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Regulation of the *bE* and *bW* genes in *Sporisorium scitamineum* using silver nanoparticles synthesized with *Carissa spinarum* extract

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Sugarcane smut is caused by the fungus *Sporisorium scitamineum.* It is a disease of economic importance in the sugarcane industry because it can cause losses that can lead to total crop failure. Bio-synthesized silver nanoparticles have been found to possess antimicrobial properties, yet they have not been explored against *S. scitamineum.* Optimization of the mixtures using ultraviolet-visual spectroscopy (UV-Vis) showed peaks in the range of 340 to 400 nm. The Fourier transform infrared spectroscopy (FTIR) analysis identified proteins as essential capping agents, and reducing sugars were responsible for reducing the silver nitrate to nanoparticles and stabilizing the nanoparticles. They have the highest antifungal activity at 5 mg/ml, while the minimum inhibitory and fungicidal concentrations were 0.078 mg/ml. The *in-vivo* assays showed a significant (P<0.05) reduction of the pathogen biomass in plants treated with the nanoparticles compared to the control plants. The application of 0.0585 mg/ml of the nanoparticles to the *S. scitamineum* resulted in a significant (P<0.05) increase in the expression of the *bE* and *bW* genes. Silver nanoparticles that were synthesized using *C. spinarum* crude extract inhibited the growth of *S. scitamineum* both *in-vitro* and *in-vivo* and had a regulatory effect on the expression of the pathogenicity genes in the fungus.

Key words: *Sporisorium scitamineum*, sugarcane smut, *Carissa spinarum*, biosynthesized silver nanoparticles, antifungal activity.

INTRODUCTION

Sugarcane**,** *Saccharum officinarum*, is a perennial grass that belongs to the Poaceae family. It is primarily grown to produce sugar, but also has other by-products which include biofuel, ethyl alcohol (ethanol), molasses, rum,

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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 has been reported to cause yield losses that range from 30 to 100%, and sometimes even leading to the elimination of some varieties (Rajput et al., 2022).

Sugarcane smut is primarily managed by the use of resistant varieties. This usually poses challenges to maintain because the *S. scitamineum* evolves to produce new strains that are not inhibited by the host resistance mechanism of these varieties (Jacques-Edouard et al., 2020). Other management practices include hot water treatment of seed cane, the use of fungicides and also 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 have deleterious impacts on the environment (Cui et al., 2020).

Silver nanoparticles have been traditionally known for their antimicrobial activities. They are produced by physio-chemical methods that include ion sputtering or pulsed laser ablation, reduction, solvo-thermal synthesis, hydrothermal and sol-gel methods. The nanoparticle synthesis methods can be broadly categorised as chemical, physical, photochemical and biological. Chemical and physical methods are generally expensive, harmful and inflammable, yet biosynthesis is cost-effective, energy-saving and environmentally benign as it uses microorganisms and plant extracts. Phytochemicals such as lipids, proteins, polyphenols, carboxylic acids, saponins, amino acids, polysaccharides, and enzymes present in plants are used as reducing, capping and stabilizing agents (Chouhan, 2018).

Bio-synthesised silver nanoparticles (b-AgNPs) have been found to have antibacterial, antifungal and antiviral properties, with no environmental concerns and development of microbial resistance (Velu et al., 2017; Ahmadi et al., 2021). These characteristics have ignited an increasing interest in the biosynthesis of silver nanoparticles. The b-AgNPs can be produced by either using microorganisms (fungi or bacteria) or plant materials. The use of plant extracts has been proven to be affordable, easy to bulk up, simple and environmentally friendly (Sanchooli et al., 2018).

Conventionally, silver has been known and used for its antimicrobial activity (Jamiu and Bello, 2018). When reduced to their nano-form, silver nanoparticles (AgNPs) possess novel and more efficient antimicrobial properties, owing to their large surface-to-volume ratio, size, shape and structure (Ahmadi et al., 2020). Bio-synthesised silver nanoparticles (b-AgNPs) have been found to have antibacterial, antifungal and antiviral properties, with no environmental concerns or development of microbial resistance. These characteristics have ignited increasing interest in the synthesis of silver nanoparticles using the green method or biosynthesis (Ahmadi et al., 2020). 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 (AgNPs) that have been synthesized with *Carissa spinarum* against the fungus *S. scitamineum in-vivo* and to investigate the gene-regulatory effects of the b-AgNPs on the *bE* and *bW* genes of the fungus. Considering the known antimicrobial applications of b-AgNPs and the availability of the medicinal plant *Carissa spinarum* in Kenya, we decided to evaluate the antifungal efficacy of the b-AgNPs on *S. scitamineum* as well as their regulatory effects on the *bE* and *bW* genes which are responsible for the pathogenicity of the fungus.

