Regulation of the bE and bW Genes in Sporisorium scitamineum using Silver Nanoparticles Synthesized with Carissa Spinarum Extract

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Abstract

Background: Sugarcane smut is a disease that is caused by the fungus *Sporisorium scitamineum.* This is a disease of economic importance in the sugarcane industry because it can cause losses of up to 50%. Current management practices have shown to be ineffective in controlling the fungal disease and hence there is a need for the development of antifungal agents that are biocompatible, non-toxic, environmentally friendly and easy to develop. Biosynthesized silver nanoparticles have been found to possess antimicrobial properties, and have not been explored in *S. scitamineum.*

Results: The synthesis of silver nanoparticles using *Carissa spinarum* yielded particles that were spherical, smooth and had a size range from 3nm to 33nm in size. Optimization of the mixtures using ultraviolet-visual spectroscopy (UV-Vis) showed peaks in the range of 340nm to 450nm. The Fourier transform infrared spectroscopy analysis identified proteins to be essential capping agents and reducing sugars were responsible for the reduction of the silver nitrate to nanoparticles and stabilizing the nanoparticles. The biosynthesized silver nanoparticles had the highest antifungal activity at 5mg/ml, while the minimum inhibitory concentration and minimum fungal concentration were 78µg/ml. The *in-vivo* assays showed a significant (at P=0.05) reduction of the pathogen biomass concentration on the plants that were treated with the nanoparticles when compared to the control plants. The application of 58.5µg/ml of the b-AgNPs to the *S. scitamineum* resulted in a significant (P=0.05) increase in expression of the *bE* and *bW* genes, while the treatment with 39 µg/ml significantly (P=0.05) increased the expression of the *bE* gene, but had no significant (P=0.05) change in the expression of the *bW* gene.

Conclusion: Silver nanoparticles that were synthesized successfully using *C. spinarum* crude extract inhibited the growth of S. scitamineum both *in-vitro* and *in-vivo.* The silver nanoparticles had a regulatory effect on the expression of the bE and bW genes in the fungus.

1. Background

Sugarcane, *Saccharum officinarum*, is a perennial grass that belongs to the *Poaceae* family. It is primarily grown for its juice which is used to make sugar. The plant has other products and by-products which include biofuel, ethyl alcohol (ethanol), molasses, rum, straw and bagasse (Yamane, 2018). Sugarcane production is challenged by several insect pests and diseases. Among these is the sugarcane smut, which is caused by the fungus *Sporisorium scitamineum* (Syd) M. Piepenbr., M. Stoll & Oberw (Comstock, 2000).

Sugarcane smut is a major challenge to sugarcane production because of its potential to spread quickly and the considerable losses that it causes (Cui et al., 2020; Jacques-edouard et al., 2020). The disease can cause losses that range between 10% and 50% which could amount to billions of dollars (Schenck *et al.*, 2013).

Sugarcane smut is primarily managed by the use of resistant varieties. This is usually difficult to maintain because the *S. scitamineum* sometimes produces new physiological strains that are not

inhibited by the host resistance mechanism (Jacques-edouard et al., 2020). Other management practices include hot water treatment of seed cane, the use of fungicides and rouging of infected sugarcane stalks or stools. The use of fungicides usually has very limited efficacy since they are unable to penetrate the waxy coat of the sugarcane, and they also pose a negative impact on the environment (Cui et al., 2020).

Biosynthesised silver nanoparticles (b-AgNPs) have been found to have antibacterial, antifungal and antiviral properties, with no environmental concerns and development of microbial resistance. These characteristics have ignited increasing interest in the synthesis of silver nanoparticles (Velu et al., 2017). The use of plant extracts has been proven to be affordable, easy to bulk up, simple and environmentally friendly (Sanchooli et al., 2018).

Sporisorium scitamineum, formerly known as *Ustilago scitamineum*, is genetically similar to the maize pathogen *Ustilago maydis* which has been extensively studied with attributes from the availability of the complete genome sequence. The reproduction process of the pathogen is mainly regulated by two loci, locus *a* and locus *b* (Yan et al., 2016).

The *a*-locus has been studied and found to regulate the pheromone and pheromone receptor system or cell-cell recognition system, while the *b*-locus encodes for homeodomain proteins that function as transcription factors (Yan et al., 2016).

The *b* locus is formed by the *bE* and *bW* genes which are responsible for the differentiation of self and non-self, a heterodimeric transcription factor which will only be active when these have been contributed by different alleles. These genes encode for the homeodomain proteins *bE* (HD1) and *bW* (HD2) which are present and conserved in the genomes of Ustilaginaceae which includes both the genera *Ustilago* and *Sporisorium* (Peters et al., 2020; Que et al., 2014).

The *bE* and *bW* genes are involved in several pathways that include the pathogenic development of the fungi, the cell cycle regulation, mitosis as well as DNA replication (Yan et al., 2016).

This study aims to investigate the efficacy of silver nanoparticles that have been synthesized with *Carissa spinarum* against the fungus *Sporisorium scitamineum in-vivo* and to investigate the gene-regulatory effects of the b-AgNPs on the *bE* and *bW* genes of the fungus.

2. Materials And Methods

2.1 Collection and identification of the fungus

This objective was conducted in Kenya during the period from 2021 to 2022. The smut-infected plants were identified at the Sugarcane Research Institute in Kisumu, Kenya. Visible sori were cut from the infected sugarcane plants and bagged to prevent any spread to healthy plants. The spores were maintained at the molecular biology laboratory (Pan African University). These spores were rinsed 3 times with distilled water and cultured in potato dextrose agar (PDA). The plates were incubated in

darkness at 28^{o}C (Cui et al., 2020; Singh et al., 2005). To purify the cultures, single colonies were transferred onto new plates and incubated in darkness at 28^{o}C (Que et al., 2014).

The fungal genomic deoxyribonucleic acid (DNA) was extracted from mycelia using a Zymo Fungal/Bacterial Genomic DNA Extraction Kit (Inqaba Biotech, South Africa), following the instructions of the manufacturer. The quality and concentration of DNA were analysed by 1% agarose gel electrophoresis and a nanodrop spectrophotometer. To verify the identity of the fungus, the DNA that was extracted from the samples was amplified on conventional PCR using the *bE*4 (5'-CGCTCTGGTTCATCAACG – 3') and *bE*8 (5'- TGCTGTCGATGGAAGGTGT – 3') primers that are specific for *S. scitamineum*(Izadi and Moosawi-jorf, 2007; Zhang et al., 2015).

Conventional PCR amplification was carried out in a 25μL volume containing 1µl of 0.1ng/µl gDNA, 12.5μL of 2x OneTaq master mix, 0.5μL of each of the upstream and downstream primers and 10.5μL of water. The PCR amplification was performed following a thermal cycling programme of 95^{o}C for 5min; 35 cycles of 95^{o}C for 30s, 52^{o}C for 30s, and 68^{o}C for 40s; and a final extension at 72^{o}C for 5min. The PCR amplicons were checked for quality in a 1% agarose gel electrophoresis and then documented.

2.2 Plant sourcing and extract preparation

The *Carissa spinarum* leaves were sourced from the Jomo Kenyatta University of Agriculture and Technology's (JKUAT) botanical garden, in Kenya.

The leaf crude extract preparation was done by cleaning the leaves with sterile water, drying them and cutting them into small pieces using a blender. Then 50g of the leaf sample was heated at 80^{o}C in 250ml of sterile water in a 500ml Erlenmeyer flask for 30 minutes. The crude leaf extract was then filtered using Whatman No. 1 and stored at 4^{o}C (Velu et al., 2017).

2.3 Biosynthesis of silver nanoparticles using *Carissa spinarum*

To synthesize the b-AgNPs, 1mM of silver nitrate was formulated by adding 0.167g of silver nitrate into 1L of distilled water. The mixture of the silver nitrate and the plant's crude extract was made at an optimised ratio of 100ml: 3ml and kept in darkness (for 24 hours) to prevent photo-reduction of the silver, at 28^{o}C in a 150rpm shaking incubator. The intensity of the colour which is indicative of nanoparticle formation was recorded between 200 to 800nm on a UV-Vis spectrophotometer using the Flight Deck, Jenway Model 6800 Spectrophotometer (Velu et al., 2017).

2.4 Optimizing the nanoparticles

The b-AgNPs were optimised under different reaction conditions which included leaf extract reaction volume (2, 3, 4, 5, 6, 7, 8, and 9 ml) and the duration of incubation of the AgNPs in darkness which was varied at 0, 2, 4, 12, 24, 48 and 72 hours (Houllou et al., 2019). While optimizing each parameter, the other parameter was kept constant.

The b-AgNPs were isolated from the optimized mixture by centrifugation at 12 000 rpm for 20 minutes. The pellet was then purified using distilled water and washed twice to ensure better separation of free entities from the AgNPs. The b-AgNPs were kept at -20^{o}C for 24 hours, moved to -80^{o}C to be kept for 48 hours, and then they were lyophilized and used for further characterization (Velu et al., 2017).

2.5 Characterization of the b-AgNPs

UV-Vis Spectra Analysis

The sample (1ml) of the suspension was collected periodically to monitor the completion of bio-reduction of Ag + in an aqueous solution. The UV-Vis spectrum of the solution was measured between wavelengths 200 and 800nm using the Jenway Model 6800 Spectrophotometer Flight Deck with a resolution of 1nm (Sanchooli et al., 2018).

FTIR Analysis: The nanoparticle characterization included ascertaining the active biomolecules responsible for the reduction; capping and stabilising by FTIR Spectrometer model 8400, Shimadzu. For the FTIR analysis, the dried b-AgNPs were added to FTIR-grade potassium bromide (KBr) in 1: 100 ratios and observed in the range of 4000 to 400 cm^{-1}.

