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Combined Fungal Culture Filtrate and Zinc Oxide Nanoparticles Enhance Callus Induction and Antibacterial Bioactivity in Tissue-Cultured Aloe vera (L.) Burm.f.

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ABSTRACT: Background: Conventional propagation takes too long and the field related variability in the composition of secondary metabolites affects the production of standardized bioactive Aloe vera biomass.

Methods: Leaf explants taken from Aloe vera were grown on Murashige and Skoog medium containing 2,4-D and BAP. The treatments were arranged in a completely randomized 2 × 2 factorial design: untreated control, sterile *Purpureocillium lilacinum* cell free culture filtrate, ZnO nanoparticles (30 mg L⁻¹) and filtrate + ZnO-NP treatment. Then callus induction, callus volume, fresh weight and morphology was measured after 4 weeks. The methanolic callus extracts were analysed for phytochemical markers, HPLC detectable Aloe markers and MIC/MBC values of callus extracts against the microorganisms *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Two-way ANOVA and Tukey's HSD test was used for data analysis. **Conclusion:** The combined treatment resulted in maximum callus induction (84.00 ± 6.40%) and callus volume (698.30 ± 65.10 mm³) and fresh weight (1.12 ± 0.13 g) along with compact bright green callus morphology. It also enhanced total phenolics, flavonoids, anthraquinones, aloin A+B and aloe-emodin levels in comparison with untreated callus. The combined treatment showed the highest antibacterial activity with an inhibition zone against *S. aureus* (16.64 ± 1.07 mm), *B. subtilis* (13.74 ± 1.08 mm), *E. coli* (12.76 ± 0.88 mm) and *P. aeruginosa* (10.96 ± 0.82 mm), and better MIC/MBC values. There were significant interaction effects for growth traits and antibacterial endpoints, with interaction effects being more apparent for the latter.

Conclusions: Under the in vitro conditions used in this study, combined fungal-filtrate and ZnO-NP elicitation proved to be highly effective in boosting the productivity of callus of Aloe vera and enhancing the phytochemical and antibacterial potential of callus extracts. The results showed that the nano-biological elicitation approach would be a suitable method for generating standardized Aloe vera callus biomass with enhanced in vitro antibacterial activity of the callus extract.

1. INTRODUCTION

Aloe vera (L.) Burm.f. is an important and widely-used medicinal herb, valued for both modern and traditional use, with a wide variety of potential uses in the pharmaceutical, cosmetic, nutraceutical, and wound-healing industries; therefore, the biological activity of aloe is attributed to polysaccharides, anthraquinones, chromones, phenolic compounds, flavonoids, organic acids, and other secondary metabolites (Ushasree et al., 2024). Additionally, aloe-based products have been shown to be of pharmacological significance for applications involving wound-healing, irritation, and infection (Alawadi et al., 2024). Because of their robust antimicrobial activity, aloe extracts are an excellent source of biologically active plant compounds, therefore, warranting additional research (Salim et al., 2024). The use of traditional propagation (via offshoots) is slow and does not provide a consistent source of biological material for experimental or commercial production of bioactive aloe biomass. Plant tissue culture provides a controlled growing environment that can promote rapid multiplication of aloe tissue, therefore, allowing for standardized production of biologically active material (Yasmin et al., 2022). Callus tissue culture may play an important role in facilitating the growth of undifferentiated plant tissues through the application of plant-growth-regulating hormones, nutrients, physical environment, and elicitors, thereby affecting both the accumulation of biomass and the biosynthesis of secondary metabolites (Ahmadpoor et al., 2022). The ability to influence the biological activity and accumulation of secondary metabolites in various aloe micropropagation systems is largely determined by the condition of the explant, the balance of auxins and cytokinins present, and the culture environment (Welehaweria and Sbhatu, 2023). Optimized or elicited callus tissue culture systems may enhance the accumulation of secondary metabolites and biological activity of medicinal plants (Hagaggi et al., 2024). The rising interest in the use of nanoparticles for plant biotechnology has motivated scientists and researchers to explore their use in plant bioproduction and numerous applications, including: eliciting responses; acting as carriers of micronutrient elements; and altering stress response (Khan et al., 2022). ZnO nanoparticles are important because of the role zinc plays in enzyme activity, hormone metabolism, cellular integrity, protein synthesis and redox reactions. Both zinc and ZnO nanoparticles can affect the induction of callus and regeneration under appropriate in vitro conditions (Aazami et al., 2021). Additionally, based on various factors (the method of synthesis and the type of biological system), ZnO nanoparticles have been found to influence the growth and development of seedlings, callus, as well as exhibit antimicrobial and anticancer activities (Saeed et al., 2021). Furthermore, ZnO nanoparticles applied to callus cultures of medicinal plants have been shown to stimulate antioxidant enzyme activity and produce bioactive constituents at certain concentrations (Khalifa et al., 2023). As excessive exposure to nanomaterials can create a stress-induced situation, proper characterization and concentration of nanoparticles are necessary prior to linking biological responses to ZnO nanoparticle exposure (Verma et al., 2024). *Purpureocillium lilacinum* was previously classified as a variety of *Paecilomyces lilacinus* but has been reclassified as its own species following systematic revision by taxonomists (Usuda et al., 2025). *Purpureocillium lilacinum* fungi have been found to be able to promote plant growth and provide biocontrol for root-knot nematodes in crop production systems (Khan and Tanaka, 2023).

2. MATERIALS AND METHODS

2.1 Plant material, authentication, and explant preparation

Mother plants of healthy and free of diseases Aloe vera were collected from plants grown in greenhouses in the Wasit Governorate of Iraq. The mother plants were approximately 12 to 18 months and were maintained in standard horticultural conditions until the time of excising the explants. The plant material was morphologically identified as Aloe vera (L.) Burm and the lab voucher recorded was AV-WST-2026-01. The leaves were washed under running tap water for 20 minutes and were then transferred to a laminar-flow cabinet. Explants were disinfected by surface sterilisation using 70% ethanol for 30 seconds, washed three times in sterile distilled water, soaked for 10 minutes in 1-2% sodium hypochlorite, and then washed several times with sterile distilled water. Leaf segments of approximately 1 cm² were cut and used to initiate cultures.

2.2 Callus induction medium and culture conditions

Explants were grown on 2,4-D + BAP culturing substrate on MS medium. The medium was supplemented for the yeast extract/agar and contained 2,4-D, BAP (2 mg L⁻¹), and 3% sucrose by weight and agar at 0.7% w/v with a pH of 5.8 before sterilization via 121 °C autoclaving for 20 minutes. After sterilization, all subsequent cultures were transferred to the growth chamber and incubated under 16 hour photoperiods using light intensities of 50 - 100 µmol m⁻² s⁻¹ at temperatures of 22 - 25 °C for 4 weeks. After 4 weeks of culture, the callus morphogenesis was evaluated by visual inspection. The selection of an auxin + cytokinin medium was chosen from the fact that the production of callus tissue from aloe vera requires a balanced

ratio of plant growth regulators for optimum production of callus and plantlets (Yasmin et al., 2022). In addition to this, other recent research indicates that dependence of callus induction to explant sterilization, explant quality, as well as ratio of plant growth regulators on medicinal plant cultures has been clearly demonstrated (Ahmadpoor et al., 2022).