MATERIALS AND METHODS

Collection and identification of the fungus

This study 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. The 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 three times with distilled water, by centrifugation at 10 000 rcf for 1 min, and cultured in potato dextrose agar (PDA) by spreading the suspended spores using a sterile swab. The plates were

incubated in darkness at 28°C (Singh et al., 2005; Cui et al., 2020). To purify the cultures, the fungal isolates were transferred onto new plates and incubated in darkness at 28°C (Que et al., 2014).

The fungal genomic deoxyribonucleic acid (DNA) was extracted from mycelia using a Zymo Fungal and

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

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

Biosynthesis of silver nanoparticles using *C. spinarum*

To synthesize the b-AgNPs, 1 mM of silver nitrate was formulated by adding 0.167 g of silver nitrate into 1 L of distilled water. The mixture of the silver nitrate and the plant's crude extract was kept for 24 h in darkness at 28°C in a 150 rpm shaking incubator (Velu et al., 2017).

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 h (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 12000 rcf for 20 min. 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°C for 24 h, moved to -80°C to be kept for 48 h, and then they were lyophilized and used for further characterization (Velu et al., 2017).

Characterization of the b-AgNPs

UV-Vis spectra analysis

The sample (1 ml) 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 800 nm 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} (Qais et al., 2019).

Transmission electron microscopy (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; Qais et al., 2019).

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 three times. The fungal 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 (9.95 ml) to produce a 0.5 McFarland standard which is equivalent to 1.5×10^8 colony forming units (CFU). The plates were incubated in darkness at 28°C for 48 h. The b-AgNPs were dissolved in distilled water to make a stock solution of 20 mg/ml. The stock solution was then serially diluted to make various concentrations (0.62, 1.25, 2.5, 5 and 10 mg/ml) that were placed on the surface of inoculated agar plates using the disc diffusion method. 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 in millimetres (Sanchooli et al., 2018; Ahmadi et al., 2021).

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) 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 200 µl was transferred into different wells on a 96 well plate. The fungal suspension was calibrated to match the McFarland turbidity standard. Then 200 µl 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 20 mg/ml) was added onto the wells and incubated for 24 h. The negative control was the fungus without the b-AgNPs but water and the positive control was the b-AgNPs without the fungus. To aid with the observation of the MIC in broth culture, a fluorescent blue dye called resazurin was used. Resazurin is reduced by metabolic activity to a pink colour (resorufin), and where there is no metabolic activity, it remains blue (Kowalska-Krochmal and Dudek-Wicher, 2021). After the 24 hours incubation, 10 µl of resazurin was added and incubated in darkness

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* *bE*- R	TGGATCAGATATGGCGTCAA GCTCTCTGCTTAGCCCTCCT	179bp	237-415
MZ773250	*bW* - F *bW - R*	GCTTTCACTCCTTGGAGCAC TTCCGATGGTGAGATTAGGC	172bp	1042-1213
KJ194461	*ITSa* - F *ITSa* - R	TGAGGGTTTTGCCATTTACC GCTTCTTGCTCATCCTCACC	150bp	456-605
DQ352817	*GAPDHa* - F *GAPDHa* - R	TTTCCGTCGTTGACCTTACC AAGATGGACGAGTGCGAGTT	166bp	692-857

Source: Authors

for 2 h, to determine the metabolic state of the fungi in the wells by colour changes. The lowest b-AgNP concentration without changing its colour to pink (remaining blue) was recorded as the minimum inhibitory concentration (Sanchooli et al., 2018). The lowest concentration of the b-AgNPs that killed 100% of the fungi is known as the minimum fungicidal concentration (MFC). 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 inhibited fungal growth (Qais et al., 2019).

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 suspended in distilled water cultured by spreading using a sterile swab on PDA and incubated at 28°C for five days. The mycelium was then transferred to a yeast extract liquid medium and incubated at 28^{o}C while shaking at 150 rpm for two days before it was used for inoculation. The inoculum suspension was calibrated using phosphate-buffered saline (PBS) solution to match the McFarland turbidity standard. The fungi were then harvested by centrifuging at 4000 rcf for 5 min. The cells were rinsed with distilled water twice before re-suspending them on 1 ml distilled water. Inoculation was done three weeks after germination following the injure and paste method (Olweny et al., 2008). The negative control was inoculated with distilled water (Yan et al., 2016; Sun et al., 2019). The success of inoculation was confirmed by conventional PCR using the *bE4* and *bE8* primers.

