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**Trichoderma longibrachiatum-mediated green synthesis of selenium nanoparticles:
Optimization, characterization, and selective *in vitro* cytotoxicity against cancer cells**

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Abstract

Biogenic production of Se-NPs presents a sustainable, less toxic substitute for traditional physicochemical synthesis. This study utilized cell-free filtrate from *Trichoderma longibrachiatum* (GenBank Accession No. PP768163.1) to serve as both the reducing agent for the sodium selenite precursor and the capping agent for the synthesized nanoparticles to enhance their stability. To maximize production efficiency, the biosynthesis process was statistically optimized using the combination of face-centered central composite design (FCCCD) and desirability function analysis. Under optimized conditions of 18.14 mM Na₂SeO₃, pH 7.16, 27.64 °C, and 7.75 days of incubation, the maximum yield reached 15.53 mM, identifying precursor concentration and incubation time as the most significant factors. Physicochemical characterization via TEM revealed spherical Se-NPs with a mean diameter of 20.16 ± 6.08 nm. The zeta potential of −25.8 mV indicated moderate colloidal stability. XRD analysis confirmed the crystalline trigonal phase, while FTIR analysis supported the involvement of fungal biomolecules in Se-NP capping and stabilization. Cytotoxicity assays demonstrated high selectivity against cancer cells, with IC₅₀ metrics recorded at 31.79 µg/mL (colon; HCT-116), 14.34 µg/mL (hepatic; HepG2), and 8.42 µg/mL (breast; MCF-7). In comparison, non-malignant WI-38 fibroblasts showed lower sensitivity, with an IC₅₀ of 81.95 µg/mL. These findings support the use of *T. longibrachiatum* as an efficient, eco-friendly platform for producing Se-NPs with differential *in vitro* cytotoxicity toward the tested malignant and non-malignant cell lines.

Keywords: Selenium Nanoparticles (Se-NPs), biogenic synthesis, *Trichoderma longibrachiatum*, Face-Centered Central Composite Design, cytotoxicity

Introduction

Nanotechnology has emerged as a cornerstone of modern materials science, emphasizing the precise manipulation of matter at the nanoscale [1,2]. Materials at the nanoscale display unique characteristics, such as increased surface area and reactivity, that distinguish them from larger bulk structure [3,4]. Consequently, these size-dependent properties allow for practical applications in medicine, agriculture, energy, and environmental remediation [5,6]. However, the practical utility of these nanomaterials is often limited by their synthesis route. Traditional physical and chemical methods require toxic reagents and high energy, creating environmental risks [7]. Consequently, scientific focus has shifted toward biological or “green” synthesis as a safer alternative [8,9]. Selenium is an essential trace element known for antioxidant protection and supporting cellular functions [10,11]. It functions as a key component of enzyme systems that limit oxidative damage caused by ROS [12,13]. Additionally, it is integral to selenoproteins

involved in maintaining redox balance and physiological activity [14]. When converted to the nanoscale, Se-NPs show higher bioavailability and lower toxicity than bulk selenium, largely due to their zero-valent state and large surface area [15,16]. Se-NPs have significant advantages over other inorganic Se-containing compounds. Consequently, Se-NPs have attracted extensive interest across agriculture, food science, and medicine due to their multifunctional advantages [17]. With their unique properties, these nanoparticles are being investigated for therapeutic uses, including cancer treatment.

Globally, cancer remains a severe public health threat, causing roughly ten million deaths in 2020 according to GLOBOCAN data [18]. In many countries, it is becoming the leading cause of early mortality [19]. Standard treatments like chemotherapy and radiotherapy face limitations, including severe side effects, low selectivity, and drug resistance [20]. These limitations create a clear need for safer, targeted therapies, particularly Se-NPs.

In cancer research, Se-NPs are valued for their ability to interact with tumor cells in a controlled manner [21]. Specifically, biogenic Se-NPs demonstrate a favorable safety profile and extensive biological activity, which supports their widespread use in therapeutic research [22]. Their anticancer effects are often linked to mechanisms of controlled oxidative stress, enabling selectivity toward cancer cells without harming healthy tissues [23]. Recent research confirms this selective activity. Specifically, nanoparticles synthesized by *Penicillium verhagenii* showed activity against MCF-7 and PC3 cells [24], while those from *Aspergillus flavus* were effective against PANC-1, HeLa, and Caco-2 lines [25].

