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ORIGINAL ARTICLE

Antibacterial activity of green silver nanoparticles on the in vitro pathogen infected *Eucalyptus pellita* plant

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Abstract

The antimicrobial activity of green silver nanoparticles (AgNPs) offers a promising approach for combating in vitro pathogen infections in *Eucalyptus pellita (E. pellita)* plants. This strategy provides an environmentally sustainable method to improve plant health and resistance. The effect of green synthesised AgNPs on the pathogen-infected in vitro regenerated *E. pellita* plantlets was examined. Firstly, shoots regenerated from cotyledonary leaf explants of *E. pellita* were examined in vitro through a direct organogenesis technique using murashige and Skoog (MS) medium supplemented with different concentrations (0.4, 1.3, 2.2, 3.1 and 3.9 µM) of 6-benzylaminopurine (BAP). The elongated shoots were rooted in vitro using ½ MS medium supplemented with different concentrations (0.5, 1, 1.5, 2 and 2.5 µM) of indole-3-butyric acid (IBA). The highest shoot formation from cotyledonary leaves was observed in the MS media supplemented with 2.2 µM BAP, followed by 3.1 µM BAP giving a mean of 7.4 and 6 shoots per explant, respectively. The highest root formation was observed in the ½ MS media supplemented with 1.5 µM IBA, yielding an average of 17.47 roots per explant. Secondly, the antibacterial effect of green AgNPs on tissue-cultured plants was examined by inoculating green AgNPs and three bacterial strains (*Bacillus* sp. strain EU_UPM1, *Pantoea dispersa* strain EU_UPM3 and *Pantoea dispersa* strain EU_UPM2). The results showed that green AgNPs controlled bacterial growth at 100 ppm concentration. The focal point of the current research is to use of green synthesised AgNPs as an efficient antibacterial agent that particularly targets in vitro pathogen infections in *E. pellita* plants. Thus, this study finds that green AgNPs possess potent antibacterial activity and could therefore be developed as a promising antimicrobial agent for the treatment of bacterial infections, including gram-positive and gram-negative bacteria.

Key Message

In vitro inoculation of green AgNPs resulted in better development and enhanced morphological characteristics in the horticulturally important *E. pellita* plantlets.

Keywords Nanoparticles · Bacterial resistance · 16 s rRNA sequence · *Eucalyptus* · Shoot induction · Root induction · Minimum inhibitory concentration

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Introduction

In recent forestry breeding programs and biotechnology research, micropropagation opened up new promises for increasing the coefficient of plant propagation (Abiri et al. 2020). Plant tissue culture is a technique in which plant tissues, organs or cells are cultured in vitro under aseptically nutritional and controlled environmental conditions, commonly for plant cloning (Oseni et al. 2018). These clones represent the true type of the chosen genotype (Hussain et al. 2012). Under such a properly monitored environment, the culture can develop and multiply (Suman 2017). An aseptic environment includes an adequate supply of nutrients, the pH value of the medium, a suitable temperature, as well as a balanced gaseous and liquid environment (Oseni et al. 2018). The plant tissue culture technique is mainly exploited for large-scale plant propagation (George et al. 2008). Besides its use as a research tool, it has acquired considerable industrial relevance in plant improvement, plant propagation, production of secondary metabolites and disease control (George et al. 2008).

Eucalyptus (group of the Myrtaceae family) is a widely distributed evergreen genus. *Eucalyptus* has a fast growth rate, including about 900 species that are highly available in Chile, Australia, Brazil, Portugal, Indonesia and South Africa (Batista et al. 2018). *Eucalyptus* has long been known for its economic value and commercial importance owing to its rapid growth, hard timber, high profitability and resistance to biotic stresses (Rezende et al. 2014; Nogueira et al. 2020). *E. pellita* is a perennial tree species with a huge densely branched crown which can grow to a height of 40 m (Menucelli et al. 2019). It has been cultivated for wood across many tropics and subtropic areas. *E. pellita* is great to be used as a shelter and windbreak (Hirsch et al. 2020). Moreover, it is ideal for coastal reforestation, and due to its ornamental value, it is commonly planted in parks (Thu et al. 2021).

Despite the wide availability of wild *Eucalyptus* trees, seed propagation has become a traditional cultivation technique with varying levels of success and achievement (Abiri et al. 2020). While this species is important for wood purposes, few reports have studied their properties and mass production techniques (Batista et al. 2018). Despite the advancement in the micropropagation strategy, the obstacles due to microbial contamination during early and late growth stages are still significant barriers to the clonal propagation of *Eucalyptus* sp. (Hirsch et al. 2020). Therefore, the first objective of the current study is to induce shoots and roots from leaf explants of *E. pellita* using cytokinin and auxin plant growth regulators (PGRs). Hence, the second objective of this study is to examine the influence of green AgNPs on the pathogen-infected in vitro *E. pellita* plant.

Nanotechnology has linked engineering, chemistry, biological sciences and materials science to create novel nanomaterials owing to its innovative nature (Salem and Fouda 2021; Dezfuli et al. 2023; Abdelfattah et al. 2023; Salem 2023). Nanotechnology has touched several industries owing to its distinct and observable effects, providing the scientific community with countless advancements in the fields of medicine, agriculture, and other domains. Nanotechnology opens a plethora of emerging applications in the field of biotechnology and agriculture, owing to its unique physicochemical properties, biocompatibility, bioactivity, antitumor, antimicrobial activity, reduce inflammation, and drug delivery agent (Mahendran et al. 2019; Salem and Fouda 2021). It has great promise in biotechnology, food technology, agricultural, environmental and medical activities (Naganthran et al. 2022; De Silva et al. 2021; Thakur et al. 2018). It entails molecular-scale structures less than 100 nm in at least one dimension (Sharma et al. 2019). Their tiny size and large surface area to volume ratio impart specific features that differ from their bulk materials (Thakur et al. 2018).

Bacterial resistance towards antibiotics has become a significant concern in environmental and public health; therefore, there has been a considerable effort to create novel bactericides (Terreni et al. 2021). Numerous investigations have studied the relevance of NPs as antimicrobial agents (Aisida et al. 2020; Simo et al. 2018; Kaviyarasu et al. 2017). The AgNPs have been studied to have antibacterial, antifungal, antiviral and anti-biofilm as well as anti-inflammatory properties (Naganthran et al. 2022; Tufail and Liaqat 2021; Majoumouo et al. 2019; Wang et al. 2015; Galdiero et al. 2011; Valodkar et al. 2010).

The synthesis mechanism of AgNPs is an essential part of nanotechnology, as they can be obtained either physically, chemically or biologically (Said et al. 2024; Salem 2022; Elakraa et al. 2022). Using chemical and physical means, including the use of toxic and hazardous compounds responsible for various environmental risks, can be quite expensive and potentially harmful to the environment (De Silva et al. 2021; Dağlıoğlu and Yılmaz Öztürk 2019). Thus, there is a need to use eco-friendly and sustainable strategies for synthesising AgNPs (Aisida et al. 2020). With more outstanding biological capabilities, AgNPs are now being synthesised using various biological entities and microorganisms such as plant extracts yeasts, bacteria and fungi (Arif and Uddin 2021; Liu et al. 2021; De Silva et al. 2020; Pirtarighat et al. 2019).