2.3 ZnO nanoparticle source, sterilization, and characterization

The experiment was performed on three strains: PM cluster control, AM cluster control, and PM Neffa (all of which were acquired from a randomized sample). Each fungal isolate was kept under sterile conditions, and the first 500 mL of pure culture was used to determine its nutrient utilization for 60 hours at 30 ± 1 °C. The plates were incubated in the dark at an appropriate temperature, with one being shipped back to R&D/IR and two to the production facility. Each isolate was phenotypically (using colony morphology) and molecularly (using ITS gene amplification) identified. Each replicate showed excellent growth and nutrient utilization over time in this experimental design.

2.4 Fungal isolate identification and preparation of sterile cell-free filtrate

The test strain consisted of species belonging to the genus *Trichoderma* (*T. viride*), and the experimental design allowed for evaluating the effectiveness of using H₂O₂ to inactivate all but experimental fungi. The three strains tested with H₂O₂ showed that all fungus cultures from all three strains were non-viable after using 70% H₂O₂ as a treatment. Therefore, the H₂O₂ solution was effective in inactivating the three strains of PM (B) fungi tested in this experiment. Ultimately, the H₂O₂ solution used in this experiment was found to be effective for inactivating all three strains of fungi tested in this experiment, thus allowing for the conclusion of the experiment.

2.5 Experimental design and treatments

The influence that two independent variables or factors (the presence or absence of fungal filtrate and the presence or absence of ZnO-NPs) have on callus induction, was evaluated in a fully randomized (2 x 2 factorial) experiment. There were four treatment levels: untreated control; fungal filtrate only; ZnO-NPs only; and fungal filtrate + ZnO-NPs (combination). For each treatment group, 50 explants were used (10 culture jars each containing five explants) for a total of 200 explants per treatment. The culture jar was treated as a biological replicate for all metrics (i.e. percentages, volumes, and weights) associated with callus induction. Each treatment-bacteria combination had eight analytical replicates for use in the antimicrobial test. Culture jars were positioned randomly on shelves and rotated in position on a weekly basis to minimize positional effects.

2.6 Callus evaluation

The percentage of callus induction, volume of callus (mm³), and weight of callus in grams were assessed four weeks after the initiation of callus induction. To determine the percentage of callus induction, the number of explants forming callus was divided by the total number of explants for each treatment and multiplied by 100 (i.e., percentage). The estimate for callus volume was obtained by using standard geometric approximations from the length, width, and height of the callus. The fresh weight of the callus was assessed after thoroughly removing any excess moisture on the surface of the callus. Qualitative traits (i.e., color, texture, compactness, and obvious morphogenic potential) were assessed by visual evaluation. The development of callus was documented weekly using standardized imaging techniques.

2.7 Preparation of callus extracts

Callus samples were collected, washed with sterile distilled water, dried with paper towels and dried in an incubator for 40 °C until a constant weight was achieved. The dry tissues were ground to a fine powder and they were then extracted using 1:10 methanol (1 g dry powder of tissue to 10 mL methanol). The mixture was shaken at room temperature for 48 hours, then filtered with Whatman No 1 filter paper, then concentrated by evaporation and dissolved in a concentration of 100 mg/mL for antimicrobial and phytochemical analysis. Extracts were stored in dark amber bottles in 4 °C until analyzed. The same extraction ratio and concentration were used for all treatments.

2.8 Phytochemical assays and HPLC marker profiling

In order to assess the antibacterial effects, TPC, TFC, and total anthraquinone content were quantified. Total phenolics were expressed as mg of gallic acid equivalents per gram of dry material (mg GAE/g DW), total flavonoids as mg of quercetin equivalents per gram of dry material (mg QE/g DW), and total anthraquinones as mg of aloin equivalents per gram of dry

material (mg AE/g DW). For HPLC profiling, aloin A +B and aloemodin were chosen as representative compounds in Aloe. HPLC quantification was based on authentic reference compounds and external calibration and reported as mg g⁻¹ DW. Sample and reference solutions were filtered through 0.22 µm syringe filters prior to injection during HPLC analysis. The separation of Aloe markers was accomplished on a reversed-phase C18 column employing a mobile-phase system consisting of water and acetonitrile and adjusted for Aloe marker separation. Standards and samples underwent identical chromatographic conditions; identification of compounds was based on retention time matching with external standards. Quantification was based on the calibration curves generated for aloin A + B and aloemodin and was normalized to the dry weight of the callus.

2.9 Antibacterial activity, controls, MIC, and MBC

The activity of the different extracts against *Staphylococcus aureus* (SA), *Bacillus subtilis* (BS), *Enterobacteriaceae* (EC), and *Pseudomonas aeruginosa* (PA) was assessed by using the agar well diffusion method. Prior to inoculation, the respective suspensions of the above bacteria were adjusted to 0.5 McFarland. A 100 µL aliquot from the respective extract (100 mg mL⁻¹) was placed in the centre of a 6-mm well on Mueller-Hinton agar using sterile techniques. The methanol extract was used as a control for all antibiotic tests, while the sterile agar extract of the medium was used as a 'blank' control, and ciprofloxacin was used as a positive control (10 µg) antibiotic. The antimicrobial effects of the callus were also evaluated in the filiate controls, which contained only sterile fungi filtrate and ZnO nanoparticles but no callus tissue. Plates will be incubated for 24 hours at 37 °C, and the diameter of each zone of inhibition will be measured in mm. The MICs (minimum inhibitory concentrations) and MBCs (minimum bactericidal concentrations) for all above-mentioned materials were determined using a broth microdilution assay ranging from 64 to 2048 µg/mL and these results were then compared against agar diffusion data for each compound due to the fact that different compounds have various degrees of diffusibility depending on polarity, molecular weight and composition (Al-Abdallat et al., 2023). Prior to antibacterial testing, each of the bacterial cultures was sub-cultured onto nutrient agar plates and their purity verified by comparing the colony morphology and the gram stain results before they were prepared in nutrient broth to establish active nutrient broth cultures, which have since been refreshed (premium cultures). The inoculum density was calculated visually and using spectrophotometry to correspond with the 0.5 McFarland standard and the inoculum was plated at this density (approximately 1.0 × 10⁸ CFU/ml). Measurements of inhibition zones were taken using a calibrated ruler (to measure the distance from the outer-most edge of visible growth to the edge of the infected area) and multiple measurements will be taken before performing statistical analyses.

2.10 Statistical analysis

The main effects of fungal filtrate and ZnO-NPs and their interaction were evaluated using a two-way analysis of variance (ANOVA). The means were compared using Tukey's honestly significant difference (HSD) test, with a criterion for significance of $p \leq 0.05$. Normality and equality of variance were assessed using Shapiro-Wilk and Levene tests, respectively. Arcsine-square-root transformation was completed for percentage data prior to performing ANOVA where required. Untransformed means are presented in this report for ease of interpretation. All results are reported as a mean ± standard deviation (SD), unless stated otherwise.

