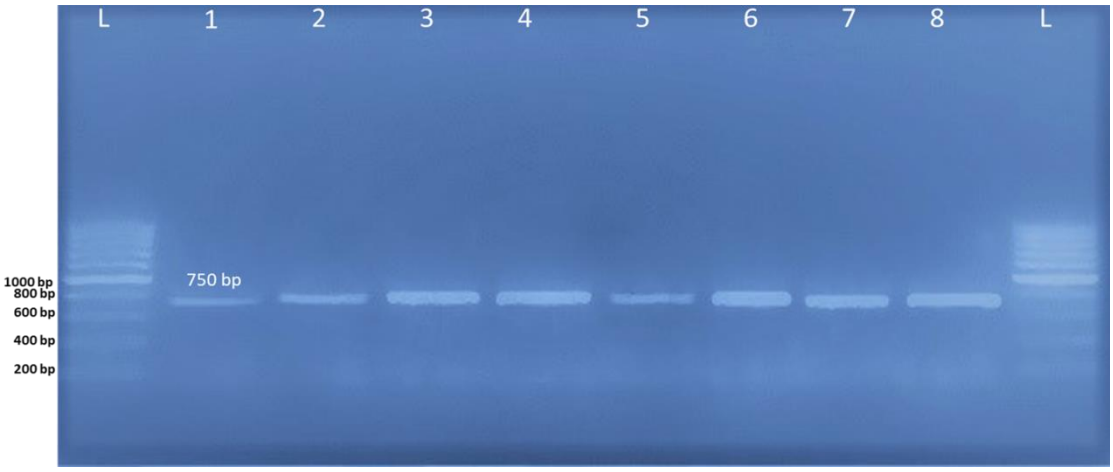

Fig. 5. PCR profile of *R. solanacearum* endoglucanase (egl) gene.

Lane L: DNA ladder. Lane (1): DNA sample extracted by physical method without additives (Control 1). Lanes (2, 3 and 4): DNA samples extracted by physical method plus 1mM Fe NPs, 40 mM Ag NPs and a combined of both nanoparticles, respectively. Lane (5): PCR product of DNA sample extracted by using ZYMO kit and no additives (Control 2). Lanes (6, 7 and 8): PCR product of DNA samples extracted by ZYMO kit plus 1 mM Fe NPs, 40 mM Ag NPs and a combined of both nanoparticles, respectively.

Discussion

Adjustments of DNA extraction and PCR technique aim to improve and enhance both of efficiency and specificity of the amplified DNA template in addition reduce the costs of high-throughput applications. Currently, various forms of metallic nanoparticles such as silver, iron, gold or zinc, play significant roles in detecting, identifying and managing different types of pathogenic bacteria (Mocan *et al.*, 2017). Here, these results revealed that the effect of nanoparticles on PCR varied according to types of nanoparticles and DNA extraction technique. Figure (5) presented the increase of *R. solanacearum* DNA template yield extracted by (physical method + 40 mM Ag NPs), (physical method + a combination of both Fe and Ag nanoparticles), (ZYMO kit+ 1 mM Fe NPs), (ZYMO kit + 40 mM Ag NPs) and (ZYMO kit + a combination of both nanoparticles). Compared to untreated samples (no Ag and Fe NPs additive). Thus, silver and iron nanoparticles enhanced PCR specificity and efficiency besides reduced nonspecific outcomes. Iron oxide and Ag nanoparticles may be useful for increasing PCR amplification by enhancing the extracted DNA template, and permitting iron consistent PCR amplification even at lower annealing temperatures. Iron oxide nanoparticles have the capability to indorse PCR productivity at low concentrations but suppress PCR amplification at high concentrations (Kambli and Kelkar-Mane, 2016). Our obtained results revealed that the application of metal oxide NPs supressed the DNA amplification faults during the PCR run.

There are two suggested potential approaches for NPs to physically interrelate with DNA polymerase in the PCR run. Firstly, excess free NPs without binding to DNA may interrelate directly with the polymerase. Secondly, the attached DNA to the metal oxide NPs is replaced modestly by the polymerase. Furthermore, metal oxide NPs may affect PCR amplification by releasing free metal ions (Innis and Gelfand, 1999). Generally, the consequence of metal NPs on PCR amplification is relying on the relations between NPs and DNA (Li *et al.*, 2013).