Various biogenic systems are used to synthesize stable Se-NPs. These systems reduce selenium salts into elemental Se⁰ or methylated forms; the latter is documented in previous literature [26,27]. Among them, fungi are particularly effective because they can reduce selenium and form Se-NPs across many species [28,29,30]. Recent studies report that several endophytic fungi can biosynthesize Se-NPs. These include species of *Trichoderma*, *Aureobasidium*, *Phoma*, *Mortierella*, *Aspergillus*, and *Fusarium* [31]. Specifically, *Trichoderma* species have gained attention for their simple, sustainable synthesis mechanisms. Their metabolism releases secondary metabolites that assist in reducing and stabilizing Se-NPs, as shown in *T. harzianum* [32]. Nandini et al. [33] described this mechanism and coined the term “Trichogenic” synthesis. Recent findings indicate that *Trichoderma* produces proteins that may enhance Se-NPs yield and ensure consistent formation [34].

In the present study, the cell-free filtrate of *T. longibrachiatum* was investigated as a fungal platform for Se-NP biosynthesis through an integrated approach combining metabolite profiling, statistical optimization of the biosynthesis conditions, physicochemical

characterization, and comparative *in vitro* cytotoxicity evaluation against malignant and non-malignant cell lines. Accordingly, this study aimed to synthesize Se-NPs using the cell-free filtrate of *T. longibrachiatum*, optimize the biosynthesis conditions using FCCCD coupled with desirability function analysis, characterize the physicochemical properties of the resulting Se-NPs, and evaluate their comparative *in vitro* cytotoxicity against HCT-116, HepG2, and MCF-7 cancer cells relative to non-malignant WI-38 fibroblasts.

Materials and Methods

Materials

The materials used for fungal cultivation and Se-NPs synthesis included potato dextrose agar (PDA), potato dextrose broth (PDB), and sodium selenite (Na_2SeO_3). All items were obtained from Millipore Sigma, Egypt.

Microorganisms and culture conditions

The fungal isolate *Trichoderma longibrachiatum* (GenBank accession no. PP768163.1) was previously obtained as endophytes from maize grains and molecularly authenticated [35]. The strain was preserved at 4 °C on PCA slants and subcultured monthly. Prior to the experiment, the fungal strain was reactivated on PDA plates at $28 \pm 2^\circ\text{C}$ for seven days to obtain actively growing mycelium.

Preparation of the culture filtrate

Mycelial discs (5 mm) from seven-day-old PDA cultures of *T. longibrachiatum* were inoculated into 100 mL of PDB contained in 250 mL Erlenmeyer flasks. Cultures were incubated at $28 \pm 2^\circ\text{C}$ for seven days under static, dark conditions. Following incubation, the contents were filtered through filter paper (Whatman No. 1 paper), followed by a fine filtration using a 0.22 μm Millipore membrane filter. The resulting sterile, acellular filtrate was frozen at -80°C , lyophilized, and stored for subsequent nanoparticles synthesis and chemical analysis.

Gas chromatography–mass spectrometry (GC–MS)

The lyophilized cell-free filtrate was redissolved in GC-grade methanol, then syringe-filtered (0.22 μm) before injection. GC–MS analysis was performed using a Shimadzu GC-2010 system coupled to a QP-2010 mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a DB-5MS capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μm film thickness). Helium was used as the carrier gas at $1.0 \text{ mL} \cdot \text{min}^{-1}$. Samples were injected in splitless mode (1 μL ; injector temperature 200°C ; solvent cut 3.0 min). The oven temperature was programmed from 70°C to 200°C at $3^\circ\text{C} \cdot \text{min}^{-1}$. Mass spectra were acquired in EI mode at 70 eV (ion source 200°C), scanning over an m/z range of 40–1000. Compound identification was based on comparison of the obtained mass spectra with reference spectra in the NIST and Wiley spectral libraries.

Relative abundances were expressed as percentages of the total integrated chromatographic peak area.

