FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 10 No. 13, July, 2026, pp 8 – 13
DOI: https://doi.org/10.33003/fjs-2026-1013-5682

Evaluation of *I* Gene Expression in Tomato Plants Against *Fusarium oxysporum* f.sp. *lycopersici* Managed Using Green Synthesized Magnesium Oxide Nanoparticles

***[1]Evans Igiehon, [1,2]Shittu Hakeem Olalekan, [1,2] Victoria Aniju Nwakaego and [1,2]Samuel Darwin Ijesuosehmwen**

[1]Department of Plant Biology and Biotechnology, University of Benin, Benin City, Nigeria.
[2]Solution Biotechnology Laboratory, Benin City, Nigeria.

*Corresponding authors' email: evansigiehon@gmail.com

Received: 25 May 2026
Accepted: 2 Jul. 2026
Published: 3 Aug. 2026

ABSTRACT
Tomato plants possess specific resistance genes known as I genes (immunity genes), which provide resistance to different physiological races of the Fusarium pathogens. The I genes in tomato plants are identified to basically encode a membrane-anchored leucine-rich repeat receptor-like protein and S-receptor-like kinase located on chromosomes 11 and 7, respectively. Once the tomato plant recognizes effectors, it activates a defense response that restricts Fusarium pathogen growth. In this study, magnesium oxide nanoparticles were synthesized using leaf extract of Moringa oleifera with magnesium oxide precursor solution and characterized using UV-Vis spectrophotometric and dynamic light scattering techniques. The in vivo study revealed that magnesium oxide nanoparticles were effective in controlling Fusarium wilt disease in the infected tomato crops. The overall percentage disease incidence in tomato crops treated with magnesium oxide nanoparticles was significantly reduced to 10%, compared to the untreated control with 100% disease incidence after 30 days of post inoculation. Also, the research highlighted that the I gene expression levels in the roots and stems of tomato plants treated with MgO nanoparticles were relatively the same with the actin gene, which serves as the reference gene, with slight variation compared to the controls. The study established that MgO nanoparticles effectively control tomato wilt disease, but through a mechanism other than I gene expression, as study results showed slight differences in expression levels between nanoparticle-treated and control tomato crops.

Keywords: *Fusarium* Pathogen, MgO Nanoparticles, *I* Gene, Level of Expression

INTRODUCTION

Fusarium species are ubiquitous soil-borne pathogens that cause a wide range of destructive vascular wilts, rots, and damping-off diseases; they are among the most toxigenic fungal phytopathogens (Okungbowa and Shittu, 2012; Srinivas *et al.,* 2019). The *Fusarium* pathogens are complex species that are highly destructive in both greenhouses and fields in wide-ranging plants (Etebu *et al*., 2013; Gordon, 2017). Among the species, *F. oxysporum* is mainly the cause of *Fusarium* wilts in crop plants (Agrios, 2005).

Fusarium oxysporum f.sp. *lycopersici* (Sacc.) WC Snyder and HN Hans, is the vascular wilt pathogen that affect tomato crops, causing considerable losses in tomato production globally (Al-Janabi *et al*., 2017; Srinivas *et al*., 2019). The control of tomato *Fusarium* wilt disease is quite difficult because of the ability of this fungus to remain dormant in the soil in the form of resting structures and chlamydospores for years without a host, and this limits the suppressive effect of crop rotation (Weeraratne and De Costa, 2018). Also, the management options of application of fungicides and other chemicals are presumes to help the tomato crops build up resistant to the pathogens, but are faced several limitations (Al-Janabi *et al*., 2017; Igiehon *et al*., 2020).