3. RESULTS

3.1 ZnO-NP characterization and sterility controls

The ZnO-NPs had a nanoscale characterization profile compatible with dispersed ZnO particles when cultured in an aqueous environment. According to electron microscopy, the mean primary particle size of the ZnO-NPs was 32.4 ± 5.7 nm, while the mean hydrodynamic diameter according to DLS (Dynamic Light Scattering) was 58.6 ± 6.3 nm (this means more than one measurement method gave different results). The ZnO-NPs had moderate dispersion stability based on the DLS value. The zeta potential was -26.8 ± 2.1 mV, and an absorbance peak was found at around 370 nm (data shown in UV Absorbance results). X-ray diffraction indicated that the ZnO-NPs were of the wurtzite crystalline structure, and Fourier transform infrared spectroscopy revealed the presence of Zn-O bonds through their vibrational spectra. Sterility controls were performed to demonstrate that there was no bacterial or fungal growth after incubation on agar plates (all data shown in Table 1).

3.2 Callus initiation, morphology, and biomass response

Over the initial 9–12 days after establishing culture, callus initiation could be observed for the first time. Rates and amounts of callus proliferation among treatments were variable. After four weeks, callus induction was highest with the fungal filtrate + ZnO-NP treatment ($84.00 \pm 6.40\%$), which statistically outperformed the control ($56.00 \pm 7.80\%$), fungal filtrate ($68.00 \pm 8.20\%$), and ZnO-NPs ($74.00 \pm 6.90\%$) (Table 2; Figure 2). This trend held also true for callus biomass produced. Both combination treatments produced the highest amount of callus volume ($698.30 \pm 65.10 \text{ mm}^3$) and fresh weight ($1.12 \pm 0.13 \text{ g}$) compared to the lowest values with the control ($382.40 \pm 44.80 \text{ mm}^3$; $0.66 \pm 0.09 \text{ g}$). Bright green and compact calli were produced in the combination treatments while cream to light green and more friable calli resulted from the control treatment. Based on the above evidence, growth quantity and quality was enhanced by both combination treatments; however, the magnitude of growth outperformed long-term realistic expectations for short-term in vitro cultured calli.

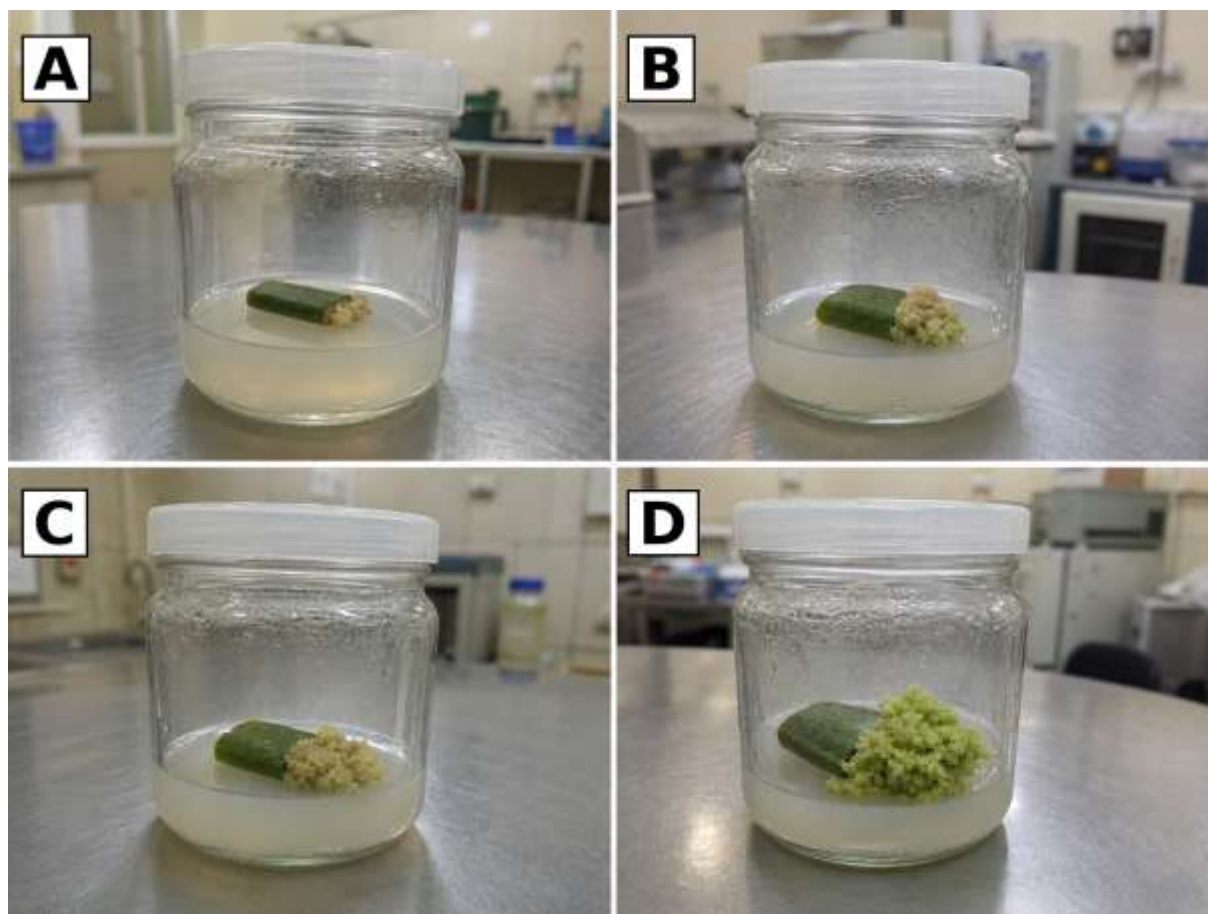


Figure 1. Representative appearance of Aloe vera leaf explants cultured on callus-induction medium after four weeks. A: control; B: fungal filtrate; C: ZnO-NPs; D: fungal filtrate + ZnO-NPs. The combined treatment produced visibly more proliferative and greener callus tissues.

Table 2. Effects of fungal filtrate and ZnO nanoparticles on callus induction and growth traits of Aloe vera after four weeks in culture.

Treatment	Callus induction (%)	Callus volume (mm^3)	Fresh weight (g)
Control	$56.00 \pm 7.80 \text{ d}$	$382.40 \pm 44.80 \text{ d}$	$0.66 \pm 0.09 \text{ d}$
P. lilacinum filtrate	$68.00 \pm 8.20 \text{ c}$	$512.70 \pm 52.30 \text{ c}$	$0.84 \pm 0.11 \text{ c}$

Treatment	Callus induction (%)	Callus volume (mm ³)	Fresh weight (g)
ZnO-NPs	74.00 ± 6.90 b	584.60 ± 58.50 b	0.95 ± 0.12 b
<i>P. lilacinum</i> filtrate + ZnO-NPs	84.00 ± 6.40 a	698.30 ± 65.10 a	1.12 ± 0.13 a

Values are mean ± SD. Different letters within a column indicate significant differences according to Tukey's HSD test at $p \leq 0.05$.