After two weeks, post-inoculation, the plants were treated with the b-AgNPs (MIC and 0.5 MIC). The extraction of DNA was done three weeks after inoculation. To treat the infected plants, the plants were completely cut off at a height of 5 cm to mimic harvesting before the treatments were applied to the vascular bundle. The treatments included; plants that were treated with water (positive control), plants that were treated with 0.078 mg/ml of b-AgNPs (MIC), plants that were treated with 0.039 mg/ml of b-AgNPs (1/2 MIC) and healthy plants as a negative control. The fungal genomic DNA was extracted and the pathogen biomass after the treatment with the b-AgNPs was quantified on a RT-qPCR using the *bE* mating- type gene-specific primers (F-CCAACGACGAAAGCGCGACG and R-GACTCTCTGCGAGCGGGCAT). The cycle conditions were: 95°C for 5 min, 95°C for 30 s and 60°C for 30 s (Nayaleni et al., 2021).

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

The fungus was cultured in nutrient broth media for 48 h. The culture (2 ml) was then transferred into tubes and was mixed with an equal amount of b-AgNPs. The three treatments were; fungi treated with 0.0585 mg/ml of b-AgNPs (75%MIC), fungi treated with 0.039 mg/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 h (Thornton and Basu, 2011) and the quality and concentration of RNA were analysed by 1% agarose gel electrophoresis and using a nanodrop spectrophotometer.

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

The primers (Table 1) 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 (Thornton and Basu, 2011).

Data analysis

For the antifungal activity of the b-AgNPs statistical analysis was 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 analyses were evaluated qualitatively.

RESULTS

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 bE4 and bE8. The three*

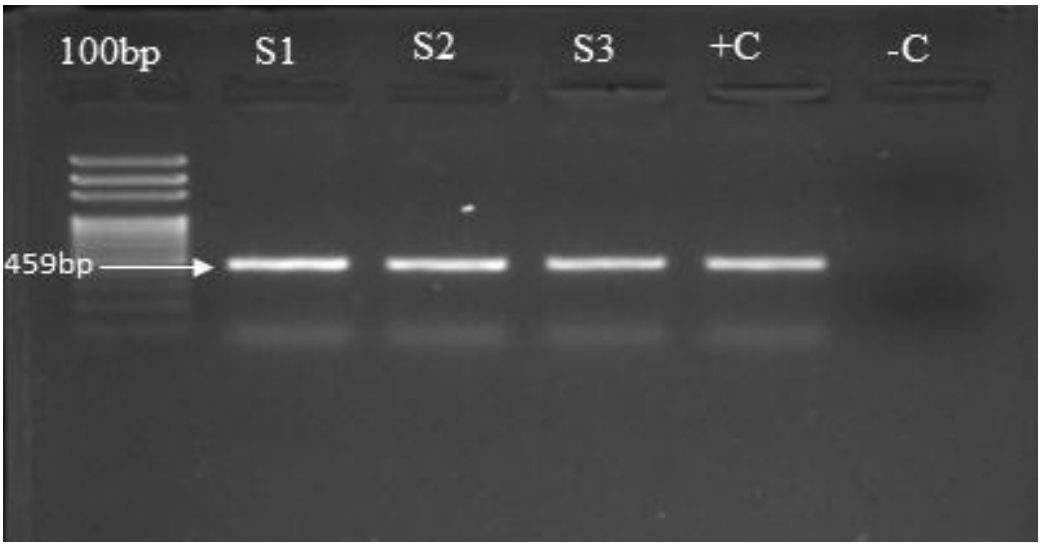

Figure 1. Gel documentation indicates the amplification of the fungal genomic DNA using the *S. scitamineum* specific primers, bE4 and bE8, that were used to confirm the identity of the pathogen. The primers had a positive amplification on the three isolates (S1, S2 and S3) and the positive control (+C) with a 459 bp amplicon size.
Source: Authors

isolates (S1, S2 and S3) were extracted and amplified along with a known positive sample (+C). All the samples had a positive amplification of a 459 bp fragment (Figure 1), which was consistent with the findings by Izadi and Moosawi-jorf (2007).

Biosynthesis and characterizing of the biosynthesized nanoparticles

Mixing the silver nitrate (100 ml) with the crude extract of *C. spinarum* (3 ml) 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 at 385 nm (Figure 2).

Fourier transform infrared (FTIR) analysis

The FTIR analysis results of the b-AgNPs (Figure 3) shows the band at 3364 cm^{-1}which corresponds to the N-H stretching of proteins' secondary amide. The peak at 1589 cm^{-1} indicates stretch vibrations for the -C=C- bond, while the benzene rings C=C and C-C are shown by the peak at 1404 cm^{-1}. The C-H bond in the pyridine ring appears at 1335 cm^{-1} and the C-OH phenols appear at 1003 cm^{-1}, while the peaks at 500 to 709 cm^{-1} show the presence of AgNPs. 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 3 and 33 nm in size (Figure 4). They were spherical in shape and smooth in texture.

