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PCR and Nanotechnology Unraveling Detection Problems of the Seed-borne Pathogen *Cephalosporium maydis*, the Causal Agent of Late Wilt Disease in Maize

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Abstract:
Late wilt disease of maize caused by the fungus *Cephalosporium maydis* is one of the most important fungal diseases in Egypt. The pathogen spreads through the remaining plant debris in soil and through apparently healthy seeds. Detection of the latent infection of *C. maydis* pathogen is of great importance step in disease management which commonly achieved by applying the standard seed health tests. The commonly used seed health tests in laboratories around the world are still lake of specificity, sensitivity, speed, simplicity, cost-effectiveness, and reliability of seed health methods. In the current study, Infection percentage of seed-borne *C. maydis* recorded 1.0 and 1.5% in 2015 and 2016, respectively from whole maize seed (Giza 2) when examined by the standard blotter method. Using the same detection method examining broken maize seeds of cv. Giza 2 gave 3.5 and 5% in 2015 and 2016, respectively. Maximum infection percentage obtained during the current study period was 6.5% which was gotten when Potato Dextrose Yeast Agar (PDYA) applied with broken maize seeds of the same cultivar. While the highest infection percentage recorded during the entire study was 11% which obtained from broken seeds of hybrid Sc 166 of season 2015 and incubated on PDYA. Relatively to other major fungi recorded, the deep freezing method used with whole seeds (Giza 2) gave the least records of *C. maydis* in 2015 and 2016; 0.0 and 1.2, respectively. On the other hand, PDYA method gave the highest record during the two successive years of investigation (18.2) obtained from 2016 broken seeds. PCR has many beneficial characteristics that make it highly applicable for detecting *C. maydis* from seeds. In PCR diagnosis study, the development of DNA extraction is one of the most important steps. These approaches include using Ag NPs and Fe NPs in DNA extraction method to enhance the quantity and purity of the DNA template for successful PCR assay.
Keywords: Maize, seed-borne, late wilt, *Cephalosporium maydis*, nano-particles, PCR.

INTRODUCTION

Seeds play a vital role in the healthy production of any crop (Nadeem *et al.,* 2017; Zaynab *et al.,* 2018). Pathogens carried on or in seeds which scientifically called seed-borne may cause heavy yield losses (Ali *et al.,* 2017). Corn (*Zea mays* L.) is the third world top cereals ranked on the fundamental of monetary value (Pervaiz *et al.,* 2011; Al-Jobory *et al.,* 2017). Late wilt disease caused by *Cephalosporium maydis* has been reported as an important seed-borne disease which transfers through soil as well as seeds due to the lack of adequate plant quarantine or ignorance of symptoms (Samra *et al*., 1962; Samra *et al*., 1963; Michail *et al.,* 1999).

The blotter and the agar plate methods are two important procedures traditionally applied in routine seed health test for seed-borne fungi detection (Warham, 1990). Those traditional diagnostic methods seem to have serious disadvantages such as time-consuming and lack of accuracy (Khiyami *et al.*, 2014). The failure to adequately identify and detect plant pathogens using conventional morphological techniques has led to the development of nucleic acid-based molecular approaches. Immuno-diagnostic tools can also be successfully employed for differential diagnosis, disease surveillance of seed-borne pathogens of quarantine importance.

Polymerase chain reaction (PCR) revolutionized pathogen detection and identification, but these methods have not yet entirely replaced traditional cultural and phenotypic tests practiced for detection of major seed-borne pathogens. PCR technology has been used to rapidly detect, characterize and identify a variety of organisms. In a PCR diagnosis study, the developments of PCR primers are one of the most important steps. PCR primers are specific to various phytopathogenic fungi. These approaches include using species-specific genes or DNA regions, or anonymous unique DNA regions to design PCR primers (Zhonghua and Michailides, 2007).