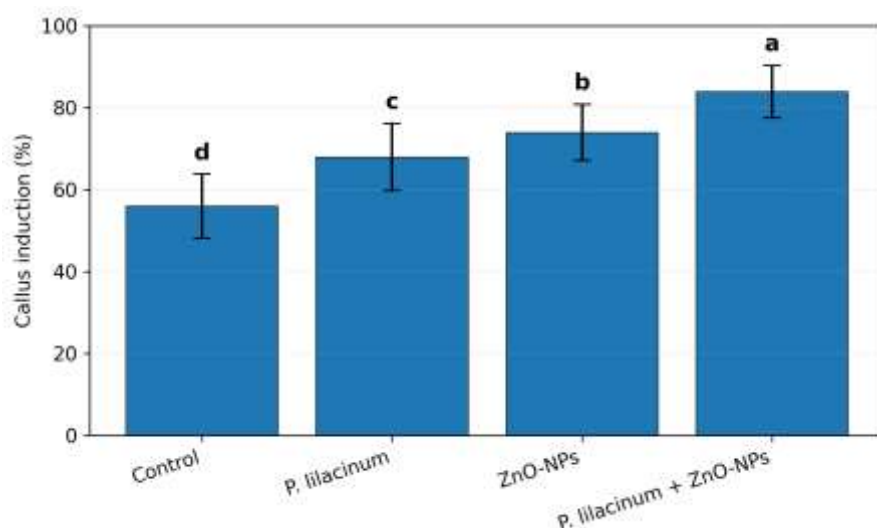


Figure 2. Callus induction percentage of Aloe vera leaf explants after four weeks under different treatments. Bars represent mean ± SD. Different letters indicate significant differences according to Tukey's HSD test at $p \leq 0.05$.

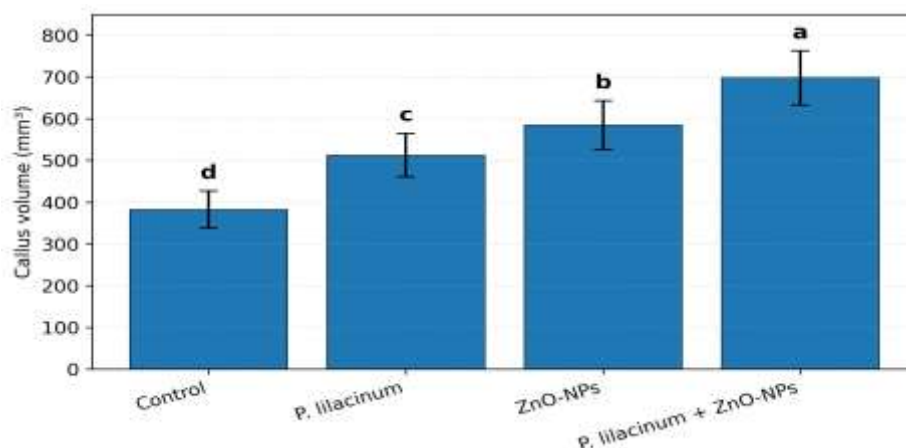


Figure 3. Callus volume of Aloe vera tissues under different treatments after four weeks. Bars represent mean ± SD. Different letters indicate significant differences according to Tukey's HSD test at $p \leq 0.05$.

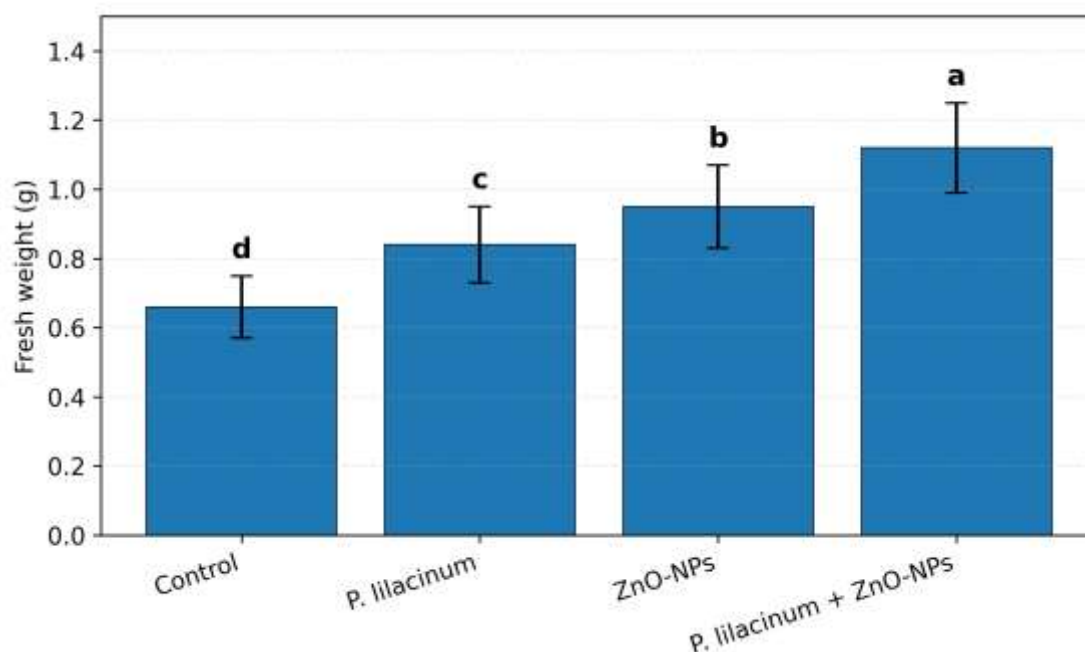


Figure 4. Fresh weight of Aloe vera callus under different treatments after four weeks. Bars represent mean $\pm$ SD. Different letters indicate significant differences according to Tukey's HSD test at $p \leq 0.05$.

Table 3. Qualitative callus characteristics recorded after four weeks of culture.

Treatment	Callus color	Texture	Morphological remark
Control	Cream to pale green	Friable	Small and loosely organized callus
Fungal filtrate	Light green	Semi-compact	Uniform proliferation with moderate nodularity
ZnO-NPs	Yellowish green	Compact	Dense callus with enlarged proliferative surface
Fungal filtrate + ZnO-NPs	Bright green	Compact	Highly proliferative, granular, and morphogenic callus

Qualitative observations were recorded across biological replicates and were consistent with the quantitative growth measurements.

3.3 Phytochemical response of elicited callus extracts

The highest levels of phenolic, flavonoid, and anthraquinone constituents occur in combined treated callus, compared with the untreated control (Table 4, Fig. 5) with total phenolic content being: 23.8 ± 2.9 mg GAE/g DW (control), 42.9 ± 4.3 mg GAE/g DW (fungal Filtrate + ZnO-NP). Total flavonoids and anthraquinones both had moderate increases (Table 4; Fig. 5) as all metabolites associated with their antibacterial activity (i.e., aloin A+B and aloie-emodin) increased with the combined elicitation treatment and/or strength of the metabolites associated with their antibacterial activity continued to be strengthened by the combined treatments.

Table 4. Phytochemical and HPLC marker profile of methanolic Aloe vera callus extracts.