Recently, the preferred approach for synthesising nanoparticles is by green synthesis using various biological entities such as (plants, algae, fungi, bacteria, viruses, and actinomycetes) which is preferred due to its safe, clean, cost-effective, and easily accessible method for large-scale NPs synthesis (Aref and Salem 2020; Al-Rajhi et al. 2022; Salem et al. 2022; Al-Zahrani et al. 2022). The investigation into

the impact of green AgNPs on *E. pellita* produced in vitro holds substantial significance for the fields of agriculture and environmental research. The results of the current research indicate that these NPs, presumably produced using eco-friendly techniques, can impact the growth and development of *E. pellita* plants. Gaining a comprehensive understanding of their influence gives useful insights into prospective uses for augmenting agricultural productivity or mitigating the effects of in vitro culture contamination. Nevertheless, it also prompts worries regarding unanticipated impacts on ecosystems and the necessity for additional investigation to guarantee the sustainable and secure application of silver NPs in agricultural activities. In summary, the study highlights the significance of maintaining a balance between innovation and environmental responsibility in contemporary agricultural practices. Therefore, the first objective of the current study is to induce shoots and roots from leaf explants of *E. pellita* using cytokinin and auxin plant growth regulators (PGRs). Hence, the second objective of this study is to examine the influence of green AgNPs on the pathogen-infected in vitro *E. pellita* plant.

Materials and methods

Plant material and surface sterilization

Seeds of *E. pellita* were provided from Sabah Softwoods Berhad (Sabah, Malaysia) and green AgNPs were biologically synthesised from *Janthinobacterium svalbardensis* strain AQ5-NA17 accession number OP643518 (unpublished) with a size range (4.34 – 15.75 nm) which obtained from Eco-Remediation laboratory, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Malaysia. All in vitro propagation studies used MS medium. The composition of the nutrient media was supplemented with 3% (w/v) sucrose and 0.7% (w/v) gelrite gelling agent for medium solidification (Aggarwal et al. 2010). The pH of the medium was adjusted to 5.8 using 0.1 N NaOH/HCl. The media were sterilised by autoclaving under 121 °C and 6.79 Ibs for 15 min. Then, they were poured into sterile (90 × 15 mm) Petri dishes with ~ 10 mL media per dish.

Seed explants were germinated in MS basal media without any PGRs. Seeds were put under the running tap water for 24 h to hydrate them. Then, soaked in 70% (v/v) ethanol for 60 s and rinsed with distilled water three times. Under the laminar flow hood, seeds were immersed in 5.25% (v/v) sodium hypochlorite for 15 min and rinsed with autoclaved distilled water five times to remove residues. The surface sterilisation procedure was conducted according to Kuppusamy et al. (2019) with slight modifications. Ten seeds per petri dish were put in touch with the medium surface containing 10 ml of full-strength MS medium (Murashige and Skoog 1962).

Shoot induction from leaf explants

Cotyledonary leaves were excised from the germinated seeds and inoculated under the aseptic conditions on MS solidified media supplemented with different concentrations (0.0, 1.0, 3.0 and 5.0 µM) of BAP. Four leaf discs were placed in a single petri dish with five replicates. Petri dishes were kept under special conditions, 25 ± 1 °C at 16:8-h light: dark photoperiod provided by a white LED bulb (300–500 lumens). After four weeks of culture, the shoot cultures were subcultured onto a fresh medium and incubated under similar culture conditions. The elongated shoots were expressed as the number of responsive leaf discs and the number of regenerated shoots per explant. The shoot induction technique was carried out following Patricia et al. (2021).

Root induction of elongated shoots

Elongated shoots were cut off and cultured in ½ MS media supplemented with different IBA concentrations (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 µM), while ½ MS media without auxin was used as a control treatment, according to Bhandari et al. (2022) with minor modifications.

Bacterial strain and identification

Three bacterial isolates were subculture and activated in nutrient broth (NB) in prior to the use. 200 µL of bacterial growth were added to 10 mL of new NB and put in orbital shaker under 25 °C. After that, Optical density ($OD_{600\ nm}$) measurement were taken every hour to reach the 0.5 McFarland standard (EUCAST 2003). The bacterial isolates used in this study were isolated from the infected in vitro *Eucalyptus* plantlets. Green AgNPs were biologically synthesised from bacterial strain *Janthinobacterium svalbardensis* strain AQ5-NA17 with accession number of OP643518 with a size range of 4.34 –15.75 nm which obtained from the Eco-Remediation Technology laboratory, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Malaysia. The genomic DNA was extracted for 16 s rRNA sequencing (Ruimy et al. 1994) using NucleoSpin® Microbial DNA Kit supplied by Qiagen following the manufacturer's protocol. Then, the DNA product amplified using PCR with the following forward and reverse primers: 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3'). The PCR products for the three isolates were purified and sent to Apical Scientific Laboratories for automated sequencing. A multiple alignment method was used on 20 16 s rRNA gene sequences that closely match the selected isolate, which were retrieved from GeneBank and aligned using clustal_W

(Thompson et al. 1994). Then, the phylogenetic trees were created using The Molecular Evolutionary Genetics Analysis (Mega) software, version 11 (Kumar et al. 2018).

Antibacterial activity of green AgNPs

The antibacterial activity of green AgNPs was studied by measuring minimum inhibitory concentration (MIC), disc diffusion assay (Gopal et al. 2018), and direct exposure of green AgNPs on the in vitro regenerated *E. pellita* plantlets. Different concentrations (80, 85, 90, 95 and 100 ppm) of green AgNPs were tested to measure the MIC. Ultrapure water and 25% of Ampicillin were used as the negative and positive controls, respectively. The tubes were incubated at 37 °C for 24 h and thereafter inspected for turbidity. Following that, a loopful of broth from each microcentrifuge tube that did not demonstrate growth was inoculated into a nutrient agar plate.

The measurement of the zone of inhibition (ZOI) in this study was performed to confirm the sensitivity of bacterial isolates toward the green AgNPs. The bacterial isolate was grown on Muller-Hinton agar medium (Mahboob et al. 2019). The broth from the non-turbidity tube was streaked on the whole surface of the medium using a sterile cotton cloth. The antibacterial susceptibility of green AgNPs was performed using filter paper discs dipped in 10 µL of distilled water (negative control), 25% Ampicillin (positive control) and green AgNPs. Then, the diameter of the inhibition zone surrounding each disc was measured in mm to evaluate the susceptibility of the tested bacteria.

The direct exposure of bacteria and green AgNPs on in vitro regenerated plants was carried out by inoculating 50 µL of 0.5 McFarland standard of the activated bacteria in nutrient broth and green AgNPs near the roots according to Vicente-Hernandez et al. (2019). The number of leaves and shoot length were measured after three weeks from the application bacteria and green AgNPs.