For the tomato crops to combat this pathogen, they possess specific resistance genes known as *I*-genes (immunity genes), which provide resistance to different physiological races of the *Fusarium* pathogens (McGrath *et al*., 1987; Huang and Lindhout, 1997). The *I*-gene family in tomato enables the plants to recognize pathogen effectors and activate defense responses (Simons *et al*., 1998). These *I*-genes are different types (*I*, *I*-2, *I*-3, and *I*-7), each conferring resistance to different pathogen races; They were incorporated into cultivated tomatoes from wild tomato species: the *I* and *I-2* genes were derived from wild tomato *Solanum pimpinellifolium* and conferred resistance to *Fol* races 1 and 2, respectively, and the *I-3* gene was derived from wild tomato *Solanum pennellii* and conferred resistance to *Fol* race 3. In addition to the *I-3* gene, resistance to *Fol* race 3 is also conferred by the *I-7* gene (Bohn and Tucker, 1940; Prihatna *et al*., 2018; Amjad *et al.,* 2018). The *I* gene has recently been identified to encode a membrane-anchored leucine-rich repeat receptor-like protein LRR-RLP, and it is located on chromosome 11; *I-2* is also located on chromosome 11 and encodes nucleotide binding leucine-rich repeat (NB-LRR); *I-3* is located on chromosome 7 and encodes an S-receptor-like kinase (SRLK); and *I*-7 is on chromosome 8 and encodes a leucine-rich repeat receptor-like protein (LRR-RLP) (Prihatna*et al*., 2018; Amjad *et al.,* 2018). Among these genes, *I*-2 has been the most extensively studied, the *I*-2 locus contains several homologous sequences but only one functional gene confers resistance to race 2 of the *Fusarium* pathogen (Simons *et al.,* 1998). The gene encodes NB-LRR protein, a class of proteins widely involved in plant immune responses. NB-LRR proteins function as intracellular receptors that detect pathogen-derived molecules known as effectors. Once these effectors are recognized, the plant activates a defense response that restricts pathogen growth (Jones and Dangl, 2006; Amjad *et al.,* 2018).

This study investigates *I*-gene expression level in the stems and roots of tomato crops that were infected with *Fusarium oxysporum* f.sp. *lycopersici,* and subsequently controlled using magnesium oxide nanoparticles (MgO NPs) green synthesized using leaves of *Moringa oleifera* plant.

MATERIALS AND METHODS

Plant Materials

Moringa oleifera and *Solanum lycopersicum* (tomato) plants were used for this study. Samples of moringa leaves were obtained from residential quarters at Uteh Community, Ikpoba Okha Local Government Area, Benin City, Edo State, while the tomato seeds from National Horticultural Research Institute (NIHORT), Ibadan Oyo State. The moringa leaves were identified and authenticated by plant taxonomist at the Department of Plant Biology and Biotechnology, University of Benin.

Chemical Precursor

Magnesium oxide (MgO) was used as precursor salt. All reagents were used without further purification. Double distilled water was employed as the solvent.

Preparation of Plant Extract

Plant extracts were prepared by adapting the method of Shittu *et al.* (2020). Healthy fresh leaves of *Moringa* were collected, and the leaves were initially rinsed with tap water to remove any soil or dirt, and then finally washed with distilled water. The leaves were chopped into small pieces and 20 grams each was put into a 250 ml Erlenmeyer flask containing 100 ml of sterile distilled water and kept in a water bath at 100 °C for 10 minutes. To obtain the aqueous plant extracts, muslin cloth and Whatman filter paper No. 1 were used to filter the extracts. The prepared plant extract was subsequently used for MgO nanoparticles synthesis.

Preparation of Precursor Solution

Magnesium oxide was prepared at a molar concentration of 0.01 M. The MgO aqueous solution was prepared by dissolving 0.04 g of precursor salt of magnesium oxide in 100 ml of double distilled water. The solution was used for the green synthesis of magnesium oxide nanoparticles.

Synthesis of Magnesium Oxide Nanoparticles

Magnesium oxide nanoparticles were synthesized by adapting the method of Igiehon *et al.* (2020). 10 ml aliquot of leaf extracts of the resulting filtrate was transferred into 30 ml of 0.01 M MgO solutions. The mixture was incubated for about 24 hours at 28 ± 2°C (Plate 1), the final product was employed in controlling fungal pathogens.

Characterization of MgO Nanoparticles

UV-Vis Spectrophotometer

The synthesized magnesium oxide nanoparticles was characterized with the help of a UV-Vis 1800 double-beam spectrophotometer (Shimadzu, Tokyo, Japan) operating with the range of 250 to 1000 nm to established the synthesis of nanoparticles. The sample was run in the range 250-600 nm. Water blank was used for the calibration of the equipment. For the data analysis purpose 2 ml of gold nanoparticles was taken in a cuvette. The UV-Vis absorption spectra of the biosynthesized MgO nanoparticles were recorded and the graph was plotted.

Dynamic Light Scattering (DLS)

Dynamic light scattering is a technique used in material physics for determining the size distribution of the nanoparticles in suspension or polymers in solution. This technique was used in the present study to determine the size distribution of the nanoparticles present in the MgO nanoparticles solution after biosynthesis. DLS also determines polydispersity, hydrodynamic sizes and aggregation of particles in the suspension.