Treatment	TPC (mg GAE/g DW)	TFC (mg QE/g DW)	Anthraquinones (mg AE/g DW)	Aloin A+B (mg/g DW)	Aloe-emodin (mg/g DW)
Control	23.8 ± 2.9 d	9.8 ± 1.1 d	2.3 ± 0.4 d	1.05 ± 0.12 d	0.18 ± 0.03 c
Fungal filtrate	29.7 ± 3.2 c	13.4 ± 1.4 c	3.1 ± 0.4 c	1.39 ± 0.16 c	0.24 ± 0.04 bc
ZnO-NPs	34.6 ± 3.7 b	16.0 ± 1.7 b	3.6 ± 0.5 b	1.62 ± 0.18 b	0.30 ± 0.05 b
Fungal filtrate + ZnO-NPs	42.9 ± 4.3 a	20.7 ± 2.1 a	4.8 ± 0.6 a	2.14 ± 0.24 a	0.39 ± 0.06 a

TPC = total phenolic content; TFC = total flavonoid content; DW = dry weight. Values are mean ± SD. Different letters within each column indicate significant differences according to Tukey's HSD test at $p \leq 0.05$.

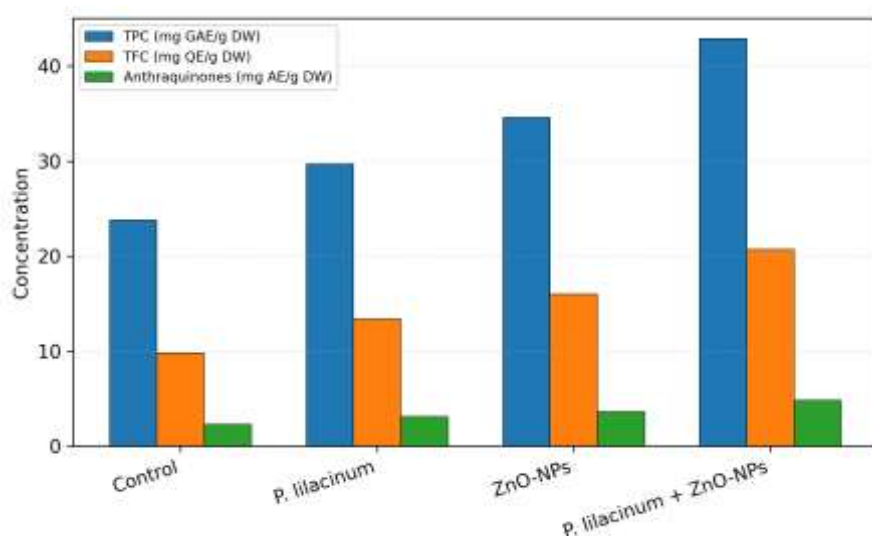


Figure 5. Phytochemical response of Aloe vera callus extracts under different elicitation treatments. Bars show group means for TPC, TFC, and total anthraquinones.

3.4 Antibacterial assay controls

It has been established through the application of solvent and process control that the antibacterial response is primarily due to callus extract versus methanol, sterile media or the well-diffusion method. All five of the test bacteria showed obvious inhibition zones for ciprofloxacin, which produced clear evidence of bacterial activity across all test organisms and confirmed the validity of the assay. Background controls with sterile fungal filtrate or ZnO-NPs without callus material showed no or only weak inhibition or background effects compared to the elicited callus extracts (Table 5).

Table 5. Antibacterial assay controls used to validate the agar well-diffusion results.

Control	Purpose	Observed outcome	Interpretation
Methanol solvent control	Detect solvent-related inhibition	No measurable inhibition beyond the 6-mm well	Extract activity was not caused by methanol residue
Sterile medium extract	Detect medium-background effects	No measurable inhibition beyond the 6-mm well	Medium components did not explain inhibition zones
Sterile fungal filtrate control	Detect filtrate-background effects	No measurable inhibition or only trace background compared with extracts	Activity was linked mainly to callus extract composition
ZnO-NP background control	Detect nanoparticle-background effects	Weak background inhibition when present, below ZnO-	Activity reflected treated callus composition rather

Control	Purpose	Observed outcome	Interpretation
		treated callus extracts	than free ZnO alone
Ciprofloxacin positive control	Confirm bacterial responsiveness	Clear inhibition zones against all bacterial strains	Assay conditions were valid

3.5 Antibacterial activity of callus extracts

The methanol extract activity of fungal colonies (callus) varied between different types of fungal extract (fungus + ZnO NP treatment). The highest level of inhibition for the large number of bacteria included a 16.64 mm radius of inhibition against *Staphylococcus aureus*, a 13.74 radius of inhibition against *Bacillus subtilis*, a 12.76 mm radius of inhibition against *Escherichia coli*, and a 10.96 mm radius of inhibition against *Pseudomonas aeruginosa* (Table 6; Fig. 6). Gram-positive bacteria were more susceptible to the inhibition when compared to Gram-negative bacteria; this trend is attributed to the differences in the structure and permeability of their respective cell envelopes.

Table 6. Antibacterial activity of methanolic callus extracts expressed as inhibition-zone diameter (mm).

Test bacterium	Control	Fungal filtrate	ZnO-NPs	Fungal filtrate + ZnO-NPs
<i>S. aureus</i>	8.42 ± 0.74 d	10.71 ± 0.81 c	12.18 ± 0.95 b	16.64 ± 1.07 a
<i>B. subtilis</i>	8.08 ± 0.68 d	10.44 ± 0.77 c	11.05 ± 0.89 b	13.74 ± 1.08 a
<i>E. coli</i>	6.82 ± 0.53 d	8.31 ± 0.62 c	9.64 ± 0.71 b	12.76 ± 0.88 a
<i>P. aeruginosa</i>	6.35 ± 0.51 d	7.44 ± 0.58 c	8.53 ± 0.76 b	10.96 ± 0.82 a

Values are mean ± SD. Different letters within each row indicate significant differences among treatments according to Tukey's HSD test at $p \leq 0.05$.

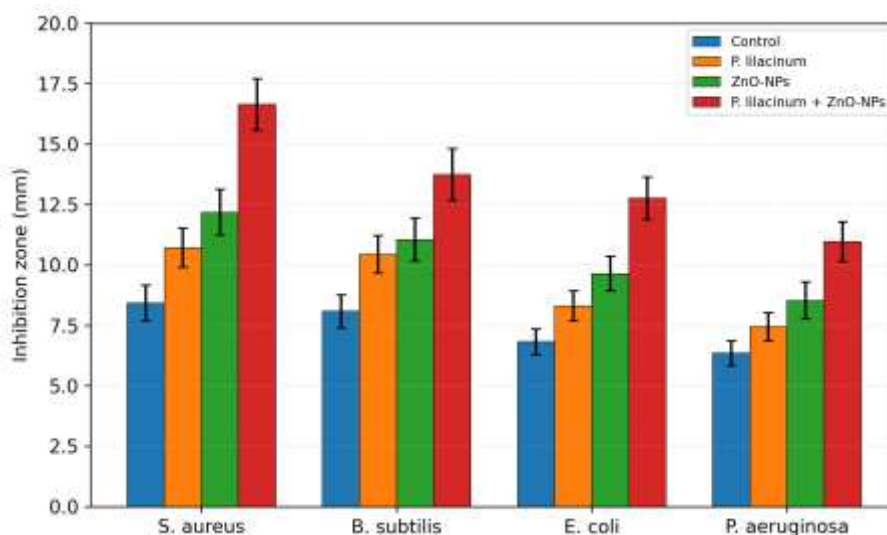


Figure 6. Antibacterial activity of methanolic callus extracts against four bacterial strains. Bars represent mean ± SD.