Statistical analysis

All experiments were conducted in triplicates, and the average diameter of the ZOI was measured. The data sets were then analysed using analysis of variance (ANOVA) according to *Tukeys'* post-test by the statistical analysis system (GraphPad Prism_5.0). Means between groups were statistically compared using Tukey's test. The significance level was set up at $P < 0.05$.

Results and discussion

Shoot induction from leaf explant

The germination of *E. pellita* seeds started from day 4 of the culture and grew cotyledonary leaves after 14 days. Then, cotyledonary leaves were gently excised at the petiole base from each seedling and transferred to a new fresh medium supplemented with plant growth regulators for multiplication. The analysis of variance reveals a statistically significant difference ($P \leq 0.05$) in both shoot length and the number of leaves. The results indicated that the full strength of the MS medium was influential in the seed germination of *E. pellita* seeds. The mean number of germinated seeds varied from 7 to 8 after 14 days 14 of culture, as shown in Fig. 1. This experiment also revealed the shortcomings of relying solely on 14 days of germination since cotyledonary leaves will develop into true leaves with a slower multiplication rate after this period. In addition, investigations on plant propagation from cotyledonary explants of *Eucalyptus* have been reported for *E. globulus* (Serrano et al. 1996), *E. grandis* (Hajari et al. 2006), *E. tereticornis* (Parthiban et al. 1999), *E. urophylla* (Tibok et al. 1995), and *E. gunnii* (Hervé et al. 2001). Similarly, Afroze et al. (2021) demonstrated that a duration of four to ten days was sufficient to assess germination characteristics for the majority of 15 tested *Eucalyptus* species.

Furthermore, all the explants responsive to initiate multiple shoots in the media supplemented with different concentrations of BAP after 8 weeks of culture. The number of responded explants were in MS media supplemented with BAP (0.4, 1.3, 2.2, 3.1 and 3.9 µM) giving a mean of 4 explants per treatment, as depicted in Fig. 1. Figure 2 shows that the maximum number of shoots induced per explant were produced in MS media supplemented with

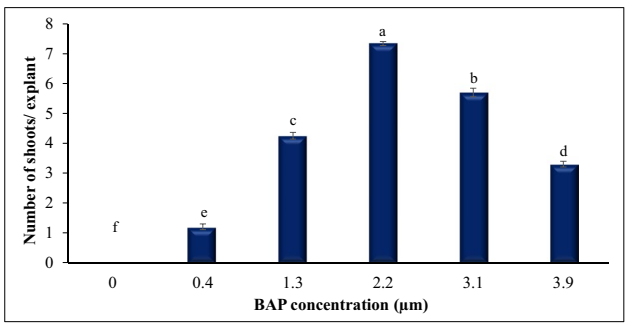

Fig. 1 Effect of different concentrations of BAP supplemented with MS medium on multiple shoot production from cotyledonary leaf explants of *E. pellita* excised from in vitro germinated seeds after eight weeks of culture. Letters above the bars indicate significant difference at ($P < 0.05$) according to *Tukeys'* post-test

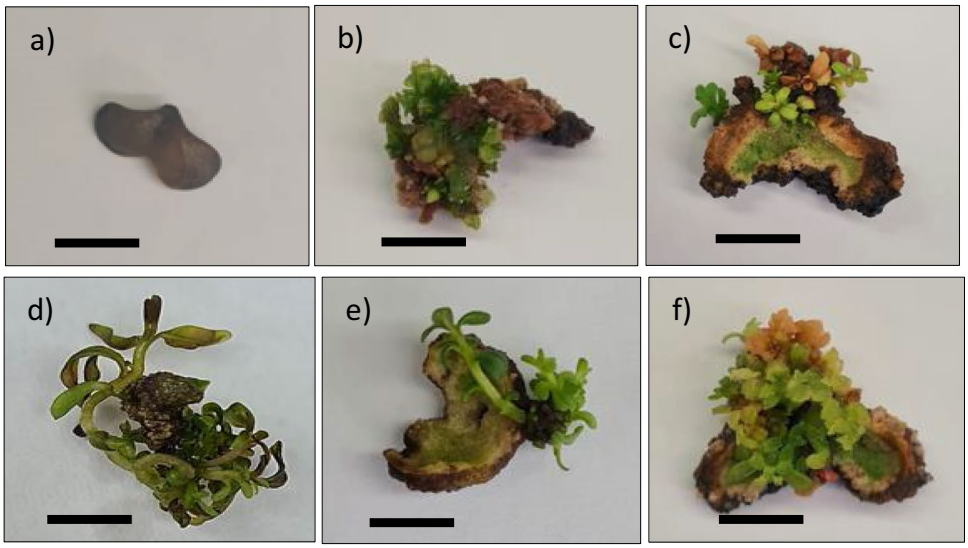

Fig. 2 In vitro effect of different concentrations of BAP (**a**) control, (**b**) 0.4 µM, (**c**) 1.3 µM, (**d**) 2.2 µM, (**e**) 3.1 µM and f) 3.9 µM on direct shoot regeneration from cotyledonary leaf explants of *E. pellita*. Bars = 1cm

2.2 µM BAP, followed by 3.1 µM BAP, giving a mean of 7.4 and 6 shoots, respectively. The multiple shoots induced per explant increased from 1.3 µM to 3.1 µM for BAP. Meanwhile, the MS media fortified with 1.3 µM, 2.2 and 3.1 µM produced highest shoots with 4.25, 7.3 and 5.7, respectively.

The cytokinin BAP stimulates axillary bud formation and cell division, while prohibiting root formation (Sutter 1996). In this study, the response to 2.2 µM BAP of cotyledonary leaf explants of *E. pellita* was considerable and fast. George et al. (2008) realised that excessive amounts of cytokinin in the culture media may harm the explant, leading to serious problems in the subsequent stages, which supports the results from 3.1 µM and 3.9 µM BAP concentrations used in this experiment. Shoots from cotyledonary nodes of *E. saligna* were successfully multiplied on MS medium containing 1.1 µM BAP (Da Silva et al. 2015). Micro-cutting of *E. benthamii x E. dunnii* was successfully induced in MS and ½ MS media containing 2.8 µM BAP and 0.3 µM NAA (Brondani et al. 2011). Bisht et al. (1999) proved that the concentration of BAP of more than 8.6 µM significantly decreased the number of proliferated buds per explant. Bennett et al. (1994) investigated that BAP concentration exceeding 2.5 µM on MS medium reduced the number of proliferated buds per explant of *E. globulus*. Similarly to the current findings, they also stated that the concentrations above 2.2 µM BAP decreased the number of regenerated buds per explant. On the contrary, it has been found that, *E. camaldulensis* cultured in MS medium without BAP showed considerably greater shoot length at 4 and 8 wees of culture (Afroze et al. 2021). Which contracdicts the current research results. This is may due to the difference a enough amount of endogenous cytokinins that might stimulate morphological growth, specifically in terms of shoot length (Afroze et al. 2021).