Pure and rapid DNA extraction is a pre-requisite step for the most advanced techniques such as genetic mapping, fingerprinting, marker-assisted selection and plant originality. Tan et al. (2013) reported that the high quality of DNA extractions is necessary for genetic studies of a variety of plants. After the evaluation of several methods, one most suitable method described by Dellaporta et al. (1983) was selected for isolating DNA with main factors of price, time required, quantity and the quality of the DNA extracted. Besides, nanotechnology became widely used in different fields, recently nanobiotechnology focused in short and quick detection of different pathogens (Iqbal *et al.,* 2018; Ismaeel *et al.,* 2017; Selim *et al.,* 2017). Nano-PCR is quite new area in the field of biotechnology in which the notion of mixing nanoparticles (NPs) into DNA extraction protocol followed by PCR for increasing DNA yield and enhancing PCR proficiency and specificity. Metallic nanoparticles have great impact on enhancing DNA extraction methods and PCR amplification. Gold, Silver, and Zinc oxide nanoparticles enhanced DNA yield and PCR sensitivity and specificity for several plant pathogenic fungi (Rehman *et al*., 2015; Al-Dhabaan *et al*., 2017).

The present investigation aimed to evaluate the use of nanoparticles in the extraction of DNA and advanced PCR-based techniques being employed in the detection of seed-borne pathogen in one of major food and feed crops; maize to overcome some disadvantages of traditional methods of detection and seed health tests.

MATERIALS AND METHODS

Seed samples

A total of four maize (*Zea maize* L.) seed samples were collected from fields known with a

long history of heavy infection of late wilt disease caused by the fungus *Cephalosporium mayids* (*Harpophora maydis*). One seed sample from hybrid Sc.166 in 2015 and another one in 2016. One more seed sample cv. Giza 2 was collected in 2015 and another one in 2016. Subsamples of the original samples were drowned and seeds were broken down into smaller pieces and served in additional examination.

Seed health testing

Detection and isolation of *C. maydis* associated with maize seeds were carried out following the standard methods published by ISTA (2014). Random 200 maize seeds were obtained from each sample and examined with a blotter, deep freezing and agar plate methods.

Blotter method

Three pieces of blotter papers were properly soaked in sterilized water and placed at the bottom of a 9 cm diameter well-labeled Petri dishes. Ten whole seeds per Petri dish or 10 broken seeds/dish were plated equidistantly under aseptic conditions in 20 replicates each. The Petri dishes containing whole seeds or broken ones were incubated at a controlled environment room at 25±2^{o}C under alternation cycle of 12hr light and 12hr darkness for 7 days.

Deep freezing method

This method was modified from the blotter method (Neergaard, 1979). Three well-moistened blotter papers were placed in Petri dishes of 9 cm diam. at the rate of 10 maize whole seeds/dish and other 10 maize broken seeds/dish were plated in 20 replicates each. Dishes were then incubated in a controlled environment room at 25±2°C under alternation cycle of 12hr light and 12hr darkness for 2 days, then 1 day at –20°C (deep freezing) and finally 4 days at 25±2°C of 12hr light/darkness cycle.

Agar plate method:

In the agar plate method, 20 ml of potato dextrose yeast agar (PDYA) was poured in glass Petri plate. After cooling, five whole seeds and five broken seeds per Petri plate were kept at equidistance and incubated at controlled environment room at 25±2^{o}C under alternation cycle of 12hr light and 12hr darkness for 7 days. Twenty replicates of each treatment were implemented.

Major fungi; *Fusaium moniliforme, F. oxysporum,* and *C. maydis* were counted and identified according to Booth (1971) and Shurtleff (1980). Frequency percentages of the aforementioned fungi were calculated and recorded. Fungi were then isolated and further purification by hyphal tip method was carried out then transferred on PDYA slants and preserved in the refrigerator according to Hildebrand (1938) and Choi et al. (1999).