TEM Analysis

The analysis to determine the morphology, size and shape of the nanoparticles was done using the JEM-2100 Electron Microscopy. The TEM sample grid with a continuous silicon oxide film was prepared, as well as the glassware and apparatus. The sample grid was then derivatized by exposing the silicon oxide to 10µl of aminopropyldimethylethoxysilane solution. The b-AgNPs were then citrate-stabilized for them to have a negative charge to attract to the positively charged TEM surface grid (Bonevich and Haller, 2010).

2.6 Antifungal activity of the b-AgNPs

The antifungal activity of the synthesized AgNPs was assayed using the disc diffusion assay method. The *S. scitamineum* was cultured in PDA media and the treatments were replicated 3 times. The plates were incubated in darkness at 28^{o}C for 48 hours. Varying concentrations of silver nanoparticles (0.62, 1.25, 2.5, 5, and 10mg/ml) were dissolved in distilled water and placed on the surface of inoculated agar plates. The positive control was the standard fungicide nystatin and the negative control was distilled water. The antifungal activity was measured by the diameter of the inhibition zone (mm).

2.7 Minimum inhibitory concentration (MIC) of the b-AgNPs

This assay aimed to determine the least amount of the b-AgNPs that inhibited the growth of the fungi completely. The fungus was cultured on nutrient media broth and 200µl was transferred into different

wells on a 96 well plate. Then 200µl of serially diluted b-AgNPs (0.0097, 0.0195, 0.039, 0.078, 0.165, 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20mg/ml) was added onto the wells and incubated for 24 hours. The negative control was the fungus without the b-AgNPs but water and the positive control was the b-AgNPs without the fungus. After the 24-hour incubation, 10µl of resazurin was added and incubated in darkness for 2 hours, to determine the metabolic state of the fungi in the wells by colour changes. The lowest b-AgNP concentration without any colour change (indicating metabolic activity) was recorded as the minimum inhibitory concentration (Sanchooli et al., 2018).

2.8 Minimum Fungal Concentration

From the MIC assays, the wells with the least concentration that had no colour change (recorded MIC) and the subsequent well (slight colour change) were then cultured on PDA to observe the least AgNP concentration that will inhibit fungal growth.

2.9 Antifungal activity of the b-AgNPs *in-vivo*

The efficacy of the b-AgNPs to control the pathogen *in-vivo* was evaluated by measuring the pathogen biomass (copy number) using qPCR. Healthy seed cane of the smut-susceptible variety CO421 was obtained from the Sugarcane Research Institute (SRI) in Kisumu, Kenya, and grown in the greenhouse.

The *S. scitamineum* teliospores that were provided by the SRI were cultured on PDA at 28^{o}C for 5 days. The mycelium was then transferred to a yeast extract liquid medium and incubated at 28^{o}C while shaking at 150rpm for 2 days before it was used for inoculation. The inoculum suspension was calibrated using phosphate-buffered saline (PBS) solution to match the McFarland turbidity standard. This standard was made by mixing 1% Barium Chloride (0.05ml) and 1% Sulphuric acid (9.95ml) to produce a 0.5 McFarland standard which is equivalent to 1.5x10^{8} colony forming units (CFU). The fungal cells were then harvested by centrifuging at 4000rpm for 5 minutes. The cells were rinsed with distilled water twice before resuspending them on 1ml distilled water. Inoculation was done 3 weeks after germination following the injure and paste method (Olweny et al., 2008). The negative control was inoculated with distilled water (Sun et al., 2019; Yan et al., 2016). The success of inoculation was confirmed by conventional PCR using the *bE4* and *bE8* primers.

After 2 weeks, post-inoculation, the plants were treated with the b-AgNPs (78µg/ml and 39µg/ml), and DNA extraction was done at 3 weeks after inoculation. To treat the infected plants, the plants were completely cut off at a height of 5cm to mimic harvesting before the treatments were applied. The treatments included; plants that were treated with water (positive control), plants that were treated with 78µg/ml of b-AgNPs (MIC), plants that were treated with 39µg/ml of b-AgNPs (1/2 MIC) and healthy plants as a negative control.

The fungal genomic DNA was extracted using a Zymo Plant Genomic DNA Extraction Kit (Inqaba Biotech, South Africa) following the manufacturer's instructions. The quality and concentration of DNA were analysed by 1% agarose gel electrophoresis and by using a nanodrop spectrophotometer.

For the qPCR quantification (SYBR Green), pure fungal DNA was diluted in tenfold serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) with a known starting DNA concentration to generate the regression line that was used to interpolate the Ct values of the DNA from the treatments (Nalayeni et al., 2021).

The qPCR amplification was done using the *bE* mating-type gene-specific primers (F-CCAACGACGAAAGCGCGACG and R-GACTCTCTGCGAGCGGGCAT) and the qPCR cycle conditions were: 95^{o}C for 5 minutes, 95^{o}C for 30 seconds and 60^{o}C for 30 seconds.

2.10 Gene expression analysis of the *bE* and *bW* genes in *S. scitamineum*

The fungus was cultured in nutrient broth media for 48 hours. The culture was then transferred (2ml) into tubes and was mixed with an equal amount of b-AgNPs. The 3 treatments were; fungi treated with 58.5µg/ml of b-AgNPs (75%MIC), fungi treated with 39µg/ml of b-AgNPs (50%MIC), and fungi treated with water as a positive control. Total RNA was extracted from these samples at 3, 6, 9 and 12 hours (Peters et al., 2020). The samples were twelve in total.

The RNA was extracted using a Zymo Quick-RNA MiniPrep Kit (Inqaba Biotech, South Africa) following the manufacturer's instructions. The quality and concentration of RNA were analysed by 1% agarose gel electrophoresis and by using a nanodrop spectrophotometer.

The extracted RNA was converted to cDNA using the FIREScript cDNA Synthesis Kit (Solis BioDyne) following the manufacturer's instructions. Upon conversion of the RNA to cDNA, the samples were amplified on conventional PCR using the *ITSa* primer to confirm the success of conversion.

The RT-qPCR quantification (SYBR Green) treatments included the fungi with b-AgNP treatments (0.059mg/ml and 0.039mg/ml) and cDNA from fungi treated with distilled water as a positive control. The qPCR cycle conditions were: 95^{o}C for 12 minutes, 95^{o}C for 15 seconds and 60^{o}C for 20 seconds.

The primers that were used for the RT-qPCR were designed based on the *bE* gene (JQ290342.1), *bW* gene (MZ773250.1) and the GAPDH gene (DQ352817.1) as a housekeeping gene. To design the primers, the FASTA sequences of the genes were downloaded using the NCBI BLAST for nucleotides and inputted into the Primer3 software to design the primers. The Primer3 software produced several primers along with their amplicon sizes and positions on the gene (Table 1). The produced primers were then tested on the primer manipulation suite to check if the primer statistics pass to be used for PCR (Thornton and Basu, 2011). The relative expression levels of the target genes were calculated using the Delta Ct method. The house-keeping gene *GAPDH* was used as a reference gene to calculate the relative expression and to determine the ratio of expression of the target genes.

Table 1
The list of primers that were designed and optimized for the relative RT-qPCR assays

Gene ID	Primer	Forward primer	Product size	Position
JQ290342	*bE - F*	TGGATCAGATATGGCGTCAA	179bp	237–415
	bE- R	GCTCTCTGCTTAGCCCTCCT		
MZ773250	*bW* - F	GCTTTCACTCCTTGGAGCAC	172bp	1042–1213
	bW - R	TTCCGATGGTGAGATTAGGC		
KJ194461	*ITSa* - F	TGAGGGTTTTGCCATTTACC	150bp	456–605
	ITSa - R	GCTTCTTGCTCATCCTCACC		
DQ352817	*GAPDHa* - F	TTTCCGTCGTTGACCTTACC	166bp	692–857
	GAPDHa - R	AAGATGGACGAGTGCGAGTT		

2.11 Data Analysis

For the antifungal activity of the b-AgNPs statistical analysis will be performed using a one-way analysis of variance, which was used to compare the differences among samples using their inhibition zones. P values ≤ 0.05 will be considered significant and all antifungal assays will be performed with 3 replications (Sanchooli et al., 2018). The MIC and MFC analysis were evaluated qualitatively.

3. Results

3.1 Identification of the fungus

To confirm the identity of the fungus, the collected isolates were screened by conventional PCR using the *S. scitamineum* specific primers *bE*4 and *bE*8. The 4 isolates had a positive amplification of a 459bp fragment (Fig. 1), which was consistent with the findings by Izadi and Moosawi-jorf (2007).

3.2 Biosynthesis and characterizing of the biosynthesized nanoparticles

Mixing the silver nitrate with the crude extract of *Carissa spinarum* resulted in a colour change of the mixture from light pale to dark brown.

UV-Vis Spectroscopy Analysis: The analysis showed absorption peaks for the b-AgNPs that were between 340 and 485nm (Fig. 2) and confirms the formation of the nanoparticles, which is consistent with the findings of Alyousef et al. (2019); Sanchooli et al. (2018) and Masum and Siddiqa et al. (2019).

Fourier Transform Infrared (FTIR) Analysis: The FTIR analysis results of the b-AgNPs (Fig. 3) shows the band at 3364cm^{-1}, 1589cm^{-1}, 1404cm^{-1}, 1335cm^{-1}, 1003cm^{-1} and at 500 to 709cm^{-1} respectively. The band formation confirms the presence of biomolecules which are responsible for the formation of the nanoparticles.

The TEM analysis was able to provide the study with the sizes, shapes and texture of the b-AgNPs. The nanoparticles that were produced ranged between 3nm and 33nm in size (Fig. 4). They were spherical in shape and smooth in texture.