Root induction from elongated shoots

Micro-cuttings of 1.5 cm *E. pellita* shoots were obtained from the in vitro induced cotyledonary leaf explants and cultured on both PGR-free ½ MS media and ½ MS containing different concentrations of IBA (0.5, 1.0, 1.5, 2.0 and 2.5 µM). The analysis of variance revealed a significant difference ($P \leq 0.05$) among the treatments in terms of root length and the number of roots, as depicted in Fig. 3. It was observed that the excised shoots of *E. pellita* demonstrated the capability to elongate shoots and produce roots on both PGR-free and auxin-supplemented ½ MS nutrient medium after six weeks of culture. However, the average length of shoots and number of roots formed were significantly higher on the hormone-enriched medium. Among the different concentrations of IBA, the number of roots increased from 0.5 µM to 1.5 µM of IBA. The maximum number of roots with an average of 17.47 roots per explant was produced on

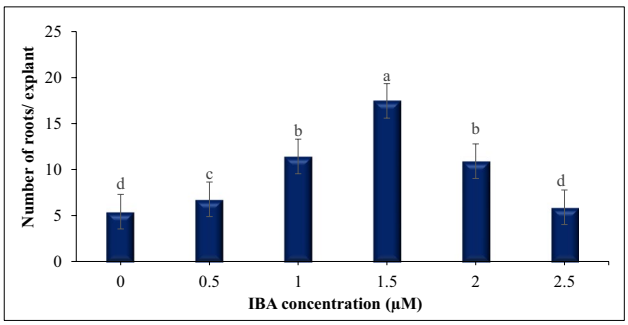

Fig. 3 In vitro effect of different concentrations of IBA supplemented with ½ MS medium on the number of roots induced from in vitro regenerated *E. pellita* shoots after six weeks of culture. Letters above the bars indicate significant difference at ($P < 0.05$) according to *Tukeys'* post-test

½ MS media supplemented with 1.5 µM IBA on week 6 of culture. However, the average number of roots declined to 11.4 and 10.9 roots at 1.0 µM and 2.0 IBA, respectively, as depicted in Fig. 3. The roots formed on the media supplemented with IBA were longer and denser as shown in Fig. 4.

The emergence of adventitious roots is a developmental stage in which new roots arise from leaves, stems or non-pericycle tissues in roots (Velada et al. 2020). Adventitious roots may be occurred naturally or under environmental stress conditions (Steffens and Rasmussen 2016). Moreover, they may be induced using a mechanical injury or, after the shoot, regeneration using the tissue culture technique (Guan et al. 2015; Porfirio et al. 2016). In this study, the root induction of *E. pellita* micro-cuttings was conducted using different concentrations of IBA.

Auxins play a key role in controlling adventitious root formation (Pacurar et al. 2014; Pop et al. 2011) and, therefore, are frequently employed as rooting inducers. Several studies have been done to investigate the role of auxins, for example, in the control of zygotic embryo development (Wolters et al. 2011), tropisms (Kimura and Kagawa 2006; Palme et al. 2006), growth of leaves and flowers (Guenot et al. 2012; Bainbridge et al. 2008), nodulation (van Noorden et al. 2007), development of shoots and roots (Lewis et al. 2011; Overvoorde et al. 2010; Dubrovsky et al. 2008), plant tolerance to stress (Min et al. 2014; Van Ha et al. 2013), as well as apical dominancy regulation (Prusinkiewicz et al. 2009; Leyser 2005). The results of this experiment showed that low concentrations of ½ MS + 1.5 µM IBA were efficient in stimulating the root formation of *E. pellita* plantlets. Microcuttings of *E. camaldulensis* were also successfully rooted in ½ MS media culture (Ho et al. 1998). Similar findings were confirmed by Ito et al. (1996) for micro cutting of *E. camaldulensis*, *E. grandis*, *E. botryoides* and *E. deglupta* using B5 medium (Gamborg et al. 1968). Nevertheless, contradictory results were reported by Barrueto Cid et al. (1999). According to Das and Mitra (1990), the best rooting media of *Eucalyptus tereticornis* axillary buds can be achieved in Knop's medium supplemented with 5.5 µM IBA. A recent study by de Oliveira et al. (2017) found that the maximum rooting percentage (35%) of *E. grandis* × *E. urophylla* AEC 224 clone was recorded on ½ MS medium + 2.46 µM IBA. Based on this experiment's results, it is noteworthy that rooting culture medium formulation should be adjusted based on the studied species.

Bacterial strain and identification

The morphological colony properties of the three bacterial isolates (EU_UPM1, EU_UPM2, and EU_UPM3) including colour, appearance and texture were recorded on agar plates and summarised in Table 1. The colony of isolate EU_UPM1 displayed creamy coloured, frosted glass appearance and dull texture. Furthermore, colonies of isolate EU_UPM2 and isolate EU_UPM3 exhibited the same yellow colour, translocated appearance and smooth

Table 1 Morphological characteristics of bacterial isolates (EU_UPM1, EU_UPM2 and EU_UPM3) on agar plates

Colony Morphology on NA			
Bacterial isolate	Colour	Appearance	Texture
EU_UPM1	Creamy	frosted glass appearance	Dull
EU_UPM2	Yellow	translocated appearance	Smooth
EU_UPM3	Yellow	translocated appearance	Smooth

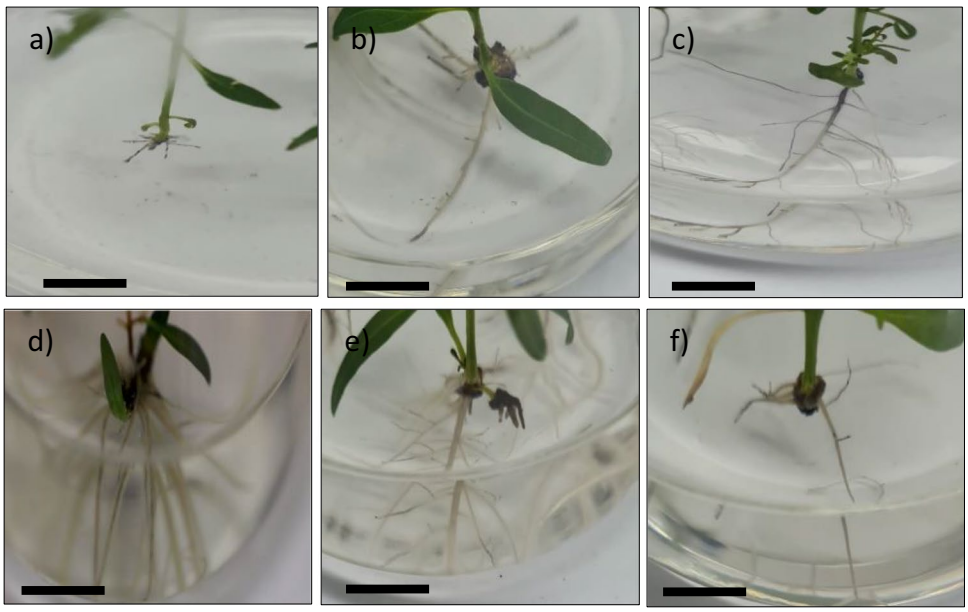

Fig. 4 Morphological appearance of *E. pellita* roots after six weeks of regenerated shoots culture in ½ ms medium supplemented with different concentrations of IBA (**a**) control, (**b**) 0.5 µm, (**c**) 1.0 µm, (**d**) 1.5 µm, (**e**) 2.0 µm and 2.5 µm). bars = 1cm