PCR ASSAY:

DNA extraction from cultures of *C. maydis*

According to Dellaporta et al. (1983), pure cultures were individually and carefully frozen in liquid N_2 and ground to a fine powder in a mortar and pestle. The powder was directly transferred to 1.5ml microfuge tube. 500 µl Dellaporta extraction buffer was added (100mM tris pH 8.0, 50mM EDTA, 500mM sodium chloride and 10mM β-mercaptoethanol) and ground with pestles. A 33µl of 20% Sodium Dodecyl Sulphate SDS (w/v) was added, vortexed and incubated at 65°C for 10 min. Then a 160 µl of 5M potassium acetate was added and vortexed, spin 10 min at 10,000 rpm. A 450µl supernatant was transferred carefully to a new Eppendorf tube avoided any debris. The potassium acetate extraction step could be repeated if it necessary to remove any debris. The aqueous layer was transferred to new Eppendorf tube, and then half of the volume of isopropanol was added and centrifuged at 15,000 rpm for 15 min. The supernatant was discarded carefully considering that large nucleic acid pellet would slide down to the bottom of the tube. A volume of 500µl ethanol 70% was added, spin 5min and

repeating the removal as much of the supernatant as possible. The pellet was air-dried for 1h, then suspended in 50µl dH_2O. The extracted DNA was then ready for PCR.

DNA extraction from maize seeds by Dellaporta buffer or adding Ag NPs or Fe NPs

DNA was extracted from two samples of maize seeds using a modified Dellaporta extraction method (Dellaporta *et al.,* 1983). Seed samples were crushed using a Warring blender, ground in intervals of 10 seconds for 4 times at maximum speed. Samples were frozen in liquid N_2 and ground to a fine powder in a mortar and pestle. The powder was transferred to 1.5ml microfuge tube. 1000 µl of Dellaporta extraction buffer was added for each maize sample and ground with pestles. Other samples were extracted by adding 1000µl Dellaporta + 400µlAg NPs or 1000 Dellaporta + 0.04gm Fe NPs. 66µl maize of 20% SDS (w/v), vortexed and incubated at 65°C for 10 min. Then 320µl for maize of 5M potassium acetate was added, vortexed and spin 10 min at 10,000 rpm. A 450µl supernatant was transferred carefully to a new Eppendorf tube avoided any debris. The potassium acetate extraction step could be repeated when it was necessary to remove any debris. An equal volume was added of PCI, vortexed for 5 min and centrifuged for 15 min at 15.000 rpm. The aqueous layer was transferred to new Eppendorf tube, then 0.5 volume of isopropanol was added and centrifuged at 15,000 rpm for 15 min. The supernatant was discarded carefully, considering that large nucleic acid pellets would slide down to the bottom of the tube. A volume of 500µl ethano70% was added, spin 5min and repeating the removal as much of the supernatant as possible. The pellet was air-dried for 1h, then suspended in 50µl dH_2O. The extracted DNA was then ready for PCR.

PCR primers

In order to develop a tool to identify fungi and classify them according to their phylogenetic group, the advantage of the sequence diversity of the intergenic spacer regions of fungi was considered. Three PCR primers were shown to amplify three fungi. Each of these primer pairs was specific for each fungus, and they did not produce PCR products of the correct size from any other fungi group. None of the primers produced PCR amplification products of the correct size from healthy plant DNA. These primers could serve as effective tools for identifying particular fungi in field samples according to Drori et al. (2013). For the amplification of *C. maydis* on maize, the primer pair A200a/ A200b was used by product 200bp.
F:5-'CCGACGCCTAAAATACAGGA3'
R: 5'- GGGCTTTTTAGGGCCTTTTT 3'