Extracellular biosynthesis of Se-NPs

The extracellular preparation of Se-NPs was carried out using the culture filtrate of *T. longibrachiatum*. The pH of the cell-free filtrate was adjusted to 8.0 before the addition of sodium selenite. The reaction mixture was prepared by mixing 9 mL of the *T. longibrachiatum* cell-free filtrate with 1 mL of an aqueous sodium selenite preparation to achieve a final sodium selenite concentration of 15 mM in a total reaction volume of 10 mL. The reaction mixture was incubated in the dark at 28 ± 2 °C with shaking at 140 rpm for seven days. During incubation, the reaction gradually developed a reddish-orange color, supporting the involvement of fungal extracellular metabolites in the reduction of selenite and the formation of Se-NPs. Once the incubation period was completed, the tubes were centrifuged at 10,000 rpm for 10 min to collect the red precipitate. The pellets were washed several times with sterile deionized water to remove any remaining medium components. The washed material was then freeze-dried and kept at room temperature until it was used for characterization and biological tests.

Experimental design for face- centered central composite design (FCCCD)

The effects of four selected variables on Se-NPs biosynthesis were evaluated using a face-centered central composite design (FCCD) comprising 30 experimental runs and the response value entered for each run represented the mean of three determinations. The investigated factors were the selenite precursor concentration (X_1), pH (X_2), temperature (X_3), and incubation duration (X_4). Every investigated factor was evaluated at three coded points (-1, 0, and +1). Midpoint settings (level zero) for the experimental design were 15 mM of sodium selenite precursor, 7 days of incubation time, a pH of 8.0, and 28 °C. A polynomial regression equation of the second degree was utilized for fitting the experimental data:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad \text{Equation (1)}$$

In this formula, the predicted Se-NPs are denoted by Y , while β_0 serves as the model constant.

The linear, quadratic, and interaction terms are represented by

β_i , β_{ii} , β_{ij} , respectively. Furthermore, the coded levels (symbolized from X_1 to X_4) are defined by X_i and X_j .

Characterization of biosynthesized Se-NPs

UV–Vis spectroscopy

The optical properties of the biosynthesized Se-NPs were assessed using a double-beam UV–Vis spectrophotometer. Absorbance was recorded in the 200–800 nm range in quartz cuvettes,

with distilled water serving as the reference. All measurements were carried out at room temperature.

Fourier transform infrared (FTIR) analysis

FTIR spectral profiling was executed via a LUMOS microscope (Bruker, Germany) to characterize the filtrate's biomolecules, thereby determining how these organic groups actively drive the biogenic reduction and stabilization of Se-NPs. The lyophilized powder of the cell-free filtrate and the purified Se-NPs were placed directly onto the sampling stage. The analysis was conducted in the automated Attenuated Total Reflectance (ATR) mode, utilizing the integrated internal reflection element. The infrared spectra were recorded over a wavenumber range of 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} , with background correction applied prior to each scan. The spectral shifts between the free filtrate and the capped nanoparticles were systematically compared to identify the active capping ligands.

Dynamic light scattering (DLS) and zeta potential

DLS and zeta potential were measured using a Zetasizer Nano-ZS90 (Malvern Instruments, UK) at the Electron Microscopy Unit (EMU), Mansoura University. Samples were briefly sonicated before analysis to ensure proper dispersion. Measurements were performed at room temperature in disposable polystyrene cuvettes for DLS and folded capillary cells (DTS1070) for zeta potential (viscosity of water 0.8872 cP at 25 °C).

X-ray diffraction (XRD)

The crystalline nature of the Se-NPs was examined using a Rigaku MiniFlex diffractometer equipped with Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$). Dried nanoparticles powder was scanned over a 2θ range of 10°–80° with a step size of 0.02° (scan speed 2° min^{-1}). The diffraction pattern was used to confirm the characteristic crystal structure of elemental selenium.

Morphological characterization and elemental mapping (SEM-EDX)

The surface morphological features of the biosynthesized Se-NPs were examined at 30 kV by a JEOL scanning electron microscope (SEM) at Electron Microscopy Unit, Mansoura University. Samples were mounted on carbon tape and sputter-coated with a thin gold layer before imaging. Furthermore, spatial elemental mapping and semi-quantitative mass percentage (wt%) evaluations were conducted on uncoated samples using an integrated EDX detector at 20 kV.