Table 2 Characteristic of bacterial isolates (EU_UPM1, EU_UPM2 and EU_UPM3)

Bacterial isolate	Gram	Shape	Catalase test	Oxidase test
EU_UPM1	Positive	rod-shaped and spore-forming	Positive	Negative
EU_UPM2	Negative	rod-shaped	Positive	Negative
EU_UPM3	Negative	rod-shaped	Positive	Negative

texture. Table 2 shows the morphological characteristics of the three bacterial isolates as seen under the light microscope. Isolate EU_UPM1 demonstrated Gram-positive, rod-shaped and spore-forming bacterium. Meanwhile, isolates EU_UPM2 and EU_UPM3 displayed Gram-negative, rod-shaped bacterium.

Genomic DNA extraction of the isolates EU_UPM1, EU_UPM2 and EU_UPM3 was successfully done using NucleoSpin® Microbial DNA Kit. The genomic DNA of the three bacterial isolates were tested using agarose gel electrophoresis as shown in Fig. 5. The high intensity of the genomic DNA band indicated the high concentration of DNA samples. Marker lane indicated the DNA ladder VC 1 kb marker used to determine the DNA size. The sizes of all genomic DNA were exhibited approximately 10,000 bp.

Based on genomic DNA results, the 16S rRNA gene was amplified using PCR with the forward 27F (5'-AGA GTTTGATCATGGCTCAG-3') and reverse 1492R (5'TAC GGTTACCTTGTTACGACTT-3') 16S rDNA primers. The 16S rRNA gene amplification of the three bacterial isolates gave rise to a fragment with a predicted size of nearly 1000 kb as confirmed in agarose gel electrophoresis.

The forward and reverse sequences of isolates EU_UPM1, EU_UPM2 and EU_UPM3 were provided from the sequencing results. The PCR products for the three isolates were purified and sent to Apical Scientific Laboratories for automated sequencing. Results from the combined 16S rRNA gene sequence were aligned using NCBI official website. The bases were compared with 20 closely related taxa of the three isolates obtained from the database of GenBank. The analysis above displayed that isolate EU_UPM1 was 94% closely related to *Bacillus subtilis* and *Bacillus cereus*. Furthermore, Isolates EU_UPM2 and EU_UPM3 were 88% closely related to *Pantoea dispersa*, with a maximum alignment of 99%. Figure 6 depicts a neighbour-joining phylogenetic tree according to the alignment of gene sequences with 20 closely related taxa to the three isolates.

Antibacterial activity of green AgNPs

Measurement of minimum inhibitory concentration

AgNPs are revolutionising the field of science through their antimicrobials. AgNPs have a broad spectrum of antimicrobial activity and can kill bacteria (De Silva et al. 2020). The minimum inhibitory concentration (MIC) of green AgNPs on EU_UPM1, EU_UPM2 and EU_UPM3 strains was examined in this study. Turbidity of bacterial cultures was detected after 24 h of incubation under aerobic conditions at 37 °C in the test tubes of 20, 40, 80 and 100 ppm containing AgNPs confirming the bacterial growth. Whereas no turbidity was detected at 100 ppm concentration, indicating the inhibition of bacterial growth. After that, the range

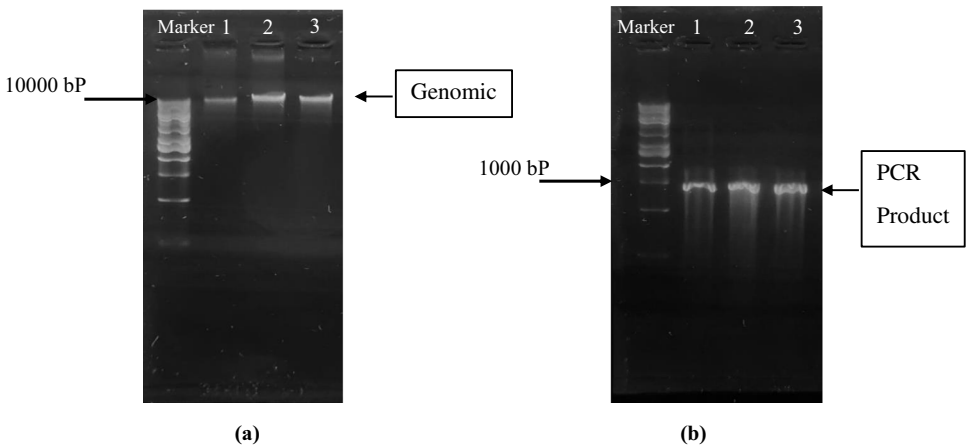

Fig. 5 Agarose gel electrophoresis. **a** Genomic DNA extraction of isolates EU_UPM1, EU_UPM2 and EU_UPM3 is indicated by the arrow. Marker lane: VC 1kb ladder marker in bp. Lane 1: indicates the isolate EU_UPM1, lane 2 indicates the isolate EU_UPM2, and lane 3 indicates the isolate EU_UPM3 (**b**) PCR product of 16SrRNA gene of the three isolates. Marker: VC 1kb ladder marker in bp; (EU_UPM1 lane: 1000 bp, EU_UPM2 lane:1000 bp, and EU_UPM3 lane:1000 bp), indicated by arrow