PCR amplification

Each PCR tube contained the following reaction mixture: 5µl of 0.2mM dNTPs, 5µl of 25mM $Mgcl_2$, 5µl 10X polymerase buffer, 2µl of 20pmol/µ of each primer from examined fungus, 0.5µl of DNA Taq polymerase (2.5 U) and 3µl DNA preparation from infected samples of maize was used as a template DNA and sterile water up to volume of 50µl. The program of PCR consisted of an initial denaturation of 2 min. at 94^{o}C followed by 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 1 min. and final extension of 72^{o}C for 5 min. All reactions were cycled on a thermocycler TECHNE (TC- 512). The amplified DNA was electrophoresed on 2% agarose gel in 1XTBE buffer at 120V for 45 min., stained with ethidium bromide (0.5/ml) and photographed using UV lamp in gel-documentation (Bio-Rad, Gel Doc XR system 170-8170). The molecular weight of the PCR products was determined by comparison with DNA markers weight VC 100bp plus DNA ladder (Vivantis) and VC 1Kb plus DNA ladder (Vivantis).

RESULTS

Data presented in Table (1) show that regardless method of detection or season of samples collection of cv. Giza 2, percentages of seed infection recorded with *C. maydis* were the

least when compared to other recorded fungi. At the same time, broken maize seeds yielded higher percentages than those obtained from whole seeds using either method of detection. In 2015, PDYA used in examining broken maize seeds yielded the highest percentage of *C. maydis* (6.0%), followed by equal records of 3.5% when blotter or deep freezing methods used to examine broken maize seeds. It is also clear from data tabulated in Table (1) that none of the three methods succeeded to express *C. maydis* satisfactorily from whole maize seeds of cv. Giza 2.

Relative to other major fungi recorded, the deep freezing method gave the least records of *C. maydis* in 2015 and 2016; 0.0 and 1.2%, respectively. Although PDYA method gave the highest record in almost equal values during the two successive years of investigation; 11.1 and 11.2%, respectively, but it recorded a little higher than one-tenth of the three fungi considered.

Table 1. Infection percentage of three major seed-borne fungi obtained from maize cv. Giza 2 collected in 2015 and 2016 seasons using three seed health tests.

Method of detection		2015			2016			Relative % of *C. maydis* to others			
		F. moniliforme	*F. oxysporum*	*C. maydis*	*F. moniliforme*	*F. oxysporum*	*C. maydis*	2015		2016	
								T	%	T	%
Blotter method	Whole seeds	23.0	17.0	1.0	26.5	13.5	1.5	41.0	2.4	41.5	3.6
	Broken seeds	24.5	18.0	3.5	27.5	16.0	5.0	46.0	7.6	48.5	10.3
Deep freezing	Whole seeds	16.5	17.5	0.0	25.0	15.5	0.5	34.0	0.0	41.0	1.2
	Broken seeds	26.0	22.5	3.5	26.0	23.0	4.0	52.0	6.7	53.0	7.5
PDYA	Whole seeds	25.5	25.5	1.5	29.5	19.5	1.5	52.5	2.9	50.5	3.0
	Broken seeds	26.5	21.5	6.0	29.0	22.5	6.5	54.0	11.1	58.0	11.2

PDYA = Potato dextrose yeast agar; T = total fungal inocula.

Data presented in Table (2) show the same trend recorded with hybrid Sc 166 as well as cv. Giza 2 in the previous table. Broken maize seeds yielded higher percentages than those obtained from whole seeds using either method of detection. It is also clear that the late wilt fungus *C. maydis* was the weakest fungus comparing to *Fusarium moniliforme* and *F. oxysporum*. The highest two values of *C. maydis* (6.5% and 6.0%) was obtained in 2016 and 2015 seasons, respectively when broken maize seeds were incubated on PDYA for 7 days. On the contrary, deep freezing method gave the minimum records of 0.0 and 0.5% from whole maize seeds collected in 2015 and 2016, respectively. While the highest infection percentage recorded during the entire study was 11% which obtained from broken seeds of hybrid Sc 166 of season 2015 and incubated on PDYA. Exceptionally, one high relative value 18.2% was calculated in contrast to other lower relative values of the total fungal inocula presented in Table (2). All other relative values obtained proved that *F. moniliforme* and *F. oxysporum* grew vigorously and aggressively than the delicate fungus *C. maydis.*

Table 2. Infection percentage of three major seed-borne fungi obtained from maize hybrid Sc166 collected in 2015 and 2016 seasons using three seed health tests.