Antifungal activity of the b-AgNPs

The different b-AgNP amounts had a significant ($P < 0.05$) difference in their ability to inhibit fungal growth. Among the treatments, 5 mg/ml had the largest inhibition zone, followed by 10, 2.5, 1.25, and 0.62 mg/ml and then followed the crude extract (Figure 5). There was a significant ($P < 0.05$) difference between the b-AgNP treatment that had the highest inhibition zone (5 mg/ml) and the standard antifungal nystatin, while the negative treatment showed no inhibition. The treatment with 5 mg/ml also had a significantly ($P<0.05$) higher inhibition zone when compared to the treatment with the crude extract.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) 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

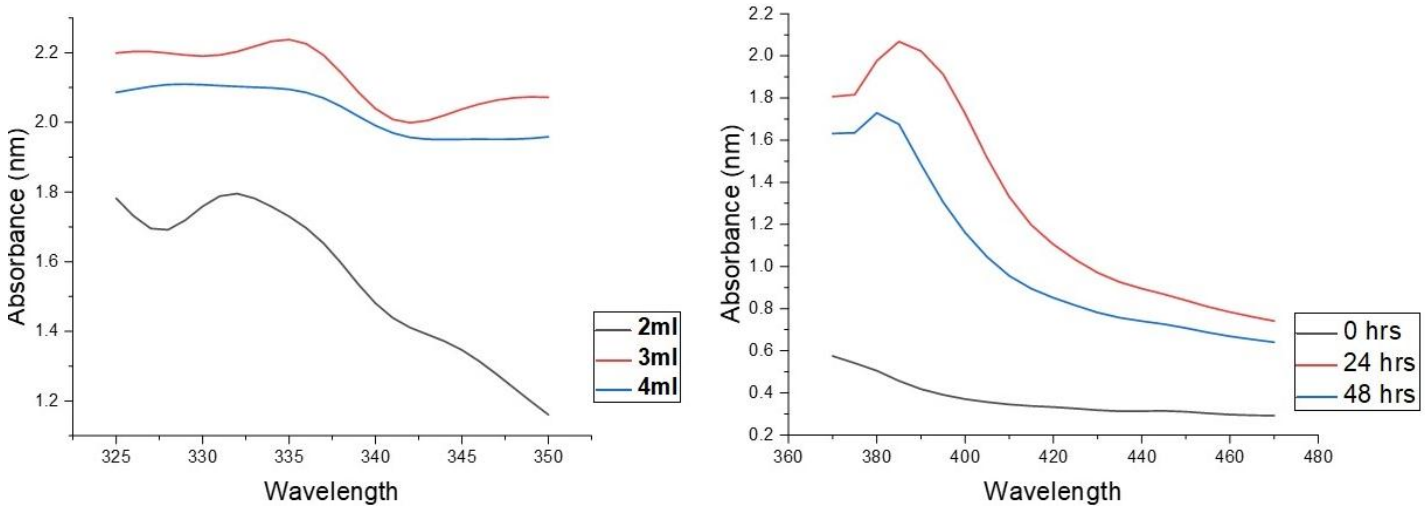

Figure 2. The optimization of the b-AgNPs by varying the incubation period, observing the absorbance at a wavelength from 200 to 800 using UV-Vis spectroscopy.
Source: Authors

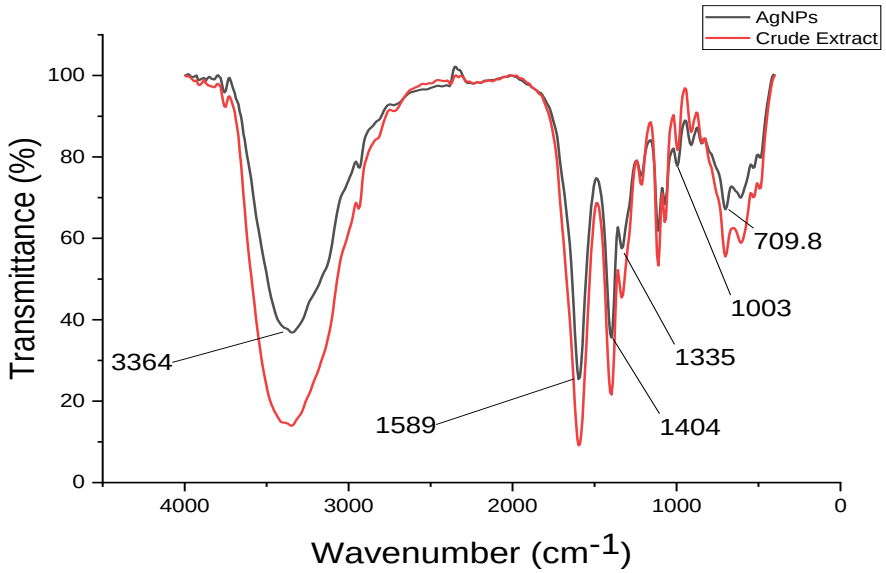

Figure 3. The FTIR results indicate the presence and sites (bands) of the biomolecules that are responsible for reducing the $AgNO_3$ to b-AgNPs well as those responsible for capping and stabilising the AgNPs.
Source: Authors