3.3 Antifungal activity of the b-AgNPs

The antifungal activity of the b-AgNPs was evaluated by measuring the inhibition zones that formed when the discs were placed on the plates that had the fungal culture. The different b-AgNP amounts had a significant (P = 0.05) difference in their ability to inhibit fungal growth. Among the treatments, 5mg/ml had the largest inhibition zone, followed by 10mg/ml, 2.5mg/ml, 1.25mg/ml, 0.62mg/ml and then followed the crude extract (Fig. 5). There was a significant (P = 0.05) difference between the b-AgNP treatment that had the highest inhibition zone (5mg/ml) and the standard antifungal nystatin, while the negative treatment showed no inhibition.

3.4 Minimum inhibitory concentration (MIC) of the AgNPs

The determination of the minimum inhibitory concentration of the b-AgNPs was observed with the use of resazurin dye. The dye turned pink in wells where there was metabolic activity (lower b-AgNP dosages) and remained blue where there was no metabolic activity (Sanchooli et al., 2018). The minimum concentration that had an absence of metabolic activity was observed to be 0.078mg/ml (Fig. 6).

3.5 Minimum fungal concentration (MFC)

On the MIC assay, the contents of the wells that were observed to be the minimum inhibitory concentration (0.078mg/ml) along with the first concentration to have a colour change to pink (0.039mg/ml) were cultured to observe the concentration that had no fungal growth (Fig. 7). The minimum fungal concentration was observed to be 0.078mg/ml, which is similar to the minimum inhibitory concentration.

3.6 The effect of the b-AgNPs on pathogen colonization *in-vivo*

The b-AgNP treatment was able to significantly (at P = 0.05) reduce the pathogen tithers in the plants that were treated with the nanoparticles 7 days after treatment. Total genomic DNA of the treatment plants was extracted and the presence of the fungal DNA was confirmed by the amplification of the *bE* primers that produced a 179bp amplicon.

The treatment with the MIC concentration of the b-AgNPs (78µg/ml) had the least fungal DNA quantity with 0.00038µg/ml, whilst the treatment with 39µg/ml of b-AgNPs had 0.005µg/ml of the fungal DNA (Fig. 8). The positive control had 0.1017µg/ml of the fungal DNA.

3.7 Gene expression analysis of the *bE* and *bW* genes in *S. scitamineum*

The gene expression assay was done to investigate if the b-AgNPs have a regulatory effect on the *bE* and *bW* genes that constitute the b locus which is responsible for the development, reproduction and pathogenicity of the fungus. Primers were designed to target these genes (*bE* and *bW*) as well as the *GAPDH* and *ITS* genes which were used as reference genes. The designed primers were optimized on conventional PCR to confirm if they amplify the *S. Sporisorium* genome and to confirm their amplicon sizes when compared to the 100bp ladder. The *GAPDHa, bE* and *bW* primers produced a positive amplification of fragments that were 166, 179 and 172bp, respectively (Fig. 9). All the primers were annealed at 52^{o}C.

The *ITSa* primers were used to verify the success of the conversion of the extracted total RNA to cDNA and a 150bp fragment was observed in all the 12 samples (Fig. 10 and Fig. 11). The amplification of all the samples warranted for the downstream RT-qPCR assays.

The application of 58.5µg/ml of b-AgNPs to the *S. scitamineum* resulted in increased expression of the *bE* gene when compared to the regular expression of the gene. The expression increased significantly (P = 0.05) from 3 hours to reach an 8-fold peak after 9 hours of exposure to the b-AgNPs. At 12 hours, the expression levels of the gene had normalized to the regular expression levels (Fig. 12).

An increased expression level was also observed on the *bE* gene when the dosage of the b-AgNPs was reduced to 39µg/ml. A significant (P = 0.05) increase to an 8-fold peak level was reached only after 3 hours, then the expression levels of the gene were reduced back to their regular expression level after 6 to 12 hours (Fig. 12).

The expression levels of the *bW* gene were also observed to increase significantly (P = 0.05) upon exposure to the b-AgNPs to reach an 8-fold peak level, after 6 hours, when compared to its regular expression. The expression level of the gene was then gradually reduced to almost the regular expression after 12 hours of exposure to the b-AgNPs (Fig. 12 and Fig. 13). When the fungus was treated with a dosage of 39µg/ml b-AgNP, the expression of the *bW* gene had no significant difference throughout the observed period of 12 hours.

The change in expression levels of the target genes was restored to the normal expression levels about 12 hours after treatment with the b-AgNPs.

4. Discussion

The colour change upon mixing the silver nitrate with the crude extract is indicative of the formation of the nanoparticles by the reduction of the $AgNO_3$ by the crude leaf extract to form b-AgNPs (Alyousef et al., 2019; Sharma et al., 2014; Velu et al., 2017). The colour change is due to the occurrence of the Surface Plasmon Resonance (SPR) phenomenon which is caused by the interaction of the conduction electrons of the silver nanoparticles (Sharma et al., 2014). The phytochemicals (lipids, proteins, polyphenols, carboxylic acids, saponins, amino acids, polysaccharides, enzymes, etc.) present in plants are used as reducing, capping and stabilising agents (Chouhan, 2018).

The FTIR analysis of b-AGNPs validates the activity of biomolecules that are in charge of the reduction and stabilization of the b-AgNPs (Khatoon et al., 2017). The bands at $3364cm^{-1}$ correspond to N-H stretching of the proteins' secondary amide. The peak at $1589cm^{-1}$ indicates stretch vibrations for the –C = C- bond, whilst the Benzene ring C = C and C-C are shown by the peak at $1404\ cm^{-1}$. The C-H bond in the pyridine ring appears at $1335cm^{-1}$ and the C-OH phenols appear at $1003cm^{-1}$. The peaks at $500cm^{-1}$ to $709cm^{-1}$ show the presence of the AgNPs (Al-zubaidi et al., 2019).

The synthesised nanoparticles were surrounded by proteins and other functional groups such as terpenoids. These results indicate the strength of the carbonyl groups from the proteins and amino acids to bind with metal, thereby capping the AgNPs. The presence of the reducing sugars could indicate their responsibility in reducing the $AgNO_3$ to AgNPs and stabilizing the AgNPs (Khatoon et al., 2017).

The TEM analysis was able to provide the study with the sizes, shapes and texture of the b-AgNPs. By their definition, the size of nanoparticles should range between 1 and 100 nm. Their nano-scale size, morphological substructure and shape are of great importance as they give the AgNPs the physicochemical properties that suit them for their multiple applications (Chouhan, 2018; Khatoon et al., 2017). The synthesis and characterisation of AgNPs made from *C. spinarum* have not been documented before this study.

The antifungal activity of b-AgNPs was also observed by (Velu et al., 2017) against the plant pathogenic fungi; *Colletotrichum acutatum, Phytophthora capsici, Phytophthora drechsleri* and *Cladosporium fulvum.* Resistance to the b-AgNPs that were synthesized from sugarcane leaves was observed on *Didymella bryoniae* (Al-zubaidi et al., 2019). The standard antifungal nystatin may have displayed significantly (at P = 0.05) higher inhibition, but the limitation of using fungicides is the failure to penetrate the waxy coat of sugarcane (Cui et al., 2020).

The antimicrobial mechanism of growth inhibition by AgNPs is still not yet fully understood. Some authors have reported that damage to the cell wall and cell membrane, while some have reported AgNPs penetrate the cells and cause damage to the cell organelles and thereby resulting in apoptosis (Al-zubaidi et al., 2019). Other reports attribute the inhibition mechanism to be caused by the effect on the microbial DNA's ability to replicate once it comes to contact with the AgNPs, inactivation of the ribosomes which ultimately fails to express proteins (Al-zubaidi et al., 2019). The increase in the inhibition effect was directly proportional to the increase in the concentration of the AgNPs. This could be caused by the

increased number of b-AgNPs that attach to the fungus until a saturation point is reached. This result corroborates the findings by (Al-zubaidi et al., 2019).

The pathogenicity of the smut pathogen is a required mechanism for it to colonize the plant and continuously draw nutrients to complete its life cycle. Su *et al*. (2013) observed a slower rate of colonization in smut-resistant sugarcane varieties when compared to the susceptible varieties. Alternative to the use of resistant genotypes for the management of sugarcane smut, fungicides could be a better alternative. The fungicides are only effective on pathogens that they will be in direct contact with on the plants' surfaces at the time of spraying, otherwise, they are ineffective in treating systemic pathogens because they are not able to penetrate the waxy coat of the sugarcane plant (Cui et al., 2020). This study has demonstrated the ability of b-AgNPs to control the smut pathogen post-colonization of the sugarcane plant.

(Peters et al., 2020) also observed an upregulation of the *sod1*, *sod2* and *katG* genes in *S. scitamineum* in the presence of hydrogen peroxide (H_2O_2) *in-vitro*, which then returned to normal expression levels after 180 minutes of exposure. The H_2O_2 was found to be produced by smut resistant sugarcane varieties, especially upon being colonized by *S. scitamineum*. The upregulation of the *bE* and *bW* genes is observed as an increase in pathogenicity of the fungus in the presence of the b-AgNPs, especially at the dosage that is below the observed lethal concentration (MIC). The significant upregulation rate of the gene's expression is returned to its regular expression level after 12 hours of treatment, which could be a signal of overcoming the inhibitory effect of the b-AgNPs.

5. Conclusion

The silver nanoparticles that were synthesized by using *Carissa spinarum* had antifungal activity against *S. scitamineum*. The b-AgNPs were found to have the highest antifungal activity at 5mg/ml, whilst the MIC and MFC were found to be 78µg/ml. When the b-AgNPs were tested on plants that were challenged by the fungal pathogen, these plants were found to have low tithers when compared with the plants that were not treated. The application of b-AgNPs was found to have a regulatory effect on the *bE* and *bW* genes in *S. scitamineum.* The application of 58.5µg/ml of the b-AgNPs to the *S. scitamineum* resulted in a significant (P = 0.05) increase in expression of the *bE* and *bW* genes, while the treatment with 39 µg/ml significantly (P = 0.05) increased the expression of the *bE* gene, but there was not significant (P = 0.05) change in the expression of the *bW* gene.