Test Fungal Pathogen

The test fungal pathogen (*Fusarium oxysporum* f.sp. *lycopersici*) used in the study was obtained from IITA, Ibadan. The pathogen was subsequently grown and maintained using Potato Dextrose Agar (PDA) at room temperature for 5 days.

Planting of Model Plant (Tomato Seeds)

The seeds of tomato (*S. lycopersicum*) were surface sterilized with 0.1% hypochlorite for 3 mins, then washed with sterilized distilled water three times. The sterile seeds were planted in plastic containers of about 17 cm width and 12 cm depth containing sterile loamy sandy soil. Subsequently, tomato seedlings were sub-planted in triplicates per plastic container and replicated in three plastic containers designed for each treatment. The plants were maintained under field conditions and were rhizoinjected every other day.

Preparation of *F. oxysporum f. sp. lycopersici (FOL)* Inoculum

The fungal inoculum was prepared with sterilized distilled water. The test fungal inoculum *F. oxysporum* f. sp. *lycopersici* maintained in PDA medium for weeks was flooded with 10 mL of distilled water in the petri dishes. The conidia (spores) were scraped out using a sterile spatula and kept in sterile 50 mL tubes. The spore suspensions were then adjusted to a final concentration of $1x10^{-5}$ spores/ml by haematocytometer under a light microscope.

Inoculation of Tomato Seedlings with *FOL* Inoculum

Tomato plant seedlings were inoculated with a test fungal inoculum using the root dip treatment method. A month old seedlings were removed from the soil, their roots were washed in distilled water, and they were placed for ten minutes in a plastic container containing a spore suspension of 1×10^{-5} mL before being replanted.

MgO Nanoparticles Treatments

The nanoparticle solutions were prepared in concentrations of 100 and 50%. The concentrations were selected on the basis of the previous *in vitro* study. The infected tomato seedlings were treated with MgO nanoparticles at concentrations of 100 and 50% after infestation on alternate days. The treatments were done through soil drench application of MgO nanoparticles solutions to the soil in the plastic containers containing tomato seedlings. Also, *Fusarium* infected tomato plants treated with distilled water, and tomato plants that were not infected by test fungal pathogen were set up as controls. Data was collected on the symptom score and percentage disease incidence.

Percentage disease incidence (PDI)

PDI of the diseased tomato plants in the experimental samples was calculated using the formula:

PDI $=\frac{n\times 100}{N}$

Where: n= number of plants showing wilts symptoms

N = Total number of plant sampled (Sharma *et al*., 2017b).

The I gene expression study

The *I* gene expression study was carried out using Quantitative Real-Time PCR according to Parmar and Subramanian, (2013) and Al-Janabi *et al*. (2017).

Total RNA extraction

Root and stem samples of each tomato plant with different treatments, along with the control tomato plants, were

collected after months of post inoculation treatments. Liquid nitrogen was used to macerate the samples, and 250 mg were obtained for RNA extraction. Then, total RNA was extracted from the samples with the Spectrum TM Plant Total RNA kit (Sigma-Aldrich, United States) according to the manufacturer's recommendations. The extracted RNA was treated with RNase-Free DNase (Promega, United States). Finally, to verify the absence of genomic DNA, a PCR was performed using the DNase-Treated RNA as a template and primers amplifying the *Actin* gene.

Concentration and purity extracted RNA

The concentration and purity check of the extracted RNA were measured using a Nanodrop spectrophotometer. The concentration and purity are measured by reading the degree of absorbance (260/280 nm).

The cDNA synthesis

The mRNA was isolated from 50–100 µg of total RNA. This was performed with the Oligotex mRNA Mini kit (Qiagen, United States). The resultant mRNA was concentrated and used in the PCR-SelectTM cDNA. First strand cDNA was synthesized from 1 mg of total RNA using the ImProm-II-Reverse transcription system according to the manufacturer's instructions.

Quantitative Real-Time PCR (qPCR)