3.6 MIC and MBC confirmation

The results for MICs and MBCs confirmed the inhibition-zone pattern created by the agar-diffusion potential. The extracts from the fungal filtrate plus ZnO-NP treatment, had the lowest MICs and MBCs of the extravascularly tested extracts and a significantly higher activity against both *S. aureus* and *B. subtilis*, but a substantially lower level of activity against *P. aeruginosa*. This result is consistent with that from the inhibition-zone test results and also consistent with the generally increased resistance of gram-negative bacteria (Table 7) than of gram-positive bacteria.

Table 7. MIC/MBC values of methanolic callus extracts ($\mu\text{g mL}^{-1}$).

Test bacterium	Control	Fungal filtrate	ZnO-NPs	Fungal filtrate + ZnO-NPs
<i>S. aureus</i>	1024 / 2048	512 / 1024	512 / 1024	256 / 512
<i>B. subtilis</i>	1024 / 2048	512 / 1024	512 / 1024	256 / 512
<i>E. coli</i>	2048 / >2048	1024 / 2048	1024 / 2048	512 / 1024
<i>P. aeruginosa</i>	>2048 / >2048	2048 / >2048	1024 / 2048	1024 / 2048

Values are presented as MIC / MBC.

3.7 Factorial ANOVA

A significant effect of both fungal filtrate and ZnO-NPs on callus induction, volume and fresh weight was confirmed using a 2-way ANOVA. There was no statistical significance for the interaction term for any of the traits associated with growth, indicating an additive rather than synergistic together (i.e., combination) effect of the 2 treatments on callogenesis (Table 8A). For the inhibition-zone diameter of all 4 bacterial strains, both fungal filtrate and ZnO-NPs had a significant effect; however, for *S. aureus*, *E. coli* and *P. aeruginosa*, interaction effects were also significant, while for *B. subtilis* they were not statistically significant (Table 8B).

Table 8A. Two-way ANOVA for the effects of fungal filtrate, ZnO-NPs, and their interaction on callus traits.

Response	Source	df	Error df	F	p
Callus induction (%)	Fungal filtrate	1	36	22.34	<0.001
Callus induction (%)	ZnO-NPs	1	36	53.36	<0.001
Callus induction (%)	Fungal filtrate × ZnO-NPs	1	36	0.18	0.6700
Callus volume (mm ³)	Fungal filtrate	1	36	48.00	<0.001
Callus volume (mm ³)	ZnO-NPs	1	36	121.26	<0.001
Callus volume (mm ³)	Fungal filtrate × ZnO-NPs	1	36	0.22	0.6402
Fresh weight (g)	Fungal filtrate	1	36	23.79	<0.001
Fresh weight (g)	ZnO-NPs	1	36	63.09	<0.001
Fresh weight (g)	Fungal filtrate × ZnO-NPs	1	36	0.02	0.8900

Table 8B. Two-way ANOVA for the effects of fungal filtrate, ZnO-NPs, and their interaction on antibacterial endpoints.

Response	Source	df	Error df	F	p
<i>S. aureus</i> inhibition zone (mm)	Fungal filtrate	1	28	112.12	<0.001
<i>S. aureus</i> inhibition zone (mm)	ZnO-NPs	1	28	231.05	<0.001
<i>S. aureus</i>	Fungal filtrate × ZnO-NPs	1	28	11.59	0.0020

Response	Source	df	Error df	F	p
inhibition zone (mm)	ZnO-NPs				
B. subtilis inhibition zone (mm)	Fungal filtrate	1	28	67.70	<0.001
B. subtilis inhibition zone (mm)	ZnO-NPs	1	28	104.35	<0.001
B. subtilis inhibition zone (mm)	Fungal filtrate × ZnO-NPs	1	28	0.29	0.5951
E. coli inhibition zone (mm)	Fungal filtrate	1	28	87.47	<0.001
E. coli inhibition zone (mm)	ZnO-NPs	1	28	217.52	<0.001
E. coli inhibition zone (mm)	Fungal filtrate × ZnO-NPs	1	28	10.93	0.0026
P. aeruginosa inhibition zone (mm)	Fungal filtrate	1	28	53.68	<0.001
P. aeruginosa inhibition zone (mm)	ZnO-NPs	1	28	140.76	<0.001
P. aeruginosa inhibition zone (mm)	Fungal filtrate × ZnO-NPs	1	28	7.78	0.0094

All tests were based on the 2×2 factorial design. p-values < 0.05 were considered statistically significant.

4. DISCUSSION

The findings of the experiments indicate that both zinc oxide nanoparticles (ZnO-NPs) and the cell-free, fungal-filtration of *P. lilacinum* resulted in positive levels of callogenic response by *Aloe vera*; moreover, the best callogenic response occurred in the conditions when both agents were used together (i.e., as a combined treatment). Biologically, *Aloe vera* tissue-culture responses are highly dependent upon the balance of growth regulators and the conditions of the in vitro culture (Yasmin et al., 2022). The callus induction and production of secondary metabolites in the cultures of medicinal plants also depend upon the condition of the explants, the efficiency of sterilization, and the presence of stress-induced signals (Ahmadpoor et al., 2022). The presence of secondary metabolites in *Aloe vera* further validates the notion that through the use of elicitors, both the quality of biomass and the biochemical potential of the biomass are altered (Ushasree et al., 2024). Additionally, while there was not a significant interaction between fungal filtrate × ZnO-NPs for growth-related characteristics, increases in the callus induction, callus volume, and fresh weight are interpreted as additive or complimentary effects rather than being statistically proven synergy. The positive effect of ZnO-NPs on the growth of *Aloe vera* has been attributed to the necessity of zinc in the activation of enzymes, the stabilization of membranes, auxin-induced metabolism, protein synthesis, and regulation of redox reactions. Zinc and ZnO-NPs are capable of promoting callus induction and regeneration under in vitro controlled conditions (Aazami et al., 2021). The impact of green vs. chemically synthesized ZnO NPs on callus development and the resulting biological effects are fundamentally dependent on both the plant growth and the manner in which the NPs

are prepared (Saeed and colleagues, 2021). Callus cultures of *Achillea* species treated with ZnO NPs have been shown to contain increased levels of bioactive compounds as well as increased levels of antioxidant enzyme activity, thereby confirming the role of ZnO NPs as in vitro elicitors of plant growth (Khalifa et al., 2023). Given that ZnO nanoparticles can also affect plants' ability to withstand stress and to respond to stress-related situations, the concentration of 30 mg L⁻¹ used in the present study should be interpreted relative to the culture conditions under which they were tested and are not intended to serve as a general recommendation (Verma et al., 2024).