Declarations

Ethics approval

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on a reasonable request

Competing interests

The authors declare that they have no competing interests

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Author’s Contribution

All authors contributed to the study and design. Material preparation, data collection and analysis were performed by BN. The first draft of the manuscript was written by BN and all authors commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

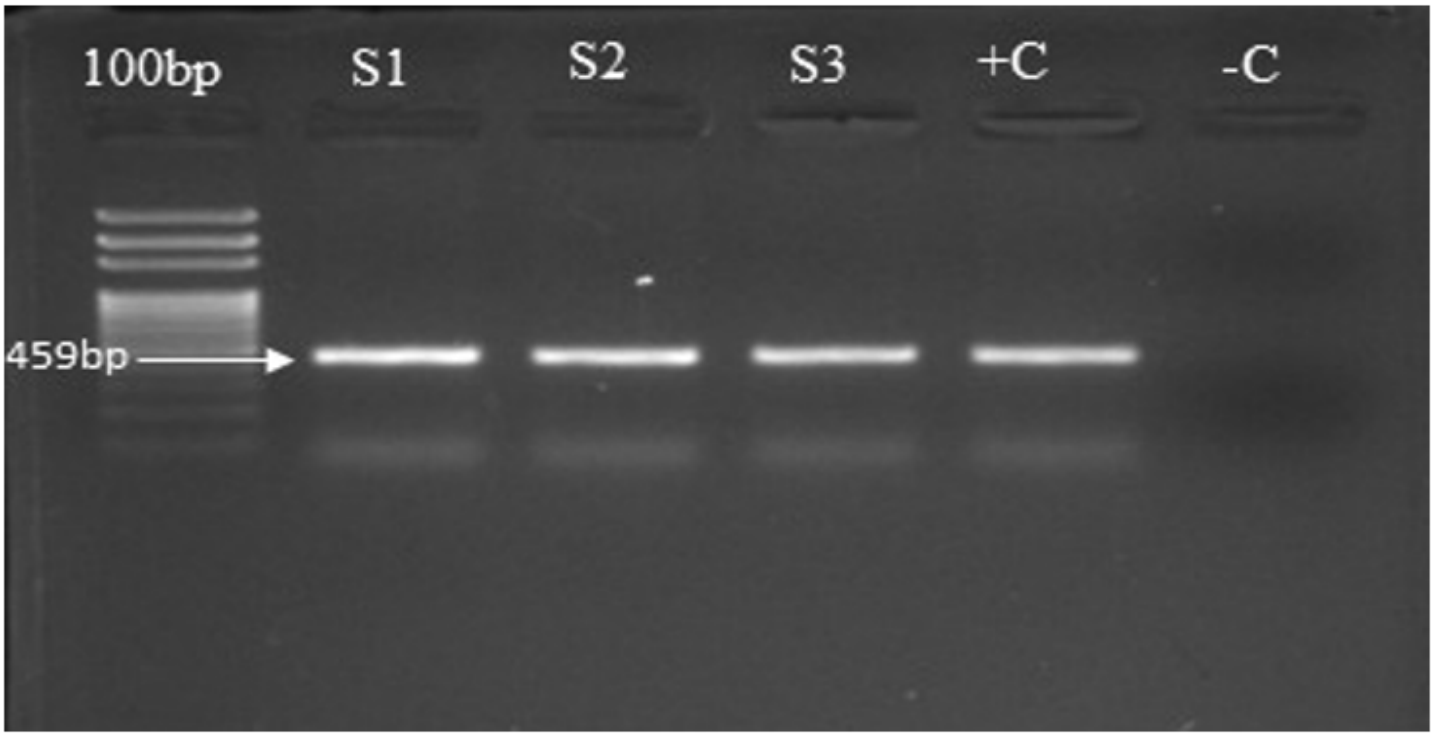

Figure 1

Gel documentation indicates the amplification of the fungal genomic DNA using the S. scitamineum specific primers, bE4 and bE8, that were used to confirm the identity of the pathogen. The primers had a positive amplification on the 3 samples (S1, S2 and S3) and the positive control (+C) with a 459bp amplicon size.

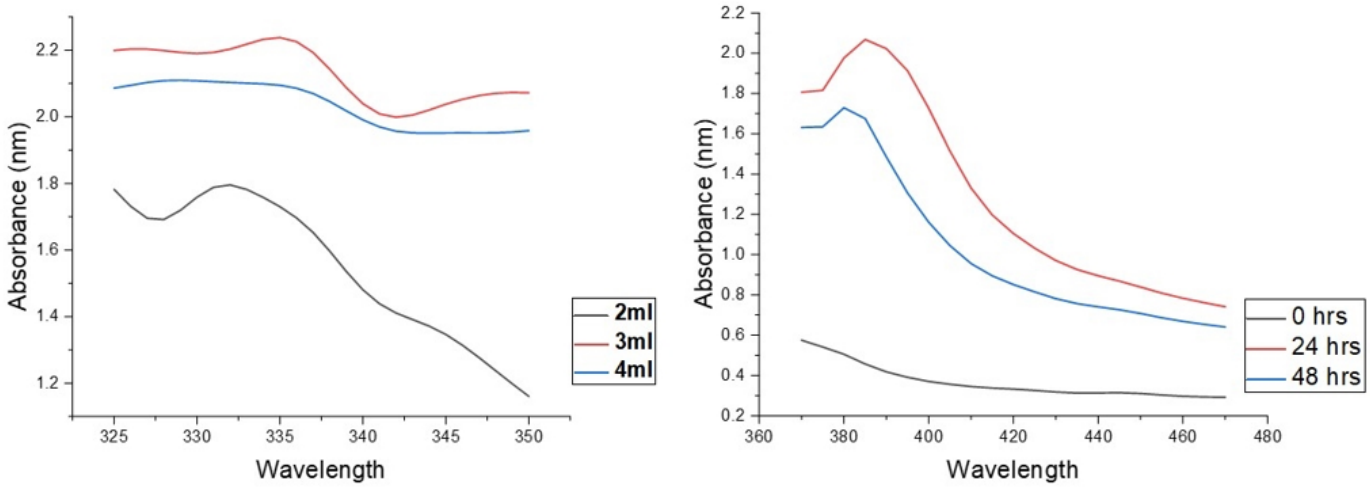

Figure 2

The optimization of the b-AgNPs by varying the incubation period, observing the absorbance at a wavelength from 200 – 800 using a UV-Vis spectroscopy.

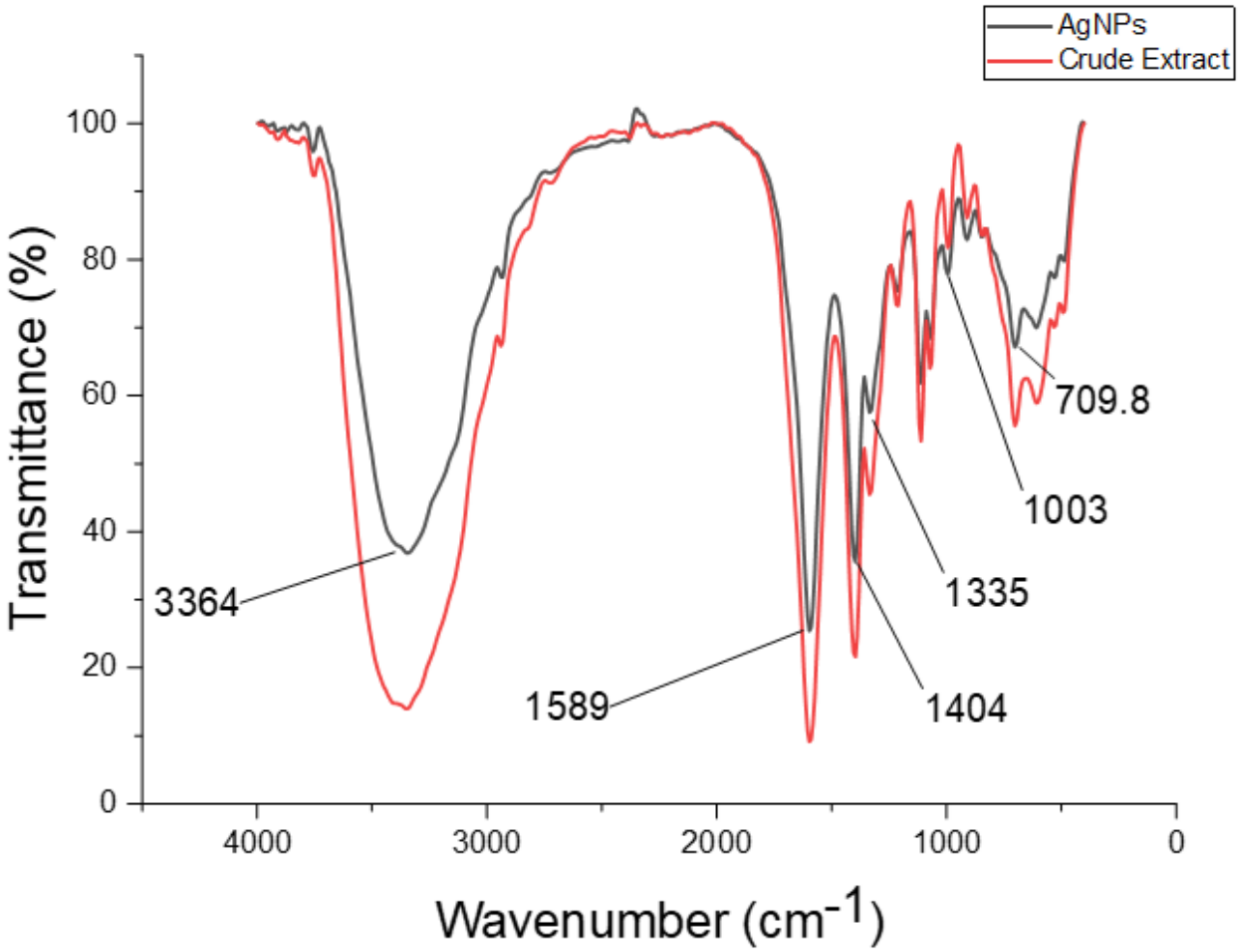

Figure 3

The FTIR results indicate the presence and sites (bands) of the biomolecules that are responsible for reducing the $AgNO_3$ to b-AgNPs well as those responsible for capping and stabilising the AgNPs.