Method of detection		2015			2016			Relative % of *C. maydis* to others			
		F. moniliforme	*F. oxysporum*	*C. maydis*	*F. moniliforme*	*F. oxysporum*	*C. maydis*	2015		2016	
								T	%	T	%
Blotter method	**Whole seeds**	24.5	19.5	3.0	24.5	17.5	1.0	47.0	6.4	43.0	2.3
	Broken seeds	26.0	21.5	2.5	28.0	19.5	5.0	50.0	5.0	52.5	9.5
Deep freezing	**Whole seeds**	26.0	21.0	1.0	24.5	28.5	0.0	48.0	2.1	53.0	0.0
	Broken seeds	27.0	23.0	3.5	30.0	29.5	2.0	53.5	6.5	61.5	3.3
PDYA	**Whole seeds**	22.5	13.0	2.0	23.5	31.5	0.5	37.5	5.3	55.5	1.0
	Broken seeds	29.5	20.0	11.0	26.5	29.5	5.0	60.5	18.2	61.0	8.2

PDYA = Potato dextrose yeast agar; T = total fungal inocula.

Transmission electron microscopy (TEM)

TEM characterization of AgNPs and FeNPs nanoparticles used in this study ranging from 14.2-29.5nm. The results showed that the biggest size was FeNPs which was in the range of 8.53-44.3nm as illustrated in Figures (1 and 2).

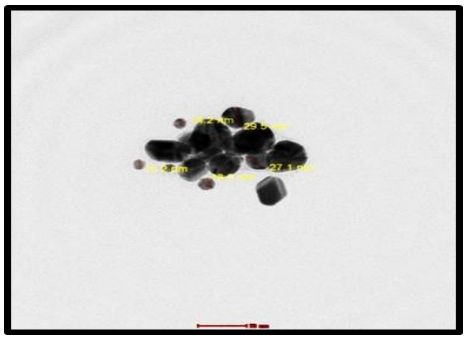

Fig. 1. Transmission electron microscopy (TEM) of Ag NPs.

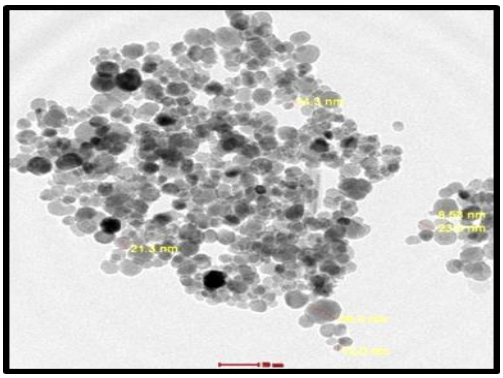

Fig. 2. Transmission electron microscopy (TEM) of Fe NPs.

Figure (3) showed that the total yield of extracted DNA template from infected Maize seeds (Giza 2) with *C maydis* by Dellaporta buffer + Ag NPs increased significantly (1269.3 ng/µl) compared to (86.9, 85.5 and 93.2 ng/µl) in the extracted DNA by using Dellaporta+ Fe NPs, positive and negative control, respectively.

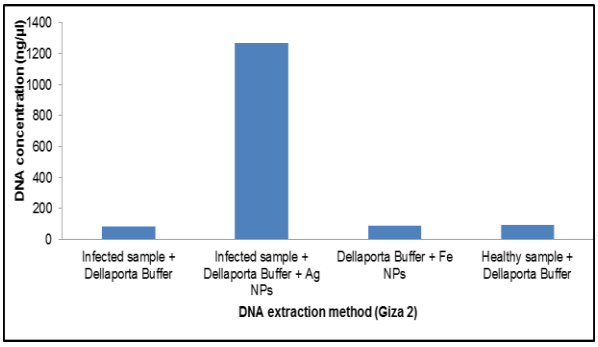

Fig. 3. DNA concentrations (ng/µl) of extracted DNA templates from infected and non-infected maize seeds (Giza 2) with *C. maydis* using Dellaporta buffer, Dellaporta buffer + Ag NPs and Dellaporta buffer + Fe NPs.