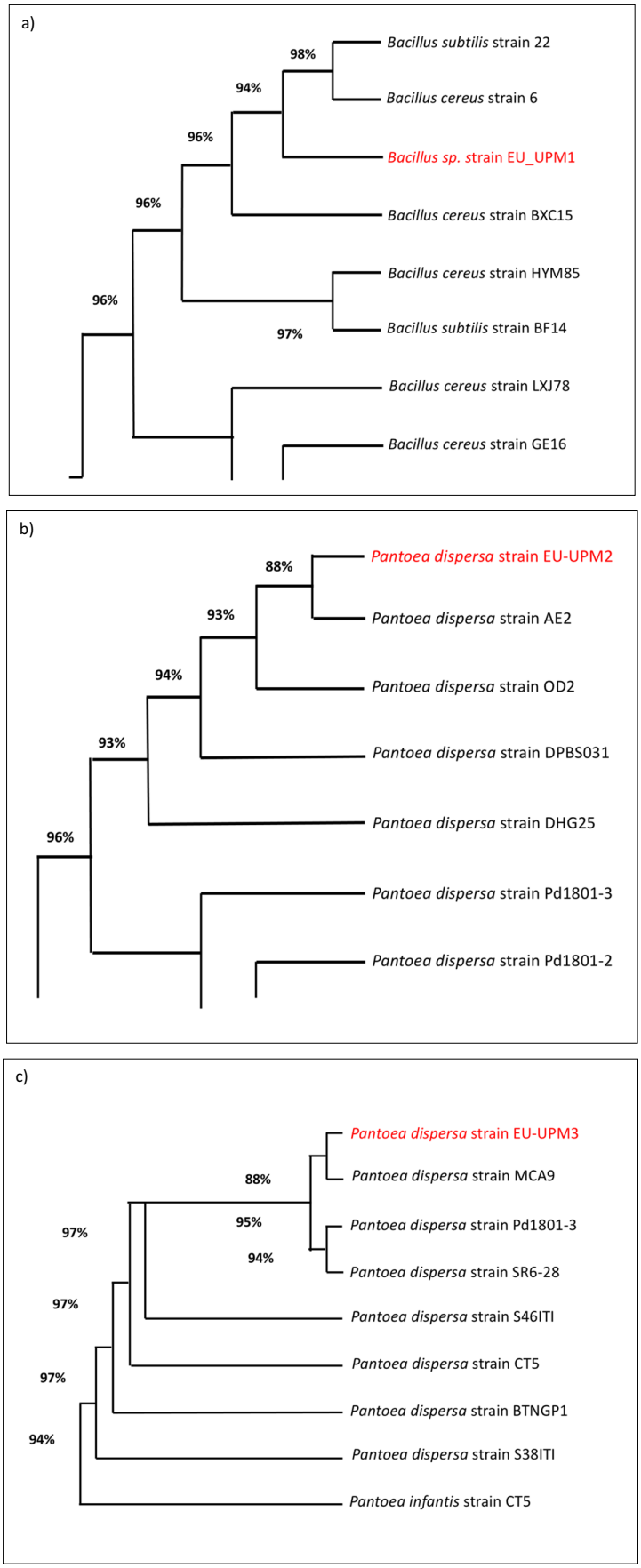

Fig. 6 Phylogenetic tree analysis based on 16s rRNA sequences. **a** strain EU_UPM1; *Escherichia coli* used as out goup, **b** strain EU_UPM2, and **c** strain EU_UPM3; *Bacillus infantis* used as outgroup for both strains.The test neighbour-joining trees were constructed using MEGA X Molecular Evolutionary Genetics Analysis software

of concentrations was narrowed to determine the specific MIC, which included 80, 85, 90 and 100 ppm. The bacterial suspension from the tube of 100 ppm was streaked on a Muller-Hinton agar plate and incubated for 24 h to confirm the absence of bacterial growth. Nevertheless, no growth of bacteria was observed in all the plates, proving 100 ppm as an effective concentration of green AgNPs to inhibit the three bacterial growths as shown in Table 3.

The antimicrobial activities of silver are mainly traced to silver ions (Chen and Schluesener 2008; Matsumura et al. 2003). AgNPs regularly produce silver ions in an aqueous microenvironment (Lok et al. 2007). Owing to their larger surface area, AgNPs demonstrate a stronger and higher bactericidal effect (Doty et al. 2005). The main reason for the effective bactericidal activities of AgNPs lies behind their ability to bind and interfere with the bacterial cellular structure (Makkar et al. 2014), considerably to their SH-groups (Zhou et al. 2012; Morones et al. 2005). In addition, AgNPs release reactive oxygen species and free radicals, which are known to cause damage to the bacterial cell wall and hinder the activity of respiratory enzymes (Loo et al. 2018). AgNPs interrupt DNA replication and inhibit the bacteria. AgNPs have a biocidal activity against several Gram-positive and Gram-negative bacteria (Prabhu and Poulose 2012).

A study found that the MIC of green AgNPs was in a concentration of 0.5 ppm against *Bacillus subtilis* (Raji et al. 2019). Another study by Ahmad et al. (2017) reported the antibacterial effect of green AgNPs against *Bacillus subtilis* at MIC 12.50 ppm. Chavan and Nadanathangam (2019) revealed that the MIC of green AgNPs against *Bacillus subtilis* and *Pantoea dispersa* were 3 and 15 ppm, respectively. This variance might be attributed to the methodology used to synthesise AgNPs.

Measurement of zone of inhibition

In this study, the green synthesised AgNPs were tested to determine their antimicrobial activity by disc diffusion method against plant pathogenic strains (EU_UPM1, EU_UPM2 and EU_UPM3). The pure cultures of bacterial organisms were sub-cultured on NB at 30 °C on an incubator shaker at 150 rpm. A concentration of 100 ppm of green AgNPs was the strongest concentration that exhibited the strongest antibacterial concentration against all tested bacterial strains. At the same time, distilled water and the antibiotic ampicillin were used as negative and positive controls, respectively. The antibacterial effect was analysed by measuring the ZOI as displayed in Fig. 7.

Based on the results of this experiment, the activity of antibiotic ampicillin was maximum for the EU_UPM2 strain (8.8 mm), followed by the EU_UPM3 strain (7.6 mm) and H4 strain 22 (7.1 mm). The green AgNPs showed antibacterial activity against all bacterial strains. Table 4 presents the ZOI of AgNPs ranging from 8.9 mm to 12.1 mm. The ZOI of AgNPs against strain EU_UPM3 was 12.1 mm, which was shown with the most susceptibility to the green AgNPs compared to the other bacterial strains. Meanwhile, strains EU_UPM1 and EU_UPM2 exhibited 8.9 mm and 11.5 mm ZOI, respectively. The best indication of the susceptibility of the tested bacterial strains to green AgNPs may be related to the plasmolysis or the detachment of cytoplasm from their outer membrane (Song et al. 2006). The antibacterial actions of green AgNPs may vary depending on the bacterial species and the size of the NPs (Nanda and Saravanan 2009).

Table 3 MIC, turbidity for various concentrations of AgNPs after 24 h

Bacterial strain / Concentration of AgNPs	20 ppm	40 ppm	60 ppm	80 ppm	85 ppm	90 ppm	95 ppm	100 ppm
EU_UPM1								
rep 1	+	+	+	+	+	+	+	-
rep 2	+	+	+	+	+	+	+	-
rep3	+	+	+	+	+	+	+	-
EU_UPM2								
rep 1	+	+	+	+	+	+	+	-
rep 2	+	+	+	+	+	+	+	-
rep3	+	+	+	+	+	+	+	-
EU_UPM3								
rep 1	+	+	+	+	+	+	+	-
rep 2	+	+	+	+	+	+	+	-
rep3	+	+	+	+	+	+	+	-

*Positive (+): Confirming bacterial growth; Negative (–): Confirming absence of bacterial growth. Replicate represented a (rep)