The use of sterile *P. lilacinum* fungal culture filtrates provides the plant tissue culture with metabolites generated by fungi that can modify plant growth, nutrient uptake, and defense metabolism, while providing a means of maintaining sterile conditions during tissue culture growth. The supported evidence of plant growth promotion and biological control by *P. lilacinum* provides additional evidence of the biological significance of indices of fungal derived metabolites in plant systems (Khan and Tanaka, 2023). Similar evidence for the promotion of plant growth has been demonstrated in **Paecilomyces** (aka **Purpureocillium**) derived isolates in the context of disease management (Liu et al., 2024). Following the application of fungi, the observed changes in the microbiome can provide further evidence that the effect of *Purpureocillium*-based treatment systems is likely to influence biological responses associated with plants (Rigobelo et al, 2024). The further evidence of the protective effect of the endophytic fungus *P. lilacinum* against the fungus *Fusarium* supports the idea that it has multiple biological activities (Kimotho et al, 2024). The increase in antimicrobial activity found in the extracts from the elicited calli indicates that either ZnO nanoparticles (ZnO-NPs) and/or the fungal filtrate have the ability to stimulate the production of, or the release of, antimicrobial metabolites from the callus tissue. Several bioactive compounds present in *aloe vera*, including phenolic compounds, flavonoids, anthraquinones, chromones, polysaccharides and acetylated mannans, are known to contribute to the biological activities of this plant (Ushasree et al, 2024). The biological activity of *aloe* extracts tested in vitro confirms their potential use as natural antibacterial materials (Salim et al, 2024). Metabolite profiling and bioactivity assessment of extracts from callus, cell suspensions, and plant tissues have demonstrated the potential value of cultured biomass for screening for bioactive compounds (Al-Abdallat et al, 2023). Examples of variations in antibacterial and antioxidant activity in medicinal plant systems have been documented in callus and leaf extracts (Hagaggi et al, 2024). Evidence of cytotoxicity is provided by callus extract indicating that callus biomass can provide a controlled source of biologically active compounds (Dominguez-Colin et al, 2024). Data from the inhibition zones were coordinated with standardized controls to account for any potential interactions or influences that may be imparted on the results through agar diffusion due to compound polarity, viscosity, molecular size and shape, residual solvents/solvent traces, and the presence of any physical entity (e.g., nanoparticles) (Al-Abdallat et al., 2023). In addition to the antimicrobial interpretation being helped through the use of controls during the experimentation (e.g., solvent control, medium blanks, ZnO-Nano particle background controls, fungal filtrate controls, positive antibiotic control, and minimally inhibitory concentrations/minimally bactericidal concentrations (MIC/MBC) confirmation), the use of these various controls enables antibacterial activity derived from callus to be distinguished from overall experimental/control influences via solvent, medium, nano particle or filtrate influences. In general terms, the ability of Gram-positive bacteria to inhibit antimicrobial activity is significantly less than that of many Gram-negative bacteria due to the difference in their envelope structure; the outer membrane of Gram-negative bacteria is an additional permeability barrier and can reduce the ability of numerous antimicrobial compounds to enter (Salim et al., 2024). Inhibitory activity against *E. coli* and *Pseudomonas aeruginosa* have also exhibited the potential to be significantly less than that of many of the other bacteria included in these studies; therefore, these two organisms provide insight into potential antibacterial activity due to their greater tendency than other bacteria to exhibit resistance to many antimicrobial agents (Al-Abdallat et al., 2023).

Both sections on the phytochemical composition and HPLC markers with respect to the biological response of *Aloe vera* contribute to providing a biological justification through matching measurable metabolic components to the antimicrobial response, rather than looking at just zones of inhibition, or the presence of an antibiotic in culture. The increases in total phenolic, flavonoid, anthraquinone, aloin A+B, and *aloe-emodin* concentrations due to elicitation support the notion that the biochemical profile of the *Aloe vera* callus extracts were modified due to elicitation. Coincident information about the concentration of phytochemicals, diameters of inhibition zones, and MIC/MBC values all provide coherent scientific rationale for the enhanced antibacterial effect associated with the combined application of treatments. This study presents a coherent biological rationale, enhanced methodology, and cautious interpretation of the factorial design used. The study's findings can be positioned as a nanobiological elicitation intervention for increasing *Aloe vera* callus biomass and improving the quality of the antibacterial *Aloe vera* extracts. This combined intervention has shown promise as it enhanced the callus

structure, growth, phytochemical markers, inhibition zone diameters, and MIC/MBC values simultaneously across one experiment, and it should not be considered an extensive antimicrobial agent or method alone.

5. CONCLUSION

The study showed that the introduction of ZnO nanoparticles (ZnO NPs) and sterile *Purpureocillium lilacinum*-derived fungal filtrate (PnF) improved callus production, biomass accumulation, phytochemical marker accumulation, and antimicrobial efficacy in tissue culture of *Aloe vera* under the parameters used in the study. The combination of the two treatments produced compact, healthy, bright green calluses with the highest percentage of induction, callus volume, fresh weight, inhibition zone values, and lowest MIC/MBC values when compared to each other. Treatment with either elicitor resulted in statistically significant main effects as determined by a factorial design analysis, with interaction effects observed primarily for the antimicrobial endpoints of the study. Thus, these data support the use of the combination of fungal filtrate and ZnO NP elicitation as an effective *in vitro* method to enhance the functional value of *Aloe vera* callus culture.

REFERENCES

- [1] Aazami, M. A., Rasouli, F., & Ebrahimzadeh, A. (2021). Oxidative damage, antioxidant mechanism and gene expression in tomato responding to salinity stress under *in vitro* conditions and application of iron and zinc oxide nanoparticles on callus induction and plant regeneration. *BMC Plant Biology*, 21, 597. <https://doi.org/10.1186/s12870-021-03379-7>
- [2] Ahmadpoor, F., Zare, N., Asghari, R., & Sheikhzadeh, P. (2022). Sterilization protocols and the effect of plant growth regulators on callus induction and secondary metabolites production in *in vitro* cultures of *Melia azedarach* L. *AMB Express*, 12, 3. <https://doi.org/10.1186/s13568-022-01343-8>
- [3] Al-Abdallat, A. M., Adayileh, B. K., Sawwan, J. S., Almaliti, J., Bustanji, Y., Alnaimat, S., et al. (2023). Secondary metabolites profiling, antimicrobial and cytotoxic properties of *Commiphora gileadensis* L. leaves, seeds, callus, and cell suspension extracts. *Metabolites*, 13(4), 537. <https://doi.org/10.3390/metabo13040537>
- [4] Alawadi, H. A., Andarzabakhsh, K., Rastegari, A., Mohammadi, Z., Aghsami, M., & Saadatpour, F. (2024). Chitosan-*Aloe vera* composition loaded with zinc oxide nanoparticles for wound healing: *In vitro* and *in vivo* evaluations. *IET Nanobiotechnology*, 2024, 6024411. <https://doi.org/10.1049/2024/6024411>
- [5] Chen, W., et al. (2024). *Purpureocillium jiangxiense* sp. nov.: Entomopathogenic potential and phylogenetic characterization. *Microorganisms*, 12(6), 1041. <https://doi.org/10.3390/microorganisms12061041>
- [6] Dominguez-Colin, J. A., Buendia-Gonzalez, L., Hernandez-Jaimes, C., Cruz-Sosa, F., & Orozco-Villafuerte, J. (2024). Cytotoxic activity of callus extract from *Vachellia farnesiana* (L.) Wight & Arn. *3 Biotech*, 14(10), 235. <https://doi.org/10.1007/s13205-024-04085-5>
- [7] Garg, M., Datta, S., & Ahmad, S. (2024). Plant tissue culture: A potential tool for the production of secondary metabolites. In *In Vitro Propagation and Secondary Metabolite Production from Medicinal Plants: Current Trends (Part 2)*, 15–63. <https://doi.org/10.2174/9789815196351124010004>
- [8] Hagaggi, N. S. A., Abdul-Raouf, U. M., & Radwan, T. A. A. (2024). Variation of antibacterial and antioxidant secondary metabolites and volatiles in leaf and callus extracts of *Phulai* (*Acacia modesta* Wall.). *BMC Plant Biology*, 24, 93. <https://doi.org/10.1186/s12870-024-04747-9>
- [9] Iqbal, Z., et al. (2022). Elicitation of the *in vitro* cultures of selected varieties of *Vigna radiata* L. with zinc oxide nanoparticles for enhancement of secondary metabolites. *Frontiers in Plant Science*, 13, 908532. <https://doi.org/10.3389/fpls.2022.908532>
- [10] Jan, S., et al. (2026). Nanotechnology in plant tissue culture: A review. *Horticultural Plant Journal*. <https://doi.org/10.1016/j.hpj.2025.02.019>
- [11] Khalifa, A. M., Eid, M. A., Gaafar, R. M., Saad-Allah, K. M., & Gad, D. (2023). Induction of bioactive constituents and antioxidant enzyme activities in *Achillea fragrantissima* (Forskal) callus cultures using ZnO nanoparticles. *In Vitro Cellular & Developmental Biology - Plant*, 59, 808–824. <https://doi.org/10.1007/s11627-023-10388-8>
- [12] Khalifa, A. M., et al. (2025). Enhancing *in vitro* production of bioactive compounds in medicinal plant callus cultures using ZnO nanoparticles. *Plant Cell, Tissue and Organ Culture*. <https://doi.org/10.1007/s11240-025-03286-x>
- [13] Khan, M., & Tanaka, K. (2023). *Purpureocillium lilacinum* for plant growth promotion and biocontrol against root-knot nematodes infecting eggplant. *PLoS ONE*, 18(3), e0283550. <https://doi.org/10.1371/journal.pone.0283550>