Transmission electron microscopy (TEM)

Ultrastructural characteristics of Se-NPs were examined using a JEOL JEM-2100 transmission electron microscope operating at 200 kV at the Electron Microscopy Unit. A drop of

nanoparticles suspension was placed on a carbon-coated copper grid, excess liquid was removed gently, and the grid was left to air-dry prior to imaging.

MTT-based cytotoxicity analysis of Se-NPs

To assess the cytotoxic potential, the standard MTT viability assay was conducted following the protocol established by Mosmann [36] and Denizot and Lang [37]. WI-38, HCT-116, HepG-2 and MCF-7 cell lines (ATCC; obtained via VACSERA, Cairo) were maintained in RPMI-1640 medium supplemented with 10 percent fetal bovine serum and 1 percent penicillin-streptomycin. Cells were seeded in 96-well plates at 1×10^4 cells per well and incubated for 48 hours at 37 °C in a 5 percent CO₂ incubator. SeNPs suspensions, prepared as described in the synthesis section, were applied at final concentrations of 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL. Doxorubicin was used as a reference drug at the same concentration range. Untreated wells served as the negative control. Following 24 hours of exposure, 20 µL of MTT solution (5 mg/mL in PBS; Sigma-Aldrich) were added to each well and plates were incubated for 4 hours. The medium was then removed and 100 µL of DMSO were added to dissolve the formazan crystals. All measurements were performed in triplicate. An EXL 800 microplate reader was employed to determine the absorbance values at 570 nm.

Cell viability was calculated using the following equation:

$$\text{Viability (\%)} = (A_{570} \text{ treated} / A_{570} \text{ control}) \times 100 \quad \text{Equation (2)}$$

Cytotoxicity was expressed as:

$$\text{Cytotoxicity (\%)} = 100 - \text{Viability (\%)} \quad \text{Equation (3)}$$

The Se-NPs selectivity index (SI) was evaluated as:

$$SI = IC_{50}(\text{WI} - 38) / IC_{50}(\text{cancer cell line}) \quad \text{Equation (4)}$$

Statistical analysis

Design optimization and data analysis were performed in Design-Expert 12 (Stat-Ease), and three-dimensional response surfaces were generated in STATISTICA (StatSoft). TEM particle diameters were measured in ImageJ 1.54g (NIH) after 200 nm scale calibration. Size distributions were plotted as 1 nm-bin histograms in OriginPro 2025b (OriginLab), which was also used to compute summary statistics (N, mean ± SD) and derive geometric metrics (GM, GSD) from ln-transformed diameters. For group comparisons, one-way ANOVA with Tukey's HSD was applied (two-sided, $\alpha = 0.05$). Replicate data are reported as mean ± SD.

Results and Discussion

Microorganisms and culture condition

Molecular identification previously confirmed the isolated *Trichoderma* strain utilized in the present study as *T. longibrachiatum* (GenBank Accession No. [PP768163.1] [35]. Morphologically, the strain showed characteristic features on PDA plates, showing fast, radial growth with dense light-green sporulation and yellowish-green reverse pigmentation after seven days at 28 °C (Fig. 1A). These morphological traits align with the mycelial characteristics descriptions of the *T. Longibrachiatum* recently described by Kim et al. [38]. The aerial mycelium showed a well-organized system of branched, septate hyphae bearing conidiophores with flask-shaped phialides under SEM (Fig. 1B–D). The conidia were slightly ornamented with fine surface textures; both the terminal and intercalary chlamydospores were observable. Such features have often been reported within the *longibrachiatum* clade [39,40]. Collectively, these cultural and ultrastructural features confirmed the identity and normal growth of the isolate, indicating its suitability for the subsequent biosynthesis of Se-NPs.