Silver NPs distinctiveness is normally established on their persistent physical possessions, which are stringently reliant on their nanometre sizes, which differ from 1 to 100 nm. Furthermore, the interesting ranges of their possessions as a result of their enormous surface area that could be accustomed or utilized for different biological applications (Hoshino *et al.*, 2007). These alterations in the physical possessions may initiate better water solubility or aiming particular biomolecule sites as well as proteins and nucleic acids (McCarthy *et al.*, 2010**).** Also, Ag NPs can bind to the phosphorus group in the DNA to increase chemical constancy and electrical conductivity, which leads to rapid amplification of the DNA strands (Rai *et al.*, 2009).

In summary, we established an extremely discriminating nano - PCR approach by applying two types of metallic nanoparticles.

The results revealed that, in the existence of suitable Ag NPs concentrations, PCR amplification may be improved depending on yield and specificity. Silver nanoparticles combined with iron oxide NPs improved both DNA yield and the final product of PCR in comparison with untreated physical and ZYMO kit DNA extraction methods. Mixtures of silver and iron oxide nanoparticles may be applied using one type of NPs for DNA extraction and another type of NPs for the PCR reaction. Our study established that the usage of metallic nanoparticles sliver and iron enhanced the extracted DNA yield from *R. solanacearum*. Consequently, applying metallic nanoparticles in the DNA extraction methodology could be effective with one type of bacteria but not the other types. Moreover, Metallic NPs could increase the total extracted DNA followed by increasing PCR final product. Also, non-specific PCR amplification products could be avoided in the existence of metallic NPs. The usage of Ag NPs, Fe NPs and a combination of them enhanced the sensitivity of the final product of PCR for *R. solanacearum*. Metallic NPs especially silver and iron oxide nanoparticles could positively enhance DNA extraction methodologies and PCR amplification.

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إستخدام الفضة وآكسيد الحديد المغناطيسى فى الصورة النانومترية لتحسين منتج تفاعل البلمرة المتسلسل لبكتريا مرض الذبول البكتيرى فى البطاطس (رالستونيا سولانسيرم)

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أصبح علم النانوتكنولوجى علما تكامليا متداخلا مع المجالات العلمية المختلفة. فالتكامل بين علم النانو والتشخيص الجزيئي امكنه تحسين التشخيص المبكر لمسببات الأمراض المختلفة. تحسين تقنيات التشخيص الجزيئية بإستخدام المواد النانومترية يزيد من كفائة وتخصصية وحساسية التقنيات البيولوجية الجزيئية المختلفة للكشف عن الأمراض فى مجال البيولوجى. يعتبر تفاعل البلمرة المتسلسل (PCR) أحد تقنيات البيولوجيا الجزيئية الحديثة الاساسية التى امكن تحسينها باستخدام المواد النانومترية تم تخليق نترات الفضة وأكسيد الحيدوز فى الصورة النانومترية وتوصيفها بإستخدام الميكروسكوب الإلكترونى التخللى وفقا للطرق القياسية المتعارف عليها، حيث أن منتصف قطر حجم جزيئات الفضة فى الصورة النانومترية تراوح بين ١٥-٢٠ نانوميتر ومنتصف قطر حجم الحديد تراوح ما بين ١٢-٢٣ نانوميتر. تم استخلاص DNA لبكتيريا *Ralstonia solanacearum* باستخدام هاتان المادتين النانومتريتين كلا على حدا ثم باستخدام مزيج منهما معا مقارنة بالاستخلاص بدونهما. و قد وجد ان استخلاص ال DNA باستخدام مزيج من المادتين معا ادى لزيادة كمية ال DNA وتبعه تحسين كفاءة الطريقة المستخدمة (PCR) و ذلك بتركيز قليل من ال DNA البكتيرى. وتشير الأبحاث والأدلة السابقة إلى أن ميكانيكية تحسين تفاعل البلمرة كان من خلال سرعة الإنتقال الحرارى فى معاملات الفضة والحديد فى الصورة النانومترية مقارنة بالكنترول. زيادة إستخلاص الحمض النووى لبكتيريا الذبول البكتيرى عن طريق إستخدام الفضة والحديد فى الصورة النانومترية ساهم بشكل فعال فى تقليل عدد دورات تفاعل البلمرة وزيادة التخصصية فى زيادة النسخ المستنسخة من الحمض النووى المستهدف وخاصة بكتيريا الذبول البكتيرى والتى تحتوى علىنسبة عالية من قواعد السيتوزين و الجوانين (مثل الجينوم البكتيري لبكتيريا *Ralstonia solanacearum*) التي لوحظ عادة أنها تسفر عن نتائج غير مرضية فى الطرق التقليدية.