Figure (4) showed typical PCR amplification of maize seeds infested with pathogenic fungus *Cephalosporium maydis* by using different extraction methods. *C. maydis* specific primers pair A200a/ A200b were used to detect the expected PCR product size (200bp). Lane1, 2 and 3 showed very distinctive strong sharp bands of DNA template extracted by using (Dellaporta buffer and Dellaporta buffer + Ag NPs) compared to no PCR product in the extracted DNA template with Dellaporta buffer Fe NPs (Lane 4) and negative control (Lane 5).

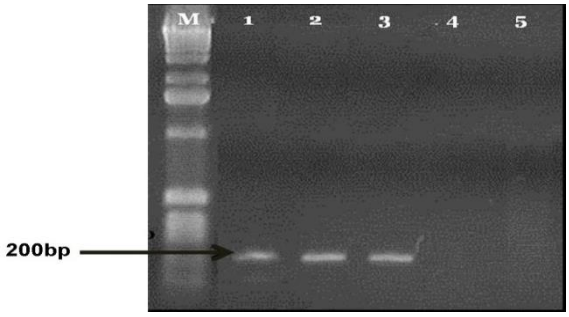

Fig. 4. Gel electrophoresis of amplified ITS-PCR for maize seed (Giza 2) pathogenic fungi *C. maydis*. M-DNA marker, VC1Kb; lane 1 – *C. maydis* DNA template extracted by Dellaporta buffer; lane 2 – Infected maize seeds with *C. maydis* DNA template extracted by Dellaporta buffer; lane 3 – Infected maize seeds with *C. maydis* DNA template extracted by Dellaporta buffer +Ag NPs; lane 4 – Infected maize seeds with *C. maydis* DNA template extracted by Dellaporta buffer Fe NPs; Lane 5- Healthy maize seeds DNA template extracted by Dellaporta buffer.

Figure (5) presented that DNA concentration from infected Maize seeds (Sc. 166) with *C. maydis* by Dellaporta buffer + Ag NPs augmented significantly to (901.5 ng/µl) compared to 136.4 ng/µl in the positive control, 121.5 ng/µl in the negative control, while Total concentration of extracted DNA by using Dellaporta+ Fe NPs was 152.9 ng/µl.

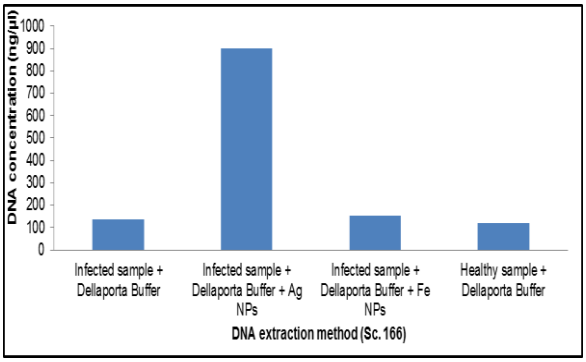

Fig. 5. DNA concentrations (ng/µl) of extracted DNA templates from infected and non-infected maize seeds (Sc. 166) with *C maydis* by using Dellaporta buffer, Dellaporta buffer + Ag NPs and Dellaporta buffer + Fe NPs.