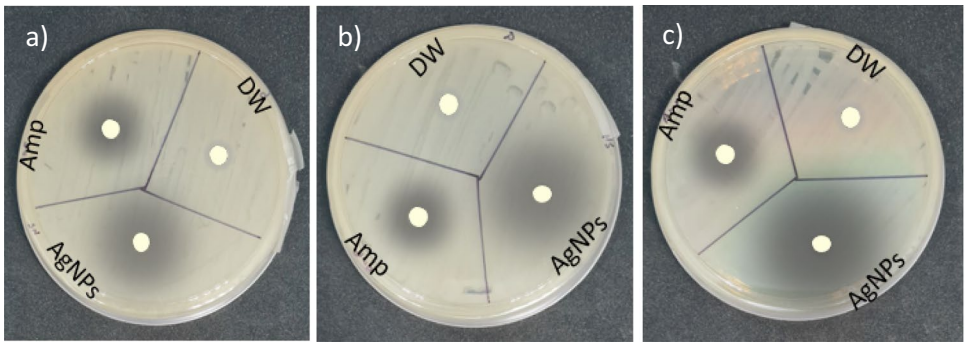

Fig. 7 Antibacterial activity of green AgNPs against three plant pathogenic bacterial strains. **a** strain EU_UPM1, **b** strain EU_UPM2 and **c** strain EU_UPM3 indicated by the disc diffusion method

Table 4 ZOI of green AgNPs against various plant pathogenic bacteria

ZOI diameter (mm)			
Bacterial strain	Distilled water	Ampicillin 25%	Green AgNPs
EU_UPM1	0	7.1	8.9
EU_UPM2	0	8.8	11.5
EU_UPM3	0	7.6	12.1

Effect of in vitro inoculation of green AgNP on *E. pellita* plantlets

The application of engineered NPs has emerged owing to their great advantages in many fields, including agriculture. AgNPs are already employed to enhance plant growth, seed germination and control of plant diseases. This study represents the influence of green AgNPs on the in vitro bacterial-infected *E. pellita* plantlets. By applying green AgNPs, *E. pellita* plantlets could enhance their antibacterial tolerance, thus extending their survivability (Fig. 8). This mechanism also supports plant growth and development. Figure 9 shows the mean number of leaves before and after applying green AgNPs. The mean number of leaves after applying the bacterial culture of strains EU_UPM1, EU_UPM2 and EU_UPM3 were 9.6, 10.6 and 8.8 leaves, respectively. Meanwhile, the mean number of leaves were 13.6 for control (without green AgNPs). After applying bacterial culture and green AgNPs, the mean number of leaves increased to reach 16.4 for strain EU_UPM1 + AgNPs, 20.8 for strain EU_UPM2 + AgNPs and 17.2 for strain EU_UPM3 + AgNPs.

The mean of shoot length of *E. pellita* plants was also recorded before and after applying green AgNPs. Figure 10 depicts that the mean length of shoots after applying the bacterial culture of strains EU_UPM1, EU_UPM2 and

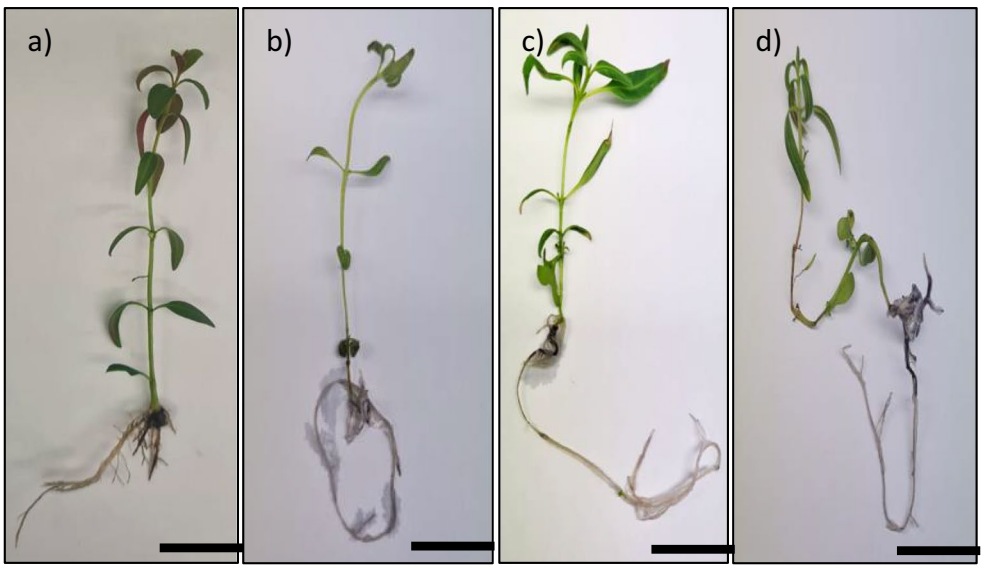

Fig. 8 Morphological alterations appearance of symptomatic effects resulted after eight days from the inoculation of bacterial growth of three plant pathogen strains. **a** *E. pellita* plantlet without bacterial inoculation shows no symptomatic effects, **b** burned leaves edges resulted from the inoculation of strain EU_UPM1, **c** yellow of plantlet stem due to the effect of strain EU_UPM2, and d) stem canker lesions appear due to the effect of strain EU_UPM3. Bars = 1 cm

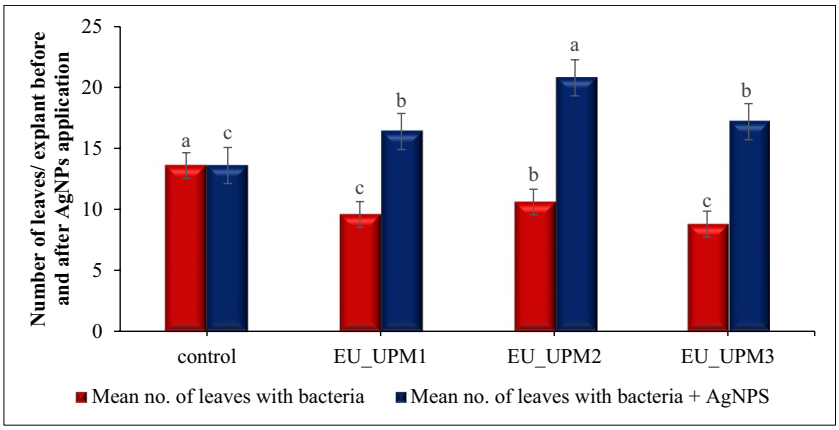

Fig. 9 In vitro effect of 50 µL bacterial inoculation without 100 ppm green AgNPs and with green AgNPs on the number of leaves of regenerated *E. pellita* plantlets. Letters above the bars indicate significant difference at ($P < 0.05$) according to *Tukeys'* post-test