The rate of gene expression activity was measured using qPCR. The primers for actin gene forward 5'-AGGCACACAGGTGTTATGGT-3' reverse 5'-AGCAACTCGAAGCTCATTGT-3' and primers for *I* gene forward 5'-CTCACCTCACTTCGCTTCA-3' reverse 5'-TAGGGCAATCCTGGATGAAC-3' forward, 5'-TAGGGAGACAGCTTGCATGCCT-3' reverse 5'-CAAGTTGAAGGATATGAGTATTAT-3' were used in the study. Real-time PCR reactions were performed using 25 ng of cDNA, 500 nM of each primer, 10 mL of the SYBR green master mix and RNase free water in a final volume of 20 mL. In the negative control, cDNA was replaced by RNase-free water. The program used for real-time PCR was 15 min at 95°C, followed by 40 cycles of denaturation for 15sec at 95°C, annealing for 30 s at 58°C and extension for 30 s at 72°C and a final cycle of 72°C 5min, at the end of which the fluorescence was measured. Two replicates of real-time PCR reactions were performed for each sample. Primer titration and dissociation experiments were performed to confirm no formation of primer–dimers or false amplicons which could interfere with the results. After the real-time PCR experiment, the Ct number was extracted for both the reference gene and the target gene with auto baseline and manual threshold. Gene expression levels relative to the *actin* gene were calculated for each cDNA sample using the following equation.

$\Delta Ct = Ct_{\text{target gene}}\ Ct_{\text{reference gene}}$; $\Delta\Delta Ct = Ct_{\text{reference gene}}\ Ct_{\text{target gene}}$; $2\Delta\Delta Ct = 2Ct$

RESULTS AND DISCUSSION

MgO nanoparticles were successfully synthesized using magnesium oxide precursor solution with moringa leaf extract. When the precursor solution and the leaf extract were added together, a colour change from pale green to brownish colloidal solutions was observed within 24 hours of synthesis at 28 ± 2°C (Plate 1). Maximum Absorbance values were recorded between the wavelengths of 390 – 410 nm with a peak value at 400 nm using spectrophotometric technique for characterisation of synthesized MgO nanoparticles (Figure1). Dynamic light scattering, technique used to determine the size distribution of the MgO nanoparticles in suspension showed average size of the biosynthesized MgO nanoparticles to be 32.54 nm with polydispersity index of 0.560 (Figure 2).

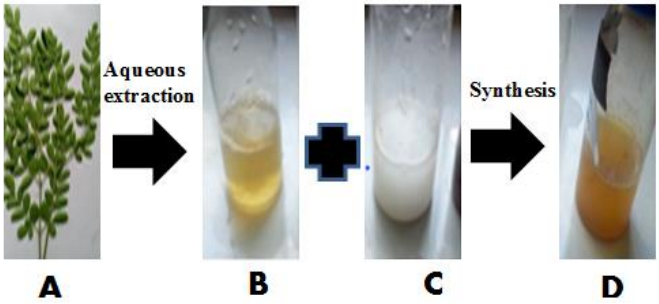

Plate 1: Schematic presentation of green synthesis of MgO nanoparticles. A: Fresh moringa leaf B: moringa leaf extract C: Magnesium oxide precursor solution D: Magnesium oxide nanoparticles

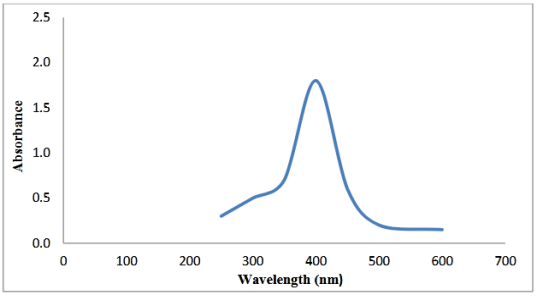

Figure 1: UV-Vis Spectra of the Green Synthesized MgO Nanoparticles after 24 Hours

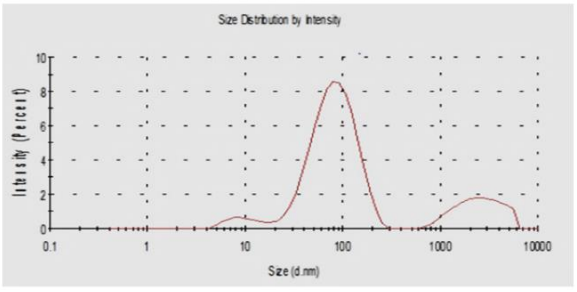

Figure 2: Dynamic Light Scattering (DLS) Pattern of Biosynthesized MgO Nanoparticle

Percentage disease incidence of *Fusarium* infected tomato plants that were rhizoinjected with MgO nanoparticles; the results revealed that there was no disease incidence at 10 days after inoculation in all the treatments and controls. Meanwhile, the untreated control had the first disease incidence of 22% at day 15 and progressed to 100% at day 35. Also, the results demonstrated that the tomato plants treated with 100% MgO nanoparticles within 35 days of post inoculation (DPI) had no significant disease incidence. However, there was a disease incidence of 18.3% on day 40 and increased to 33% on day 60 (figure 3).