Figure (6) showed typical PCR amplification of infected maize seeds with pathogenic fungus *C. maydis* compared to negative and positive control by using different extraction methods. *C. maydis* specific primers pair A200a/ A200b were used to detect the expected PCR product size (200bp). Lane1and 3 showed very distinctive strong sharp bands of DNA template extracted by using (Dellaporta buffer and Dellaporta buffer + Ag NPs) compared to no PCR product in the extracted DNA template with Dellaporta buffer Fe NPs (Lane 4) and negative control (Lane 5). While extracted DNA from infected maize seeds with *C. maydis* showed faint band.

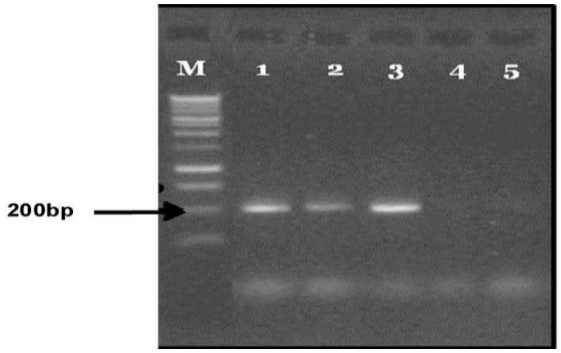

Fig. 6. Gel electrophoresis of amplified ITS-PCR for maize seed (Sc. 166) pathogenic fungi *C. maydis*. M-DNA marker, VC1Kb; lane 1) *C. maydis* DNA template extracted by Dellaporta buffer; lane 2) Infected maize seeds with *C. maydis* DNA template extracted by Dellaporta buffer; lane 3) Infected maize seeds with *C. maydis* DNA template extracted by Dellaporta buffer +Ag NPs; lane 4) Infected maize seeds with *C. maydis* DNA template extracted by Dellaporta buffer Fe NPs; lane 5) Healthy maize seeds DNA template extracted by Dellaporta buffer.

DISCUSSION

Cephalosporium maydis causes late wilt disease the major limiting factor in production in maize which is transmitted by the soil as well as the seeds. Current results proved that traditional seed health tests could not be enough to depend on their results since they gave markedly varied results. These results are in harmony of those obtained by El-Abbasi (1990) and Mathur and Cunfer (1993) who pointed out the importance of adequate plant quarantine, correct diagnosis of symptoms and/or methods of detection and isolation of such dangerous pathogen which could be transferred through seeds. Therefore, most countries have to examine seed samples carefully and/or have to treat seeds with fungicides. Moreover, Khiyami et al. (2014) discussed the common disadvantage of the traditional diagnostic methods is that they are time-consuming and lack accuracy**.** They also mentioned that the failure to adequately identify and detect plant pathogens using conventional culture-based morphological techniques has led to the development of nucleic acid-based molecular approaches.

Modification of DNA extraction methods and PCR amplification may enhance sensitivity and specificity of PCR product plus increasing the usage of DNA in variable modern techniques. Application of nanotechnology in biotechnology may change the view towards using current techniques. Nanotechnology could be very strong and effective tools for quick detection and diagnosis for plant pathogenic fungi.

In the current study, metallic nanoparticles were applied to enhance the DNA extraction methods compared to standard protocols. Application of silver nanoparticles in the extracted DNA from infected Maize seeds with *C. maydis* showed that increasing the DNA yield and PCR product compared to other extraction methods.

Ag NPs distinctiveness depends on physical properties which rely on silver nanosize and shape. Furthermore, the fascinating Ag NPs properties attributable to the enlarged surface area that may be largely applied for variable biological treatments. These alterations in the physical properties may lead to increasing water solubility or directing specific biomolecule sites such as proteins and nucleic acids (McCarthy *et al.,* 2010). Moreover, Ag NPs can enhance electrical conductivity and chemical stability by binding to the phosphorous group in the nucleic acid DNA, which has led to quick amplification of the DNA strands (Rai *et al.,* 2009).

Fe NPs did not show any differences with the standard protocols so that further studies are needed to discover the negative results of Fe NPs compared to Ag NPs.

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CONFLICT OF INTEREST

All the authors have declared that no conflict of interest exists.

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