absence of metabolic activity was observed to be 0.078 mg/ml.

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

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 titres in the plants that were treated with the nanoparticles seven days after treatment. The 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 179 bp amplicon.

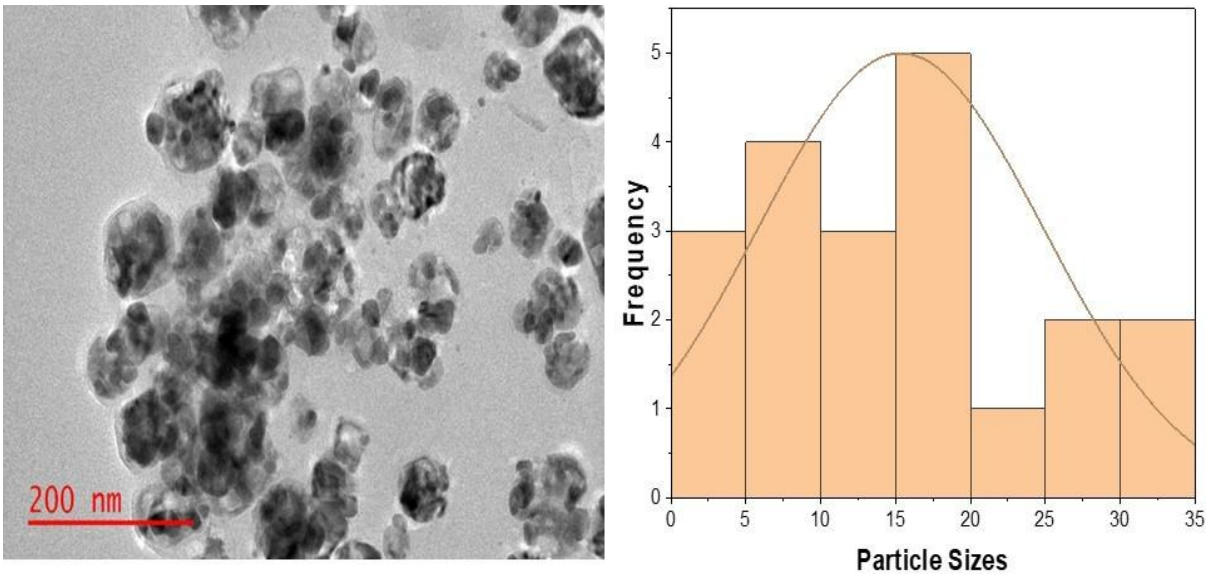

Figure 4. The TEM results indicating the sizes and shapes of the b-AgNPs that were produced by using *C. spinarum.*
Source: Authors