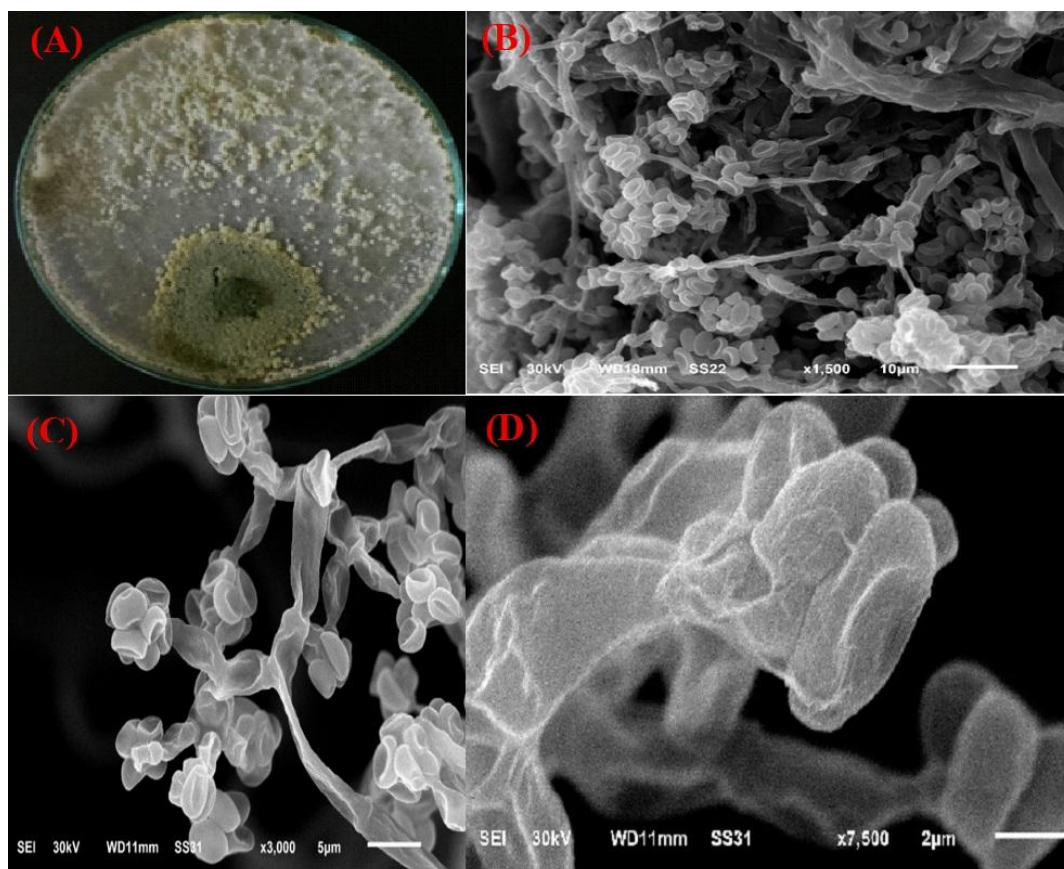
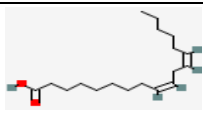
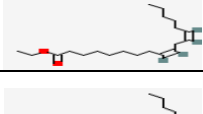
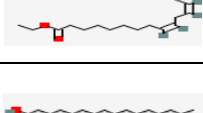
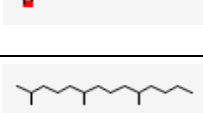
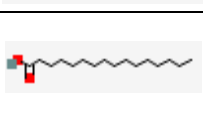
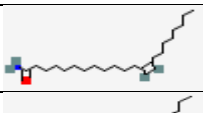
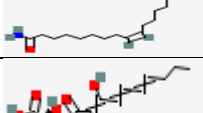
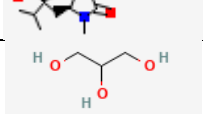
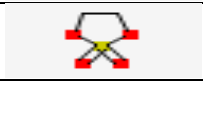


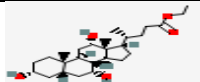
Figure 1. Morphological features of *T. longibrachiatum*. (A) Colony on PDA after 7 days at 28 °C. B–D, SEM micrographs showing branched, septate hyphae and conidiophores bearing flask-shaped phialides; (B) 1,500× (scale bar = 10 µm), (C) 3,000× (scale bar = 5 µm), (D) 7,500× (scale bar = 2 µm).

GC–MS analysis of the cell-free filtrate of *T. longibrachiatum*

GC–MS analysis of the cell-free filtrate of *T. longibrachiatum* revealed twenty-five chromatographic peaks, with the twelve peaks with the highest relative peak areas highlighted in Fig. 2 and all detected peaks listed in Table 1. The detected profile comprised mainly fatty acids, fatty acid esters, alkanes, polyols, amides, and fungal secondary metabolites. Similarly, previous GC–MS profiling of *T. longibrachiatum* filtrates revealed metabolites belonging to hydrocarbons, esters, acids, and terpenes [41]. The most prominent peak assignments included linoleic acid (17.31%), ethyl linoleate (16.54%), palmitic acid (9.55%), tetradecane (7.38%), erucylamide (6.22%), and an oleamide derivative (5.65%). Other detected metabolites, including glycerin (2.04%) and 1-octen-3-one, further illustrated the chemical diversity of the fungal filtrate.