- [14] Khan, T., Ullah, N., Khan, M. A., Mashwani, Z.-R., Nadhman, A., et al. (2022). Biological and biomedical applications of biosynthesized zinc oxide nanoparticles. *Journal of Nanobiotechnology*, 20, 127. <https://doi.org/10.1186/s12951-022-01367-z>
- [15] Khan, M. A., et al. (2024). Impact of zinc oxide nanoparticles on biosynthesis, antioxidant activity and callus culture responses in *Nigella sativa*. *Plant Nano Biology*. <https://www.sciencedirect.com/science/article/pii/S277311124000524>
- [16] Kimotho, R. N., Zheng, X., Li, F., Chen, Y., & Li, X. (2024). A potent endophytic fungus *Purpureocillium lilacinum* YZ1 protects against *Fusarium* infection in field-grown wheat. *New Phytologist*, 243, 1899–1916. <https://doi.org/10.1111/nph.19935>
- [17] Kumari, P., et al. (2023). Potential use of *Aloe vera* and *Cyclea peltata* as media additives for *Anthurium* tissue culture. *Sri Lankan Journal of Technology*. <https://www.seu.ac.lk/sljot/publication/v4n2/Potential%20use%20of%20Aloe%20vera%20%28Aloe%20barbadensis%20Miller%29%20and%20Kessibissan%20%28Cyclea%20peltata%20L.%29%20as%20media%20additives%20for%20Anthurium%20tissue%20culture.pdf>
- [18] Liu, Z.-J., Shan, L.-L., & Chen, X.-F. (2024). Identification and growth-promoting effect of *Paecilomyces lilacinus*, a biocontrol fungus for walnut rot disease. *PLoS ONE*, 19(12), e0314160. <https://doi.org/10.1371/journal.pone.0314160>
- [19] Mousavi Kouhi, S. M., et al. (2018). Application of ZnO nanoparticles for inducing callus in plant tissue culture. *International Journal of Nanoscience and Nanotechnology*, 14(2), 133–141. https://www.ijnnonline.net/article_31221.html
- [20] Rigobelo, E. C., Carvalho, L. A. L., Santos, C. H. B., Frezarin, E. T., Pinheiro, D. G., Nicodemo, D., et al. (2024). Growth promotion and modulation of the soybean microbiome INTACTA RR PRO with the application of the fungi *Trichoderma harzianum* and *Purpureocillium lilacinum*. *Scientific Reports*, 14, 21004. <https://doi.org/10.1038/s41598-024-71565-2>
- [21] Saeed, F., Younas, M., Fazal, H., Mushtaq, S., Rahman, F. U., Shah, M., et al. (2021). Green and chemically synthesized zinc oxide nanoparticles: Effects on in-vitro seedlings and callus cultures of *Silybum marianum* and evaluation of their antimicrobial and anticancer potential. *Artificial Cells, Nanomedicine, and Biotechnology*, 49(1), 450–460. <https://doi.org/10.1080/21691401.2021.1926274>
- [22] Salim, R. H., Salloum, A. M., Alsalameh, S. A., Khazem, M. R., & Hajeer, M. Y. (2024). Antimicrobial properties of *Aloe vera* ethanol extract as a denture disinfectant: An in vitro study. *Cureus*, 16(5), e59916. <https://doi.org/10.7759/cureus.59916>
- [23] Sara, S., et al. (2025). Optimizing callus formation in *Aloe vera* L.: Exploring the impact of different hormonal concentrations. *Journal of Experimental Biology and Agricultural Sciences*. <https://hal.science/hal-05002464>
- [24] Sprute, R., et al. (2021). Invasive infections with *Purpureocillium lilacinum*: Clinical characteristics and outcome of 101 cases from FungiScope and the literature. *Journal of Antimicrobial Chemotherapy*, 76(6), 1593–1603. <https://doi.org/10.1093/jac/dkab060>
- [25] Ushasree, M. V., Jia, Q., Do, S. G., & Lee, E. Y. (2024). New opportunities and perspectives on biosynthesis and bioactivities of secondary metabolites from *Aloe vera*. *Biotechnology Advances*, 72, 108325. <https://doi.org/10.1016/j.biotechadv.2024.108325>
- [26] Usuda, D., et al. (2025). *Purpureocillium lilacinum*: A minireview. *Journal of Fungi*. <https://pmc.ncbi.nlm.nih.gov/articles/PMC12418003/>
- [27] Verma, N., Kaushal, P., & Sidhu, A. K. (2024). Harnessing biological synthesis: Zinc oxide nanoparticles for plant biotic stress management. *Frontiers in Chemistry*, 12, 1432469. <https://doi.org/10.3389/fchem.2024.1432469>
- [28] Welehaweria, M., & Sbhatu, D. B. (2023). In vitro micropropagation of *Aloe elegans* Tod. using offshoot cuttings. *BMC Research Notes*, 16, 215. <https://doi.org/10.1186/s13104-023-06490-0>
- [29] Yasmin, S., Hasan, M. J., Hossain, M. S., Saha, S., & Khatun, F. (2022). Auxin and cytokinin synergism in micropropagation for mass production of *Aloe vera*. *Biotechnologia*, 103(3), 301–310. <https://doi.org/10.5114/bta.2022.118672>
- [30] Zhang, Y., et al. (2024). Recent advances in nanoparticle elicitation of medicinal plant cell and callus cultures. *Frontiers in Plant Science*. <https://www.frontiersin.org/>