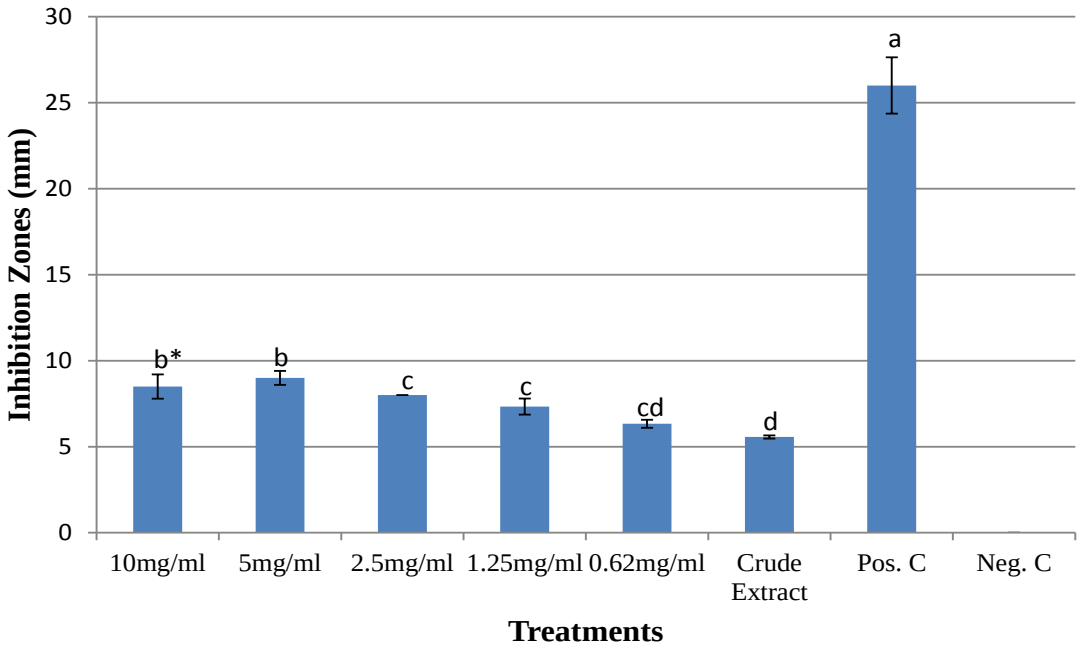

Figure 5. The inhibition zones of b-AgNPs tested *in-vitro* on *Sporisorium scitamineum* at 0.62, 1.25, 2.5, 5 and 10 mg/ml. Nystatin and distilled water were included as positive and negative control, respectively. The error bars indicate the standard deviation of the treatment means. *Means followed by the same letter (per treatment) are not significantly different at P<0.05.
Source: Authors

The treatment with the MIC concentration of the b-AgNPs (0.078 mg/ml) had the least fungal DNA quantity with 0.00038 ng/ml, whilst the treatment with 0.039 mg/ml of b-AgNPs had 0.005 ng/ml of the fungal DNA (Figure 6). The positive control had 0.1017 ng/ml of fungal DNA.

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

The optimization of the *GAPDHa, bE* and *bW* primers produced a positive amplification of fragments that were

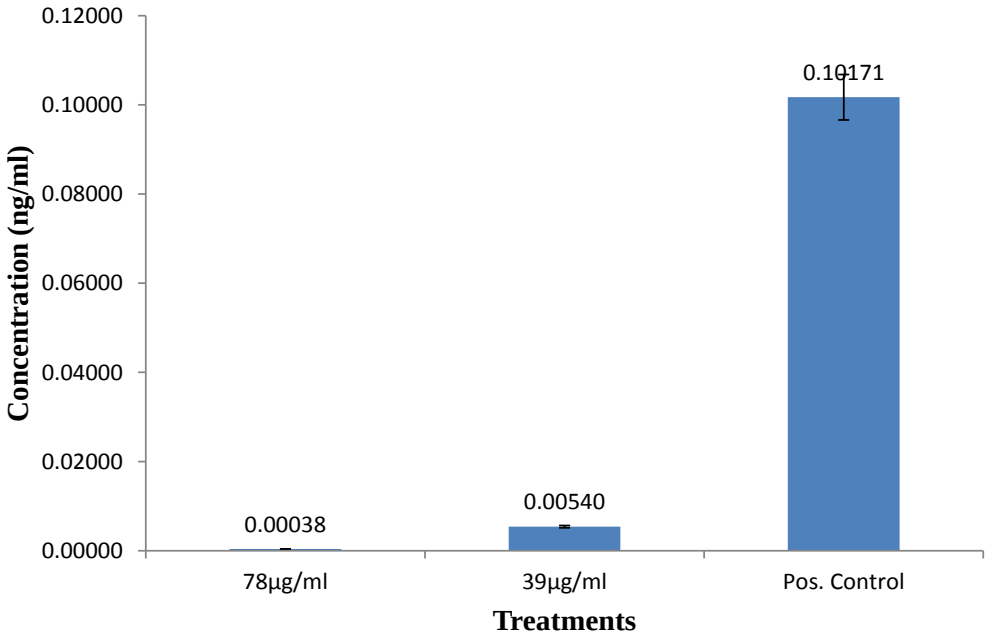

Figure 6. The pathogen biomass quantification in the plants at 7 days after treatment with varying b-AgNP concentrations compared to the positive control.
Source: Authors