Table 1. Top 12 metabolites identified by GC–MS analysis in the *T. longibrachiatum* cell-free filtrate (by relative peak area).

No.	RT (min)	Compound	Formula	Chemical structure	MW (g/mol)	Area (%)	Chemical class
12	22.19	Linoleic acid	C ₁₈ H ₃₂ O ₂		280.45	17.31	Polyunsaturated fatty acids (PUFAs) (C18:2)
15	25.93	Ethyl linoleate	C ₂₀ H ₃₆ O ₂		308.51	16.54	Ester (PUFA derivative)
20	34.02	Linoleic acid ethyl ester (Ethyl linoleate)	C ₂₀ H ₃₆ O ₂		308.51	10.25	Ester (PUFA derivative)
14	23.71	Palmitic acid (hexadecanoic acid)	C ₁₆ H ₃₂ O ₂		256.43	9.547	Saturated fatty acid (C16:0)
5	14.63	Tetradecane, 2,6,10-trimethyl-	C ₁₇ H ₃₆		240.47	7.384	Alkane (hydrocarbon)
9	18.51	n-Hexadecanoic acid (Palmitic acid)	C ₁₆ H ₃₂ O ₂		256.43	7.245	Saturated fatty acid (C16:0)
16	27.26	Erucylamide	C ₂₂ H ₄₃ NO		337.59	6.216	Fatty acid amide
18	29.62	Oleamide, TMS derivative	C ₂₁ H ₄₃ NOSi		353.67	5.653	Fatty acid amide (TMS)
2	10.45	Harzianic acid	C ₁₉ H ₂₇ NO ₆		365.43	4.662	Fungal secondary metabolite
4	13.24	Glycerin	C ₃ H ₈ O ₃		92.09	2.041	Polyol
22	34.95	Ethylene sulfate	C ₂ H ₆ O ₄ S		126.13	2.03	Sulfate ester

19	32.81	Ethyl iso- allocholate	C ₂₆ H ₄₄ O ₅		436.63	1.912	Bile acid ester
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This metabolic profile provides a biochemical basis for the biogenic synthesis mechanism. Long-chain fatty acids are widely recognized for their role in biogenic nanoparticle formation [32,42]. Specifically, the prevalence of unsaturated fatty acids (e.g., linoleic acid) and their esters is consistent with the evidence presented by Afzal et al. [43], identifying these molecules as potential reducing agents.

It is postulated that the oxidation of double bonds and carboxyl groups facilitates the electron transfer required to reduce selenite ions to elemental selenium. Furthermore, saturated fatty acids (e.g., Palmitic acid) and fatty acid amides likely act as capping ligands. According to the lipid-capping hypothesis proposed by Bulgarini et al. [44], the hydrophobic acyl chains of these lipids interact with the selenium surface to form a steric barrier, thereby potentially reducing agglomeration and contributing to colloidal stabilization. Polyols like glycerin may further assist in maintaining this stability. Additionally, the analysis detected Harzianic acid (4.66%), a *Trichoderma* metabolite known to function as a siderophore with metal-chelating properties [45,46]. Overall, the identified metabolic profile is consistent with the potential involvement of *T. longibrachiatum* metabolites in the reduction, capping, and stabilization of Se-NPs, aligning with established reports of fungal-mediated nanoparticle synthesis.