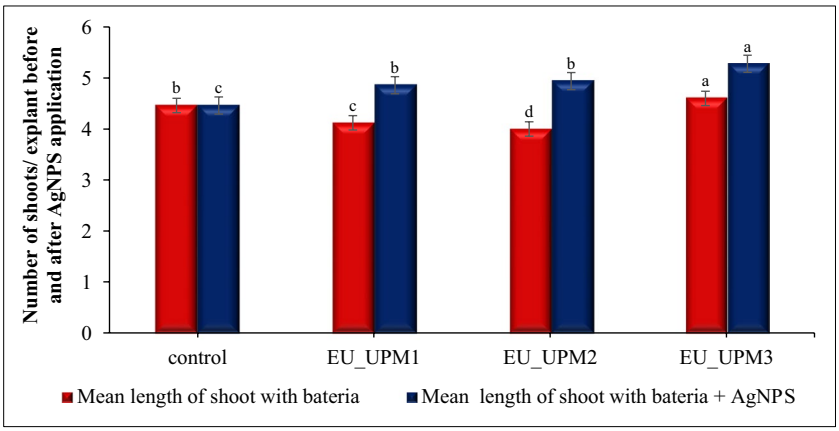

Fig. 10 In vitro effect of bacterial inoculation without 100 ppm green AgNPs and with green AgNPs on the shoot length of regenerated *E. pellita* plantlets. Letters above the bars indicate significant difference at ($P < 0.05$) according to *Tukeys'* post-test

EU_UPM3 were 4.12 cm, 4.0 cm, and 4.6 cm, respectively. Further, the mean length of shoot in the control treatment was 4.46 cm. After applying bacterial culture and green AgNPs, the mean length of shoots increased to reach 4.86 cm for strain EU_UPM1 + AgNPs, 4.94 cm for strain EU_UPM2 + AgNPs and 5.28 for strain EU_UPM3 + AgNPs.

Figure 11 demonstrates no morphological effects after 3-weeks of inoculation of three pathogenic bacterial strains and green AgNPs. Compared to the control treatment (without bacteria or green AgNPs), other treatments showed healthy and developed plants with longer shoots and a greater number of leaves.

Research on the bactericidal effect of NPs is highly desirable in light of the alarming rise in the number of resistant strains of bacteria to strong antibiotics (Morones et al. 2005). Silver has long been recognised to exhibit high toxicity towards many types of microorganisms (Liau et al. 1997), which attracted considerable attention to the well-known function of silver ions and silver-based compounds, namely AgNPs. Nevertheless, the bactericidal mechanism of AgNPs is still partially understood. It has been hypothesised that silver ions powerfully interact with thiol groups of specific enzymes and block them (Matsumura et al. 2003). Experimental studies suggested that the DNA of treated bacteria with silver ions is incapable of replicating (Feng et al. 2000).

Other researchers found morphological changes in the cell membrane and emerging tiny aggregates of silver and sulphur (Yuan et al. 2013). Although silver ions are beneficial and successful in bactericidal applications, nanotechnology offers a feasible alternative for emerging novel bactericides owing to of its distinctive characteristics (Morones et al. 2005). Metallic nanosized particles possess distinct physical characteristics dissimilar from their ions and bulk materials (Alimunnisa et al. 2017), thus giving them remarkable properties including high catalytic activity from their structure with highly active features (Feng et al. 2015).

Conclusion

The current investigation demonstrates the effect of green AgNPs on the pathogen-infected *E. pellita* plantlets. *E. pellita* was regenerated by in vitro culture of cotyledonary leaf explants excised from the aseptically germinated seeds. The explants were able to mass produce shoots in

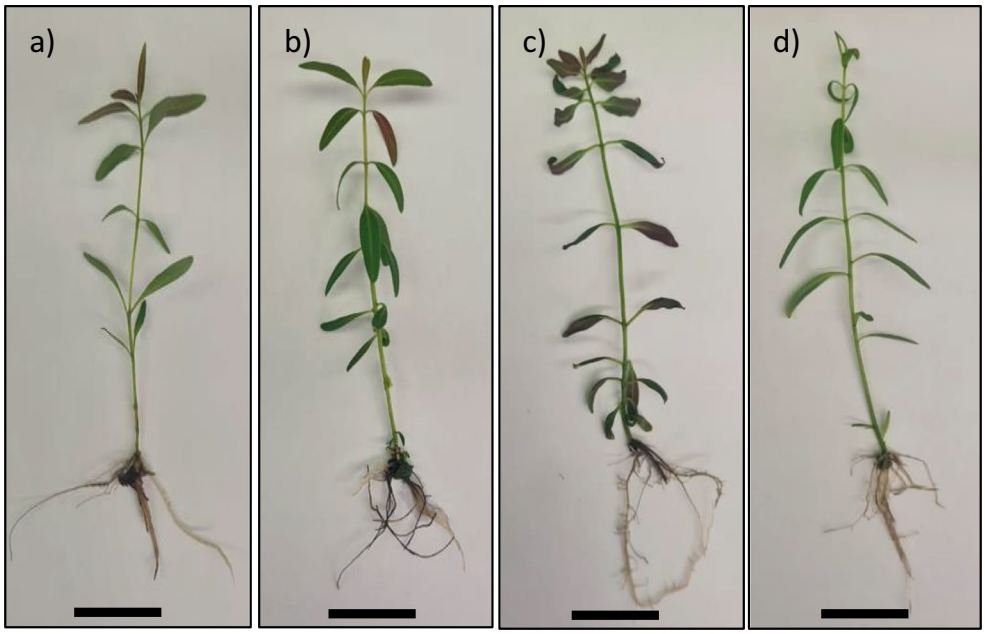

Fig. 11 Morphological appearance of *E. pellita* plantlets. The morphological alterations observed after 3 weeks of inoculation of 50 µL bacteria + 100 ppm green AgNPs (**a**) *E. pellita* plantlet without any inoculation, (**b**) *E. pellita* plantlet with the inoculation of 50 µL EU_UPM1 strain + 100 ppm green AgNPs, (**c**) *E. pellita* plantlet with the inoculation of 50 µL EU_UPM2 strain + 100 ppm green AgNPs, and d) *E. pellita* plantlet with the inoculation of 50 µL EU_UPM3 strain + 100 ppm green. Bars = 1 cm

the MS medium supplemented with 2.2 µM BAP growth regulator after four weeks of culture. The regenerated shoots successfully generated roots in the ½ MS medium supplemented with 1.5 µM IBA. Inoculation of 50 µL 0.5 (McFarland standard) of bacterial suspension near the roots of the plantlets, was an effective amount to show morphological alterations under the in vitro conditions. The effective concentration of green AgNPs was 100 ppm, controlling bacterial growth. Thereby, the findings of the current investigation indicate that the green AgNPs controlled the bacterial infection of *E. pellita* plantlets under in vitro conditions. The new protocol developed in this study could be adopted in future for rapid micropropagation of free pathogen-infected *Eucalyptus* species.

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Author contributions H.S. investigation analyzed data and wrote the paper; S.A.A. conceptualization, methodology, designed research, supervision and project administration; N.A.S. conceptualization, methodology, designed research, supervision and project administration; R.S. supervision and project administration; A.A. methodology and supervision; A.N. methodology; C.D.S. methodology; R.A. methodology; all authors participated in revising the paper.

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Data availability The data supporting the findings are available from the corresponding author upon request.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the contents of this article.

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