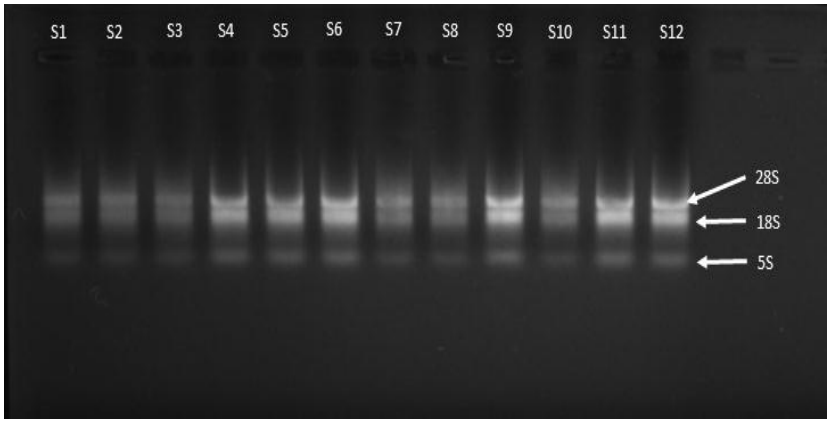

Figure 7. Integrity of the RNA that was extracted from the 12 samples showing the e28S, 18S and 5 S rRNAs.
Source: Authors

166, 179 and 172 bp, respectively. All the primers were annealed at 52°C.

The *ITSa* primers were used to verify the success of the conversion of the extracted total RNA to cDNA and a 150 bp fragment was observed in all the 12 samples (Figures 7 and 8). The amplification of all the samples warranted for the downstream RT-qPCR assays.

The application of 0.0585 mg/ml (3/4 MIC) 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 three hours to reaching an 8-fold peak after nine hours of exposure to the b-AgNPs. At 12 h, the expression levels of the gene had normalized to the regular expression levels (Figure 9).

An increased expression level was also observed on the *bE* gene when the dosage of the b-AgNPs was reduced to 0.039 mg/ml. A significant ($P<0.05$) increase to an 8-fold peak level was reached only after three hours, and then the expression levels of the gene were reduced back to their regular expression level after 6 to 12 h (Figure 9).

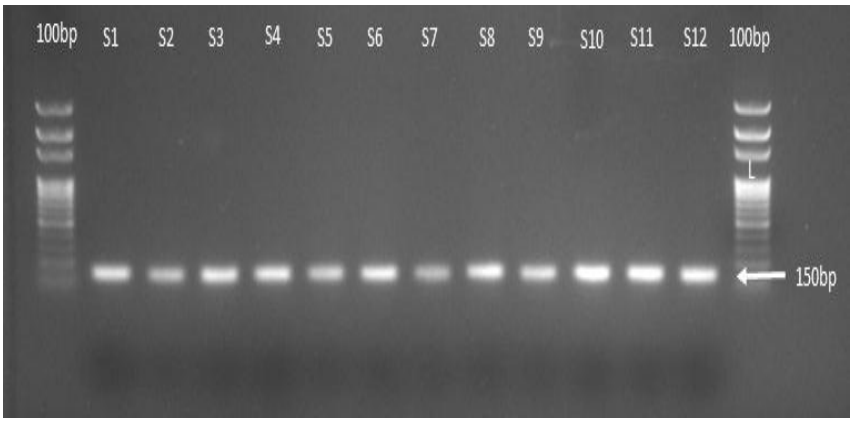

Figure 8. Amplification of the *ITSa* primer to confirm the synthesis of cDNA from the extracted RNA. The amplification produced a 150 bp fragment in all 12 samples.
Source: Authors