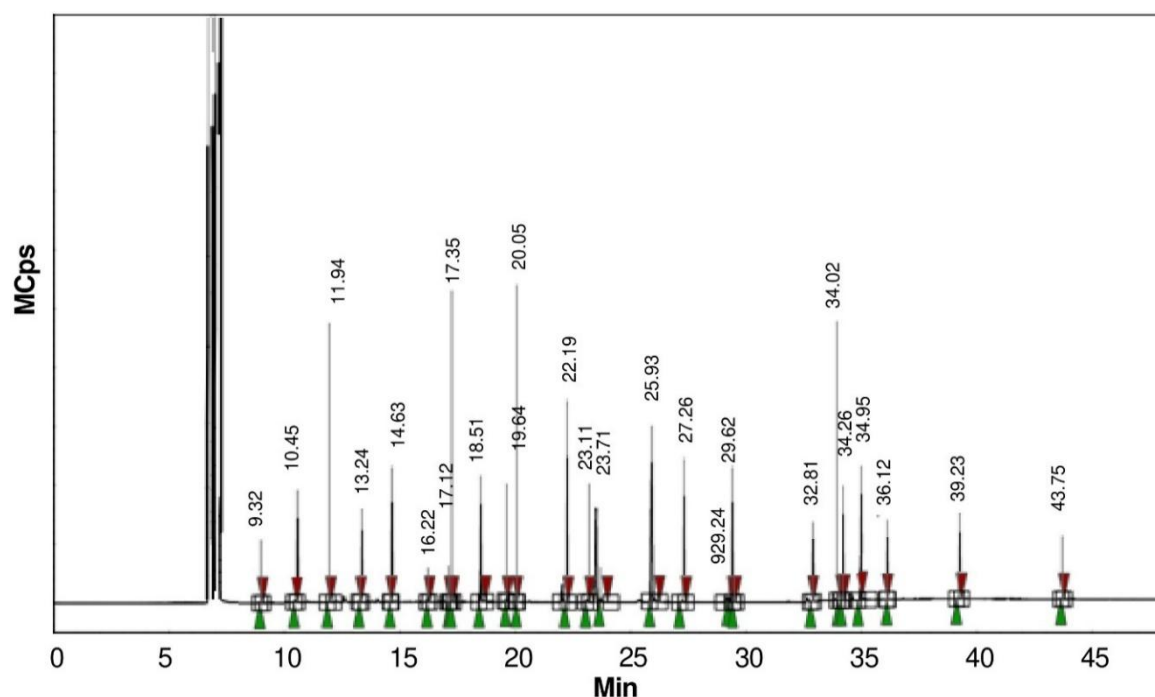


Figure 2. Total ion chromatogram (TIC) of the cell-free filtrate of *T. longibrachiatum*.

Extracellular biosynthesis and spectroscopic analysis of Se-NPs

UV–Vis spectroscopy was used as a primary indicator to monitor the early stages of elemental selenium (Se-NPs) formation. To the naked eye, the success of the synthesis was revealed by a gradual chromatic shift in the reaction mixture (sodium selenite + filtrate) to a distinct reddish–orange color (Fig. 3A). In contrast, the control solutions (sodium selenite and the filtrate alone) remained unchanged, confirming that the reduction process was driven exclusively by the fungal metabolites. This specific coloration aligns with the previous result using *Trichoderma* filtrates that reported the appearance of a red-orange color as a visible indication of the formation of Se-NPs [47]. The coloration is due to the reduction of Se^{4+} to Se^0 and is in line with what is commonly reported in trichogenic systems [48].

The synthesized Se-NPs exhibited a prominent surface plasmon resonance (SPR) peak centered at 225 nm (Fig. 3B), which falls well within the 200–400 nm absorption range reported for trichogenic Se-NPs [33]. Recently, UV–visible spectroscopy of Se-NPs synthesised by *Trichoderma* spp. were also reported to exhibit a maximum peak at 259 [49].

As well, Se-NPs formed by endophytic fungal strains showed a UV-maximum absorption peak at 265 nm, due to plasma resonance of the Se-NPs [31]. Piacenza et al. [50] observed peaks at 232 nm and 253 nm depending on dilution, illustrating that synthesis and measurement parameters can influence the absorbance profile.