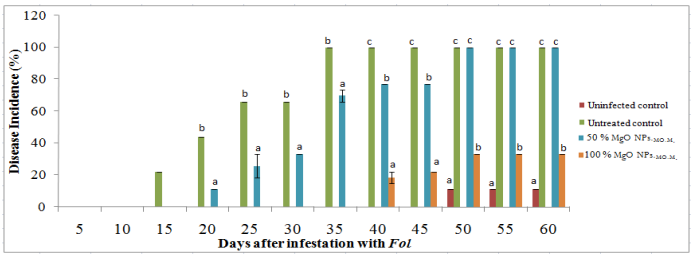

Figure 3: Percentage Disease Incidence in Tomato Plants Treated with MgO Nanoparticles Compare to the Controls

Figure 4 shows the level of expression of the *actin* gene (reference gene) in tomato plants rhizoinjected with MgO nanoparticles as well as the controls. The *actin* gene was relatively expressed in both the roots and stems of all tomato plants, with no difference in its expression level in the root and stem samples both of both MgO NPs treated and the controls. The expression levels were (roots 9.2) and (stem 8.9).

The results of expression level of resistance gene (*I* gene) in tomato plants rhizoinjected with MgO nanoparticles against *Fol* infected tomato plants in root and stem samples (Figure 5), it revealed that the *I* gene was relatively expressed in both the root and stem of the tomato plants, with expression levels of (root 10.0) and (stem 10.0) in tomato plants that were rhizoinjected with 100% MgO nanoparticles. Similar expression levels of (root 10.0) and (stem 9.8) were recorded in tomato plants treated with 50% MgO nanoparticles. The results also showed that both untreated and uninfected controls had *I* gene expression levels of (root 9.4 and stem 9.3) and (root 9.4 and stem 9.3) respectively.

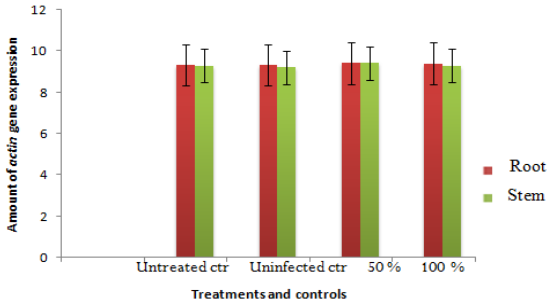

Figure 4: The Amount of *actin* (Reference Gene) Expression in the Stem and Root of Tomato Plants Treated with MgO NPs and the Controls

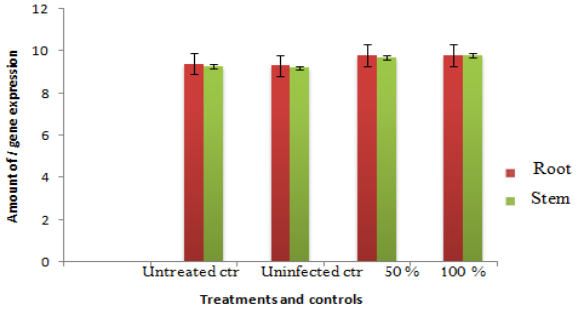

Figure 5: The Amount of *I* Resistant Gene Expression in the Stem and Root of Tomato Plants Treated with MgO NPs and the Controls

Discussion

Currently, there is increasing interest in the field of nanotechnology; its application in the control of pathogenic diseases is fundamental to cost effective management. In the present study, MgO nanoparticles were synthesized using leaf extract of *M. oleifera* with MgO precursor solution of within 24 hours at room temperature (28 ± 2ºC). The colour change with the colloidal appearance of the solutions signaled phyto-reduction of precursor solutions by the aqueous leaf extract to MgO nanoparticles. This mechanism of plant assisted reduction has been attributed to the presence of phytochemicals such as terpenoids, flavonoids, ketones, phenols, polyphenols, alkaloids, aldehydes, amines, steroids, and carboxylic acids present in plants/plant extracts which act as reducing, and capping agents (Sharma *et al*., 2017; Igiehon *et al*., 2020).

In this research the percentage disease incidence was significantly reduced in the infected tomato plants controlled using MgO nanoparticles at 100% concentration compared to the untreated control after 8 weeks of infection. The significant reduction in disease incidence (33%) was observed in tomato plants rhizoinjected with 100% MgO compared to the other treatments and the control. The observation from this result clearly showed that applications of MgO nanoparticles could have significantly improved photosynthetic efficiency, which ultimately improved growth and help to fight pathogenic infestation. This is corroborated in similar research conducted by Surega, (2015) found that green synthesized AgNPs caused complete suppression of disease incidence caused by *F. oxysporum* f.sp. *lycopersici* at the early stage of tomato and improved the growth of the plants.