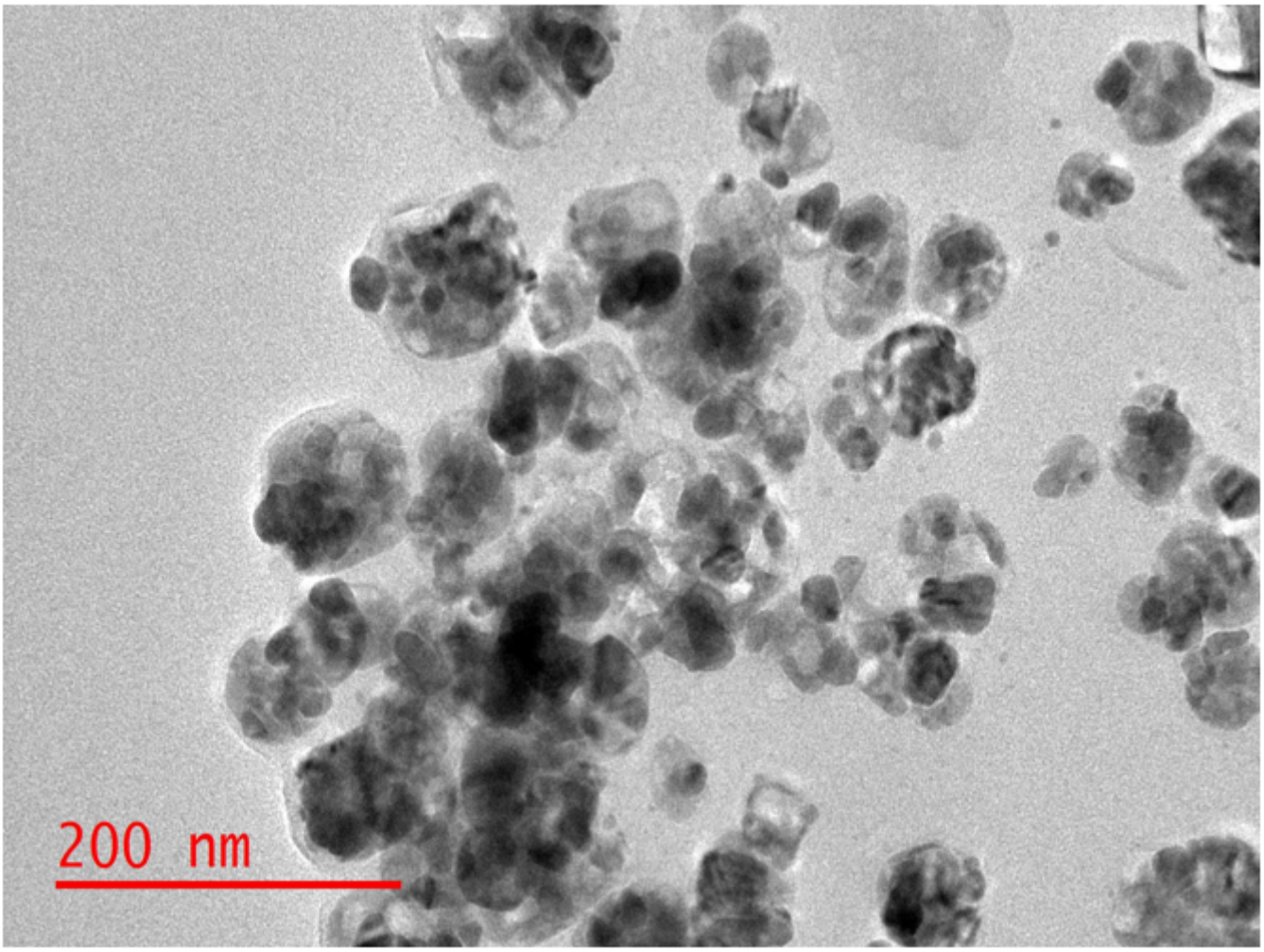

Figure 4

The TEM results indicating the sizes and shapes of the b-AgNPs that were produced by using C. spinarum

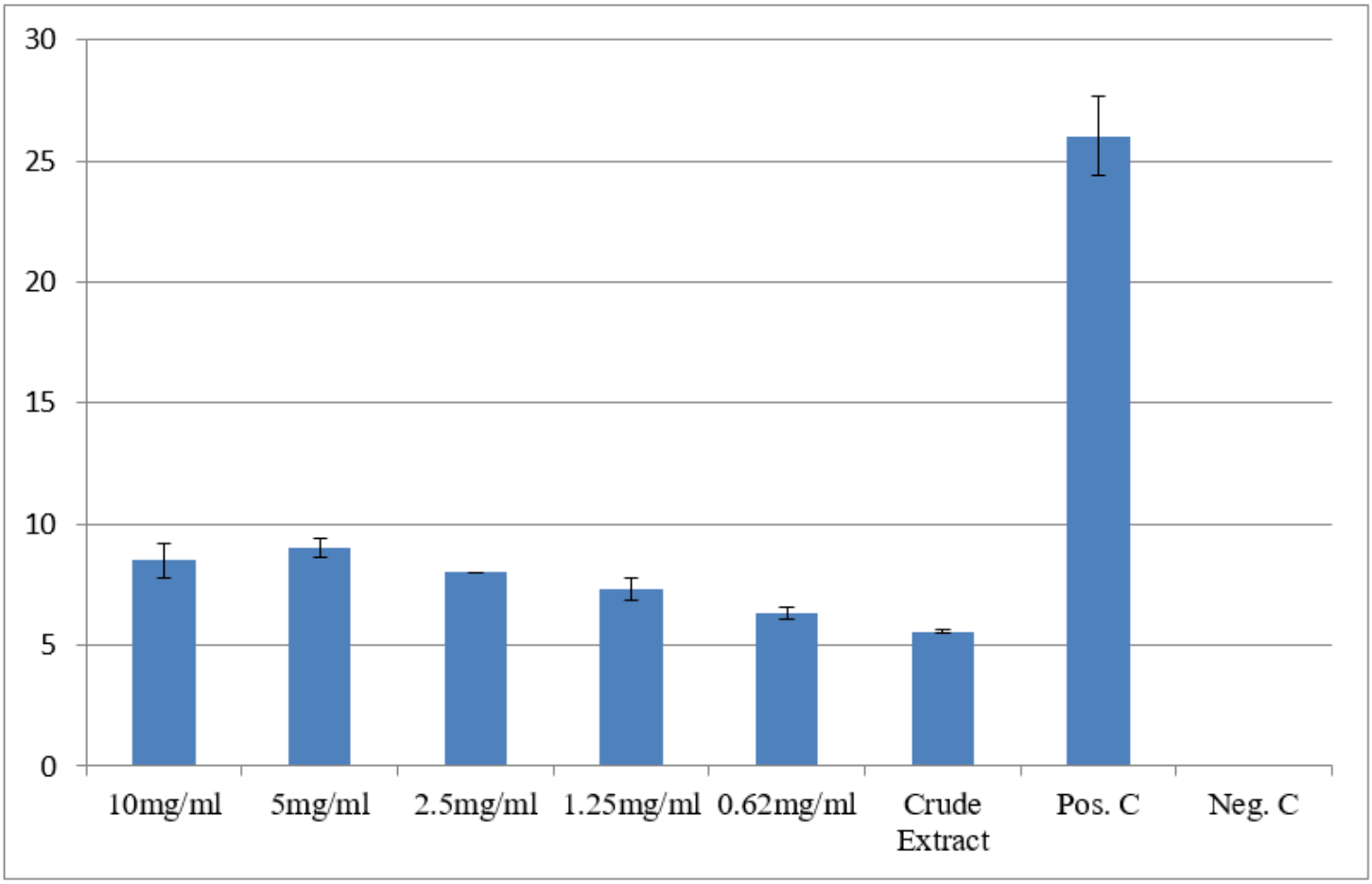

Figure 5

The inhibition zones of b-AgNPs tested in-vitro on Sporisorium scitamineum at 0.62, 1.25, 2.5, 5 and 10mg/ml. Nystatin and distilled water were included as positive and negative control, respectively.