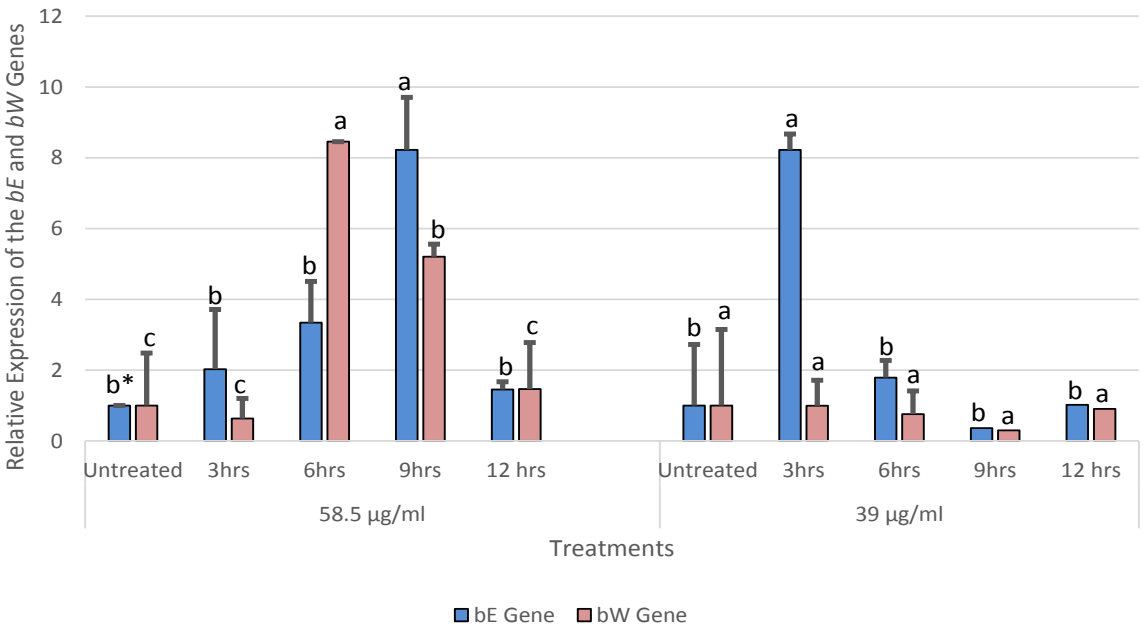

Figure 9. The relative expression levels of the *bE* and *bW* genes after treatment with 58.5 and 39 µg/ml of b-AgNPs. Total RNA was extracted from these treatments at 3, 6, 9 and 12 h after treatment. The level of significance was P=0.05. *Means followed by the same letter (per treatment) are not significantly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

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 six hours, when compared to its regular expression. The level of expression of the gene was then gradually reduced to almost the regular expression after 12 h of exposure to the b-AgNPs (Figure 9). When the fungus was treated with a dosage of 0.039 mg/ml b-AgNP, the expression of the *bW* gene had no significant difference throughout the observed period of 12 h.

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

DISCUSSION

The colour change upon mixing the silver nitrate with the

crude extract is due to the occurrence of the surface plasmon resonance (SPR) phenomenon which is caused by the interaction of the conduction electrons of the AgNPs (Sharma et al., 2014). The phytochemicals such as lipids, proteins, polyphenols, carboxylic acids, saponins, amino acids, polysaccharides and enzymes that are present in plants are used as reducing, capping and stabilising agents (Chouhan, 2018). During the optimization of the b-AgNPs, the peaks were observed with the absorbance spectra of AgNP solutions which range between 300 and 600 nm (Qais et al., 2019).

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 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; Inam et al., 2021). Inam et al. (2021) confirmed that the biomolecules that are identified by the FTIR are the ones responsible for the stability of the b-AgNPs. The biosynthesis method produces the most stable AgNPs, especially when compared to chemical synthesis (deMelo et al., 2020; Inam et al., 2021; Vala et al., 2021).

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 (Khatoon et al., 2017; Sanchooli et al., 2018). 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 resazurin assay qualitatively observes and differentiates dead and live cells by turning pink when it is exposed to metabolic activity. Resazurin is reduced by metabolic activity to a pink colour (resorufin), and where there is no metabolic activity, it remains blue (Kowalska-Krochmal and Dudek-Wicher, 2021). The colour change is caused by the reduction of the resazurin (blue) by the mitochondrial reductase into resorufin, a fluorescent pink colour. The minimum inhibitory concentration is the lowest value of the b-AgNPs that resulted in the complete death of the fungus, while the minimum fungicidal concentration is the lowest concentration of the b-AgNPs that kills 99.9% of the fungus, and verified by zero growth when cultured on media. This study observed the MIC and MFC of the b-AgNPs of *Carissa spinarum* to be 0.078 mg/ml.

The antimicrobial mechanism of growth inhibition by AgNPs is still not yet fully understood. Some authors have reported 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 ability of microbial DNA to replicate once it comes to contact with the AgNPs, inactivation of the ribosomes which ultimately fails to express proteins. 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. (2016) 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. It is important to further determine the cytotoxicity of the b-AgNPs on the plant cells as well as their effects on the agronomic properties of the sugarcane.

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 min of exposure. Hydrogen perioxides was found to be produced by smut-resistant sugarcane varieties, especially upon being colonized by *S. scitamineum*. The up-regulation of the *bE* and *bW* genes is observed as an increase in the pathogenicity of the fungus in the presence of the b-AgNPs, especially at the dosage that is below the observed minimum inhibitory concentration. The significant upregulation of the gene expression is returned to its regular expression level after 12 h of treatment, which could be a signal of overcoming the inhibitory effect of the b-AgNPs. This shows that the growth inhibition of the *S. scitamineum* upon exposure to

the b-AgNPs is caused by their effect on the genes that are responsible for the pathogenicity of the fungi.

Conclusion

The AgNPs that were synthesized by using *C. spinarum* had antifungal activity against *S. scitamineum*. The b-AgNPs were found to have the highest antifungal activity at 5 mg/ml, whilst the MIC and MFC were found to be 0.078 mg/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 0.0585 mg/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 0.039 mg/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. The *bE* and *bW* genes are responsible for the pathogenicity in *S. scitamineum,* and the regulatory effect of the b-AgNPs on these genes could be part of the basis of the antifungal activity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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