It was worthwhile to look at the percentage disease incidence before the evaluation of *I* gene expression in the tomato plants. Studies have demonstrated that the ability of plants to detect pathogen invasion and activate effective defense responses is critical to the plant's innate immune system (Jones *et al*. 2015). This can be achieved by a perception mechanism in plant immunity whereby the plant resistance (R) proteins detect specific effector (Avr) proteins produced by the pathogen, and this recognition results in effector-triggered immunity (ETI), a rapid plant defense response which prevents successful infection (Jones *et al*. 2015). This ETI defense response occurs only between particular plant cultivars and a specific pathogen strain based on the presence

of corresponding R and Avr proteins, and hence is also referred to as race-specific or gene-for-gene resistance (Jones *et al*. 2015).

In this study, the *I* gene in the tomato plants were effectively targeted and quantified using real-time quantitative PCR (qPCR). The qPCR technique has been shown to be more efficient, simple, and cost-effective than other methods commonly used to quantify gene expression (Al-Janabi *et al*., 2017; Parmar and Subramanian 2013). The *actin* gene that served as the reference gene was expressed in all the tomato plants, both the ones rhizoinjected with MgO nanoparticles and the controls, and also the *actin* gene was moderately expressed in the root and stems with no variation in its expression. The expression of the *actin* gene in all tomato plants was due to the ability of the gene to be expressed in all cells and tissues, as well as the fact that its expression is not usually affected by experimental or disease conditions (Al-Janabi *et al*., 2017).

The rhizoinjected tomato plants with 100 and 50% MgO nanoparticles to manage *Fusarium* wilt infection were found to have displayed *I* gene that was moderately expressed in both the root and stem and that they were relatively the same. Compared to the controls, it shown slight variation in gene expression levels in the roots and stems. The finding in this work is relatively new, no current scientific literature has directly published a study specifically on *I*-gene expression level in tomato crops induced by nanoparticles during *Fusarium* infection**.**

Although the mechanism of action of how MgO nanoparticles trigger resistance in plants against pathogenic diseases has not been clearly explained. In this research work, it was found that MgO nanoparticles suppressed *Fusarium* wilt disease in tomato plants, this could be as a result of slightly enhanced gene expression levels in tomato plants compared the negative control; or more importantly, is possible that MgO nanoparticles worked as an inducer, causing the genes involved in resistance to be turn on.

Few studies have revealed the control of *Fol* using MgO nanoparticles that corroborated the finding of this research. Imada *et al*. (2014) reported that when the roots of tomato seedlings were treated by drenching with MgO nanoparticle suspension prior to inoculation, there was a rapid cellular accumulation of reactive oxygen species (ROS) such as superoxide O^-, hydrogen peroxide H_2O_2 and hydroxyl radicals OH-, and this ROS does not only limit pathogen ingress but also plays a role in activating the local and systemic defense responses like the salicyclic acid (SA), jasmonic acid (JA), and ethylene (ET) signalling pathways.

Also, there are reports of callose deposition and tylose formation inducing defense response in the xylem against vascular wilt pathogens. The MgO nanoparticles might have increased the rate of tylose formation to prevent the pathogen from colonizing the vascular tissue (Mes *et al*., 2000). It was reported that MgO nanoparticles could have released several of these thousands of Mg atoms as Mg is an essential mineral element for plant health. It has been used for balanced nutrition and participation in a wide range of physiological functions like synthesis of amino acids and cell proteins, uptake and migration of phosphorus involving defense against biotic and abiotic stress in plants (Huber and Jones, 2013; Ahmed *et al*., 2016).

CONCLUSION

In this study, the synthesis of MgO nanoparticles was achieved using an eco-friendly and cost- effective method. It was found that the MgO nanoparticles were effective in the control of *Fusarium* wilt disease in tomato crops, with a significant reduction in the percentage disease incidence. However, the mode of action of the MgO nanoparticles in controlling the wilt disease in tomato crops could not be tied to *I* gene expression levels, simply because the *I* gene expression study revealed that the *I* gene expression levels in the tomato crops, both the MgO nanoparticles treated and the control, were relatively the same, with only slight variations. This finding can be improved further by conducting more detailed expression studies to determine how the *I* gene is expressed in tomato plants.

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