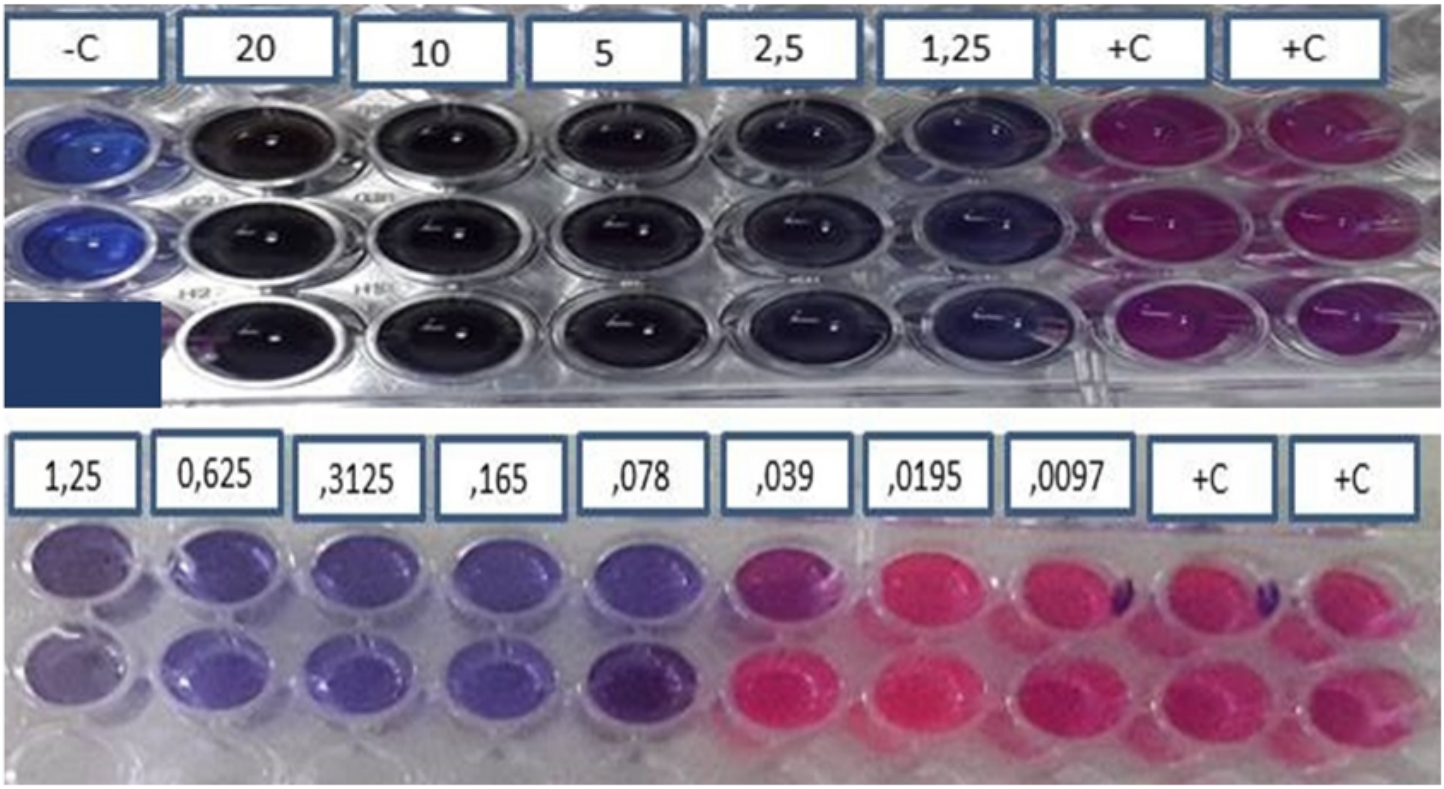

Figure 6

The MIC (mg/ml) determination by resazurin dye turning pink where there is metabolic activity and remaining blue in the absence of any metabolic activity.

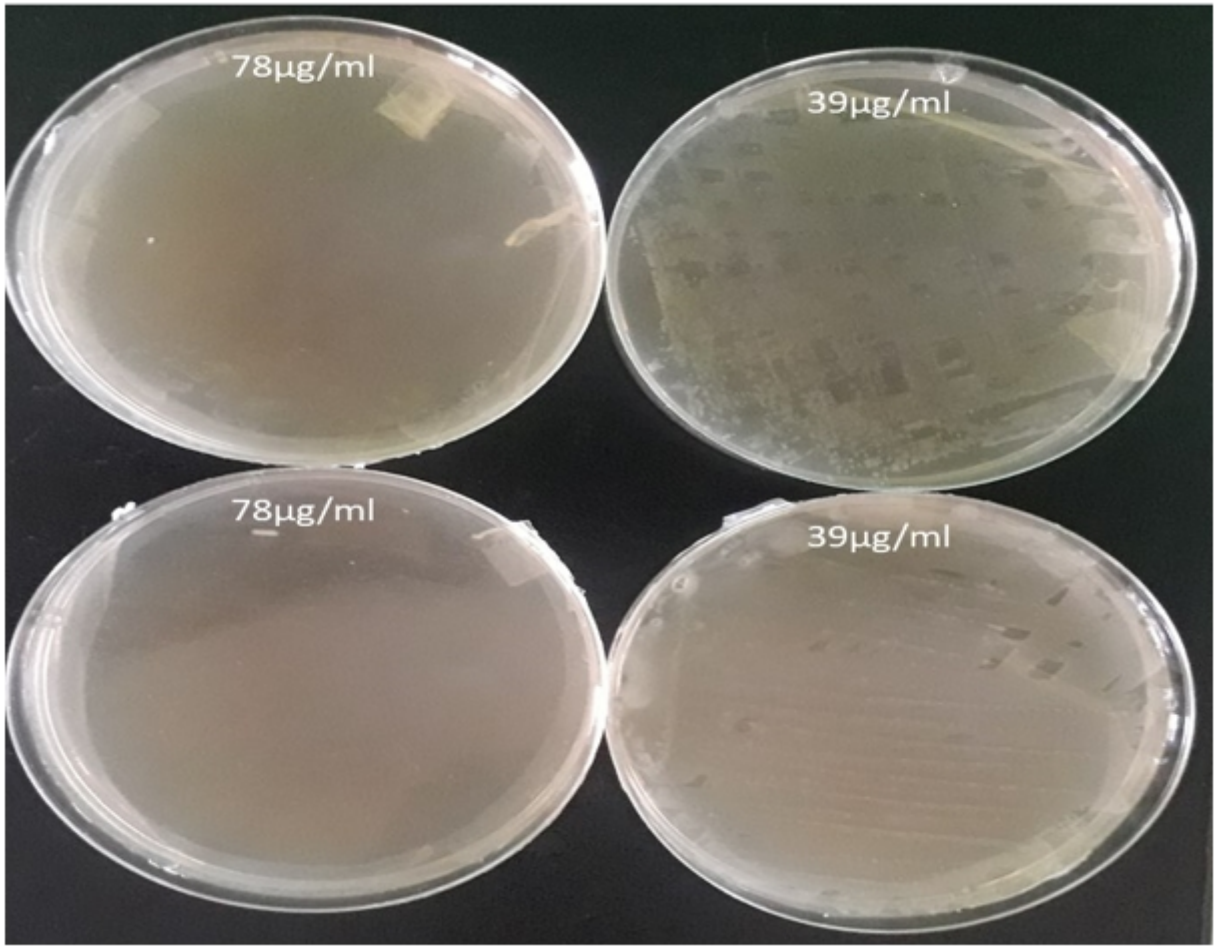

Figure 7

The determination of the MFC (mg/ml) by culturing the well with the MIC reaction and the first well that showed metabolic activity.

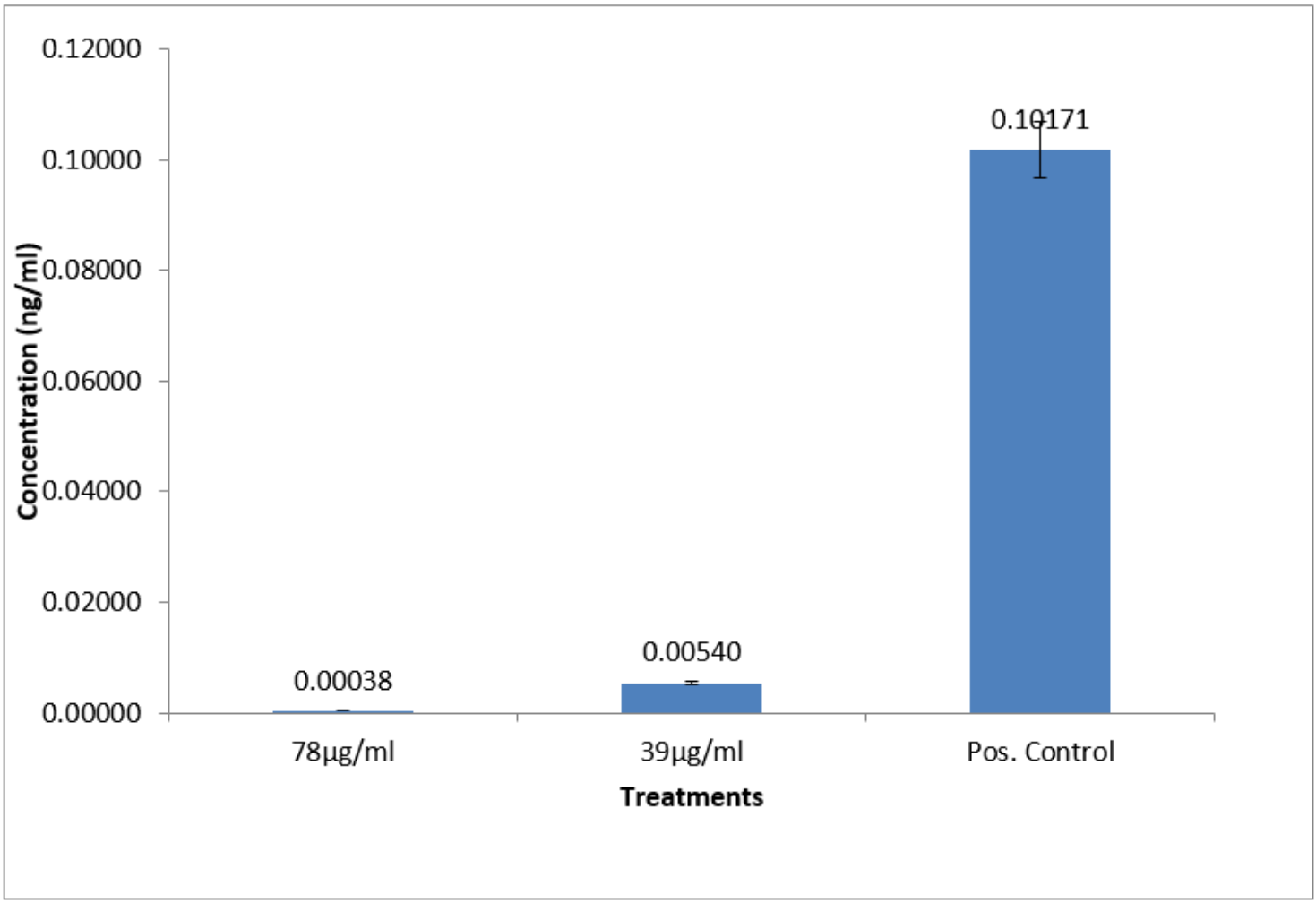

Figure 8

The in-planta pathogen biomass quantification at 7 days after treatment with varying b-AgNP concentrations compared to the positive control.

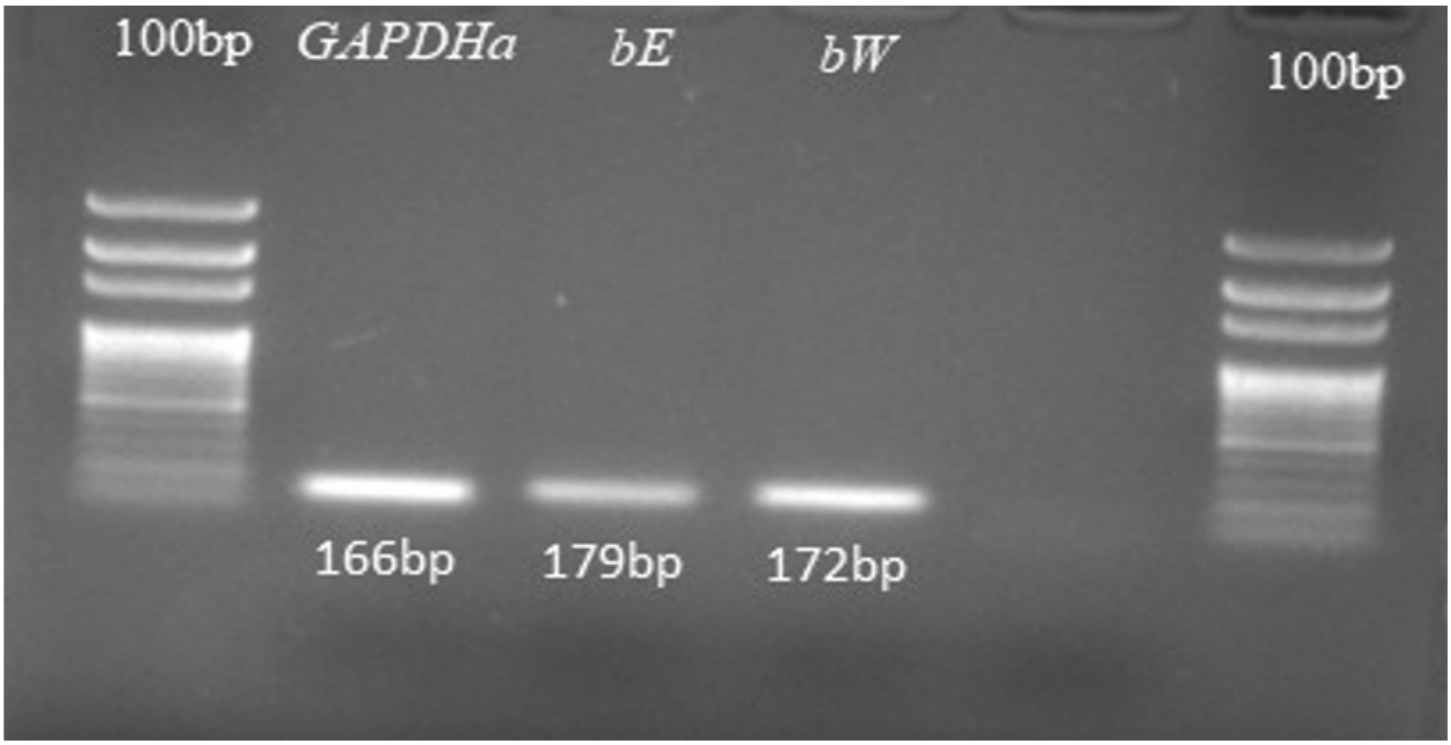

Figure 9

The optimizing of the designed primers that were used for the RT-qPCR. The optimized primers were the house-keeping gene (GAPDHa) as well as the target genes (bE and bW). The GAPDHa, bE and bW primers produced a positive amplification of fragments that were 166, 179 and 172bp, respectively

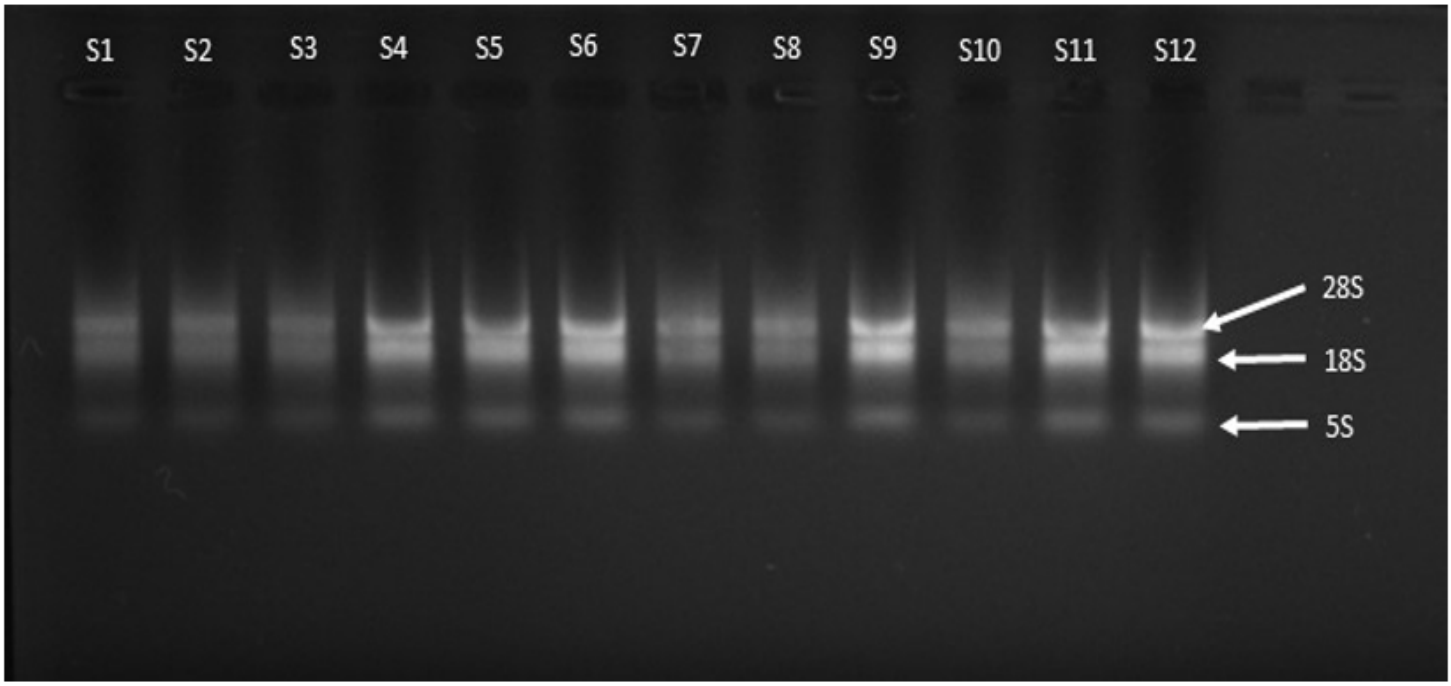

Figure 10

Integrity of the RNA that was extracted from the 12 samples showing the e28S, 18S and 5 S rRNAs

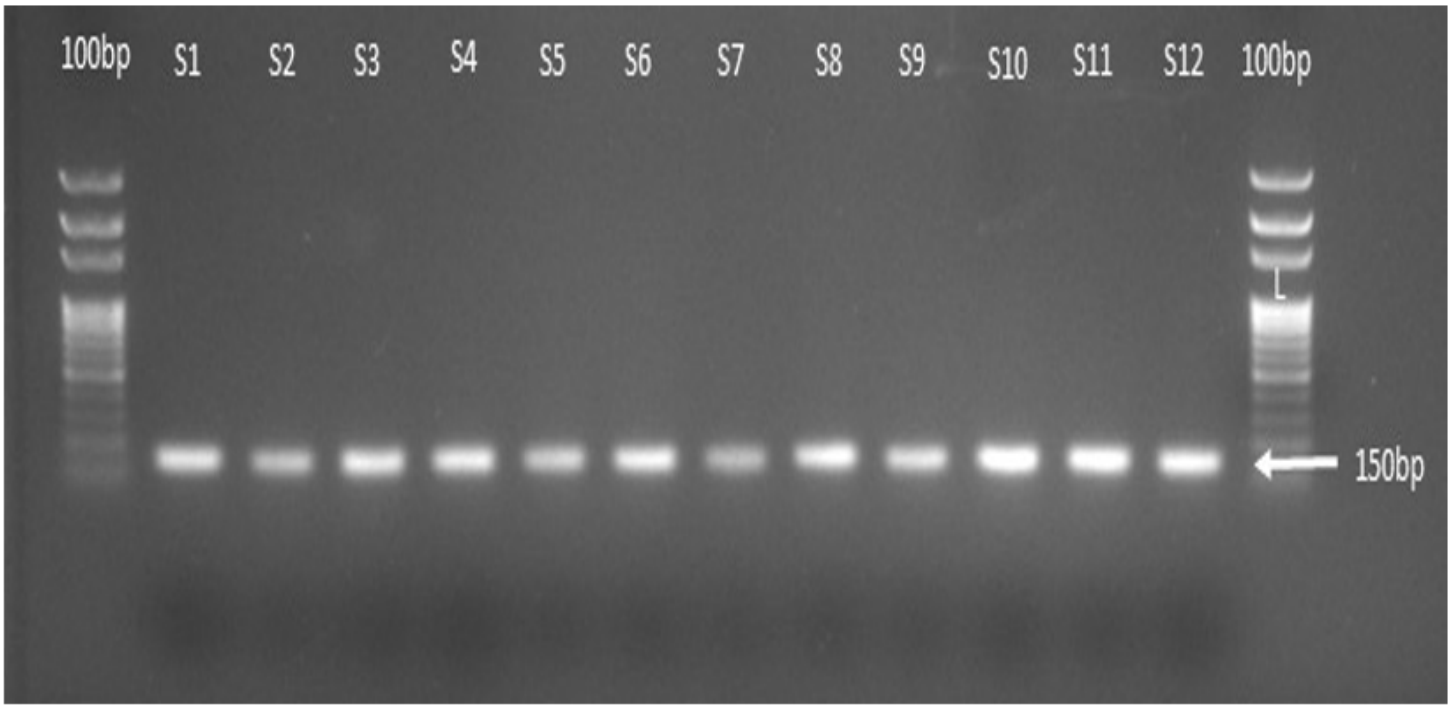

Figure 11

Amplification of the ITSa primer to confirm the successful synthesis of cDNA from the extracted RNA. The amplification produced a 150bp fragment in all 12 samples.

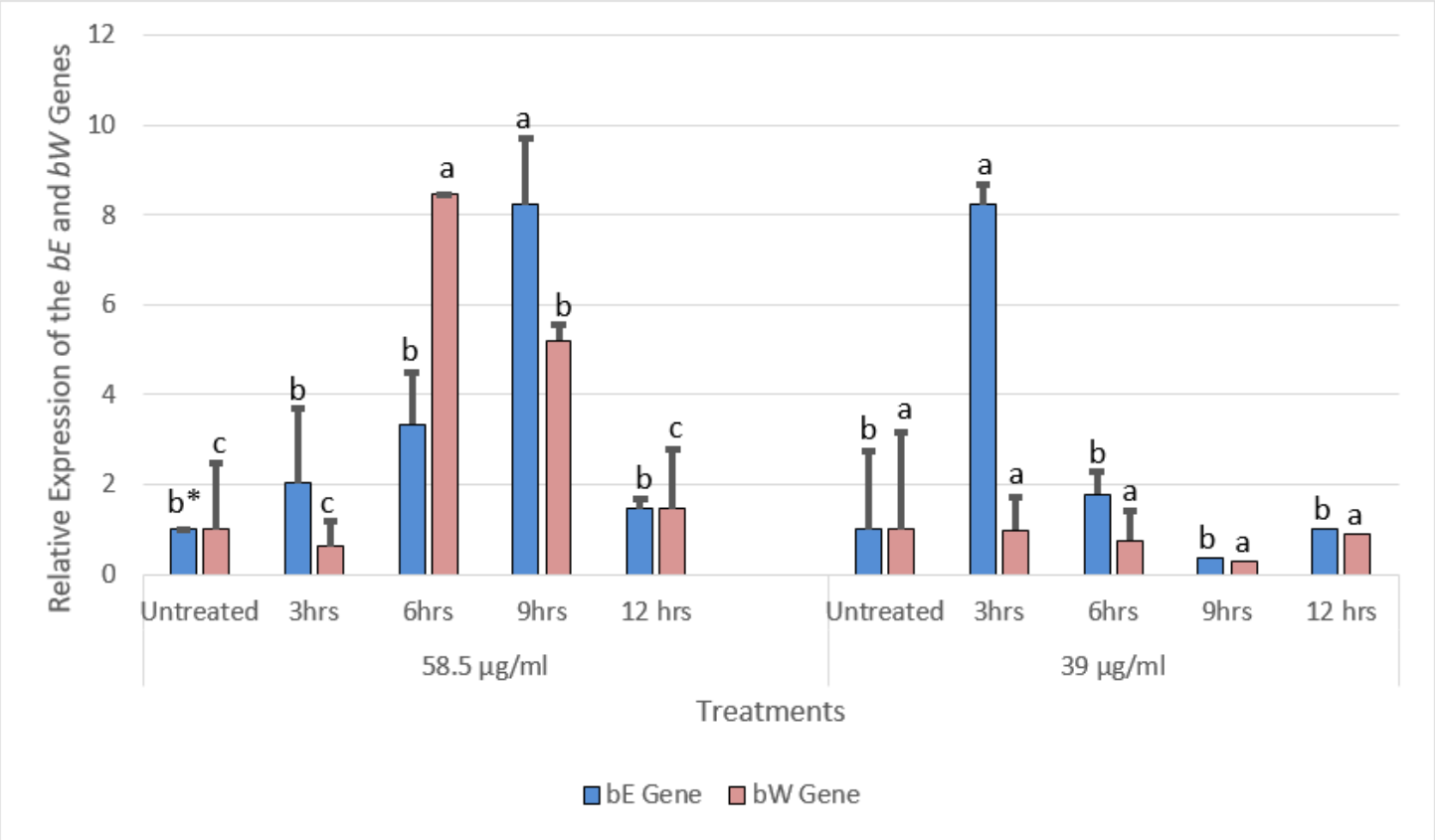

Figure 12

The relative expression levels of the bE and bW genes after treatment with 58.5µg/ml and 39µg/ml of b-AgNPs. Total RNA was extracted from these treatments at 3, 6, 9 and 12 hours after treatment. The level of significance was P=0.05. * Means followed by the same letter (per treatment) are not significantly different at P=0.05.

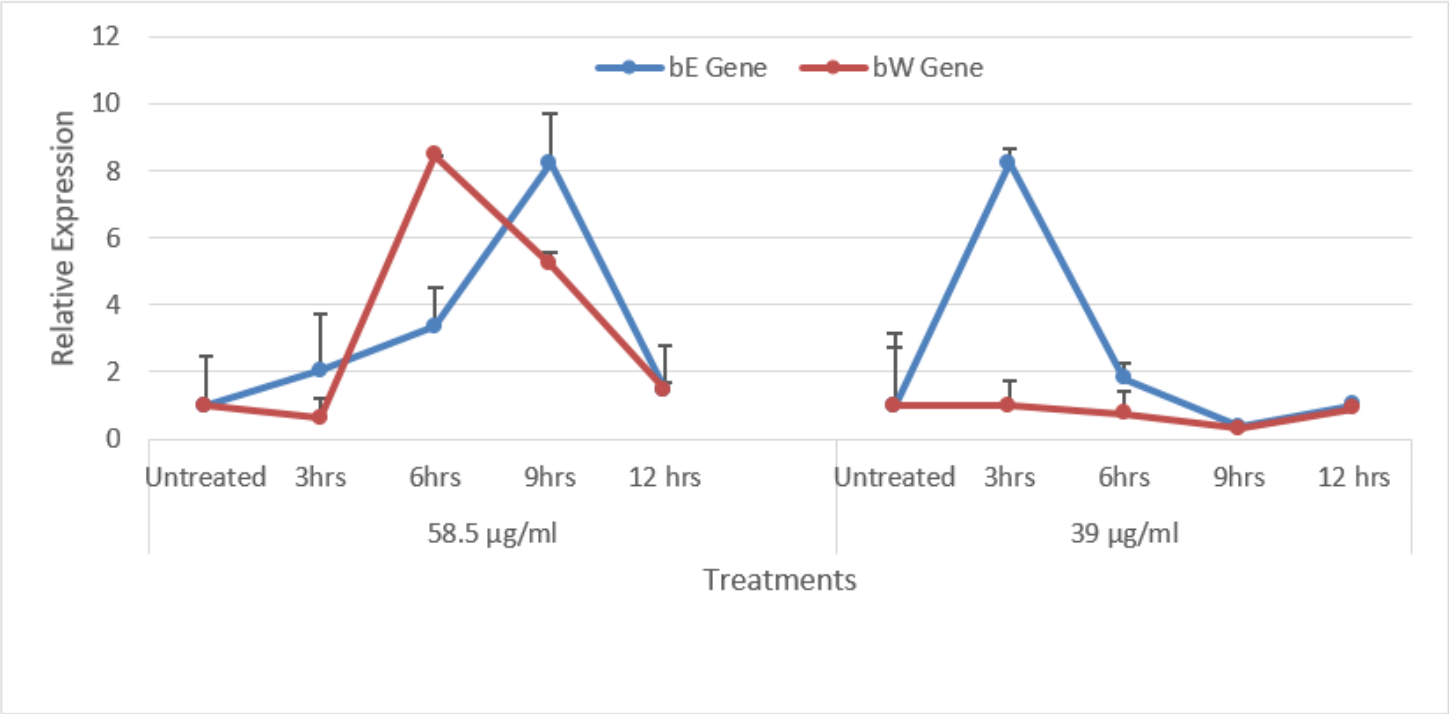

Figure 13

The relative expression rates of the *bE* and *bW* genes after treatment with 58.5µg/ml and 39µg/ml of b-AgNPs. Total RNA was extracted from these treatments at 3, 6, 9 and 12 hours after treatment. The level of significance was P=0.05.

Supplementary Files

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