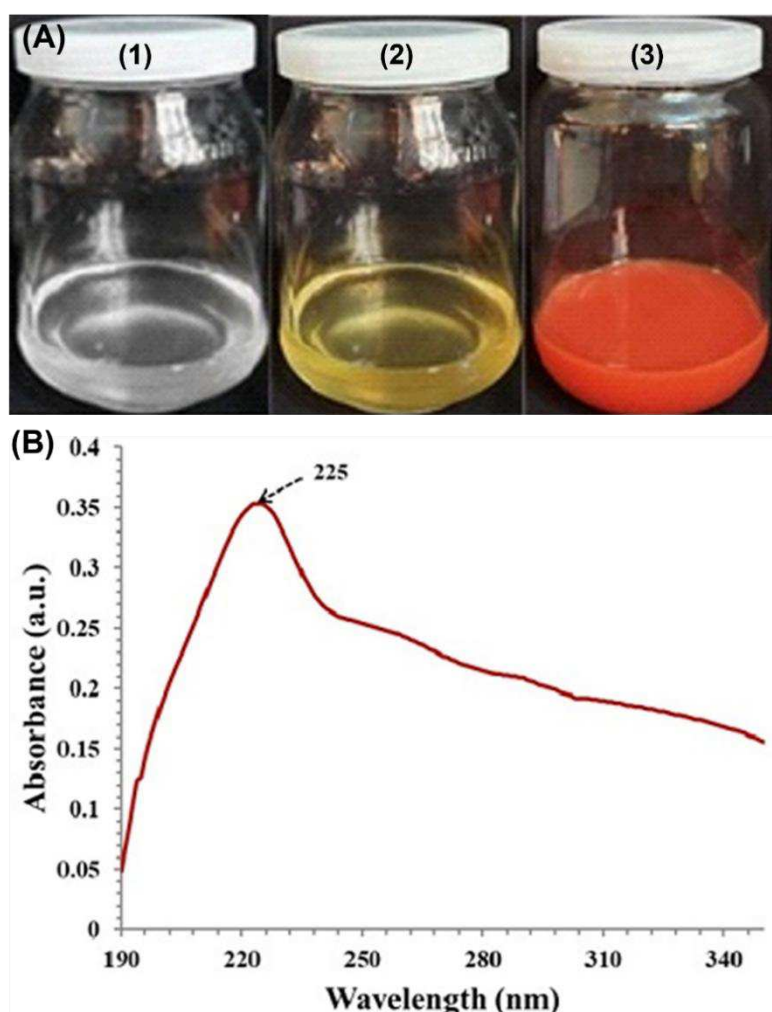


Figure 3. Biosynthesis of Se-NPs by the *T. longibrachiatum* filtrate. (A) Visual observation of the reaction components: (1) 15 mM sodium selenite solution, (2) cell-free filtrate, and (3) the synthesized Se-NPs. (B) UV-visible absorption spectrum of the biosynthesized Se-NPs.

FCCCD-mediated systematic optimization of Se-NPs production

The systematic optimization of the biosynthesis process was carried out using a FCCCD. The results for all 30 trials are listed in Table 2, which investigated four distinct parameters: concentration of sodium selenite (X_1), initial pH (X_2), incubation temperature (X_3), as well as the reaction period (X_4) on Se-NPs biosynthesis by *T. longibrachiatum*. Table 2 details the full comparison between the actual experimental results and the predicted values. Run orders 2, 10, 20, 23, 25, and 30 consistently yielded the highest Se-NPs amount. The peak biosynthetic value of 15.32 mM was achieved in Run 23, utilizing the central design conditions: 15 mM sodium selenite, pH 8, 28 °C, and a 7-day incubation period. The narrow variation among these center-point replicates confirms the high reproducibility of the system and indicates that the true optimum lies within this central experimental region.

Table 2. FCCCD experimental design and response data for *T. longibrachiatum*-mediated Se-NPs biosynthesis.

Std	Run	Type	X ₁	X ₂	X ₃	X ₄	Se-NPs biosynthesis (mM)		Residuals
							Actual	Predicted	
21	1	Axial	0	0	-1	0	13.85	13.68	0.1668
25	2	Center	0	0	0	0	14.34	14.39	-0.0512
3	3	Factorial	-1	1	-1	-1	3.01	3.05	-0.0422
8	4	Factorial	1	1	1	-1	11.34	10.74	0.5955
9	5	Factorial	-1	-1	-1	1	4.2	4.51	-0.3067
10	6	Factorial	1	-1	-1	1	12.98	12.74	0.2443
23	7	Axial	0	0	0	-1	12.94	13.21	-0.2677
1	8	Factorial	-1	-1	-1	-1	3.98	3.55	0.4276
15	9	Factorial	-1	1	1	1	3.81	3.23	0.5778
27	10	Center	0	0	0	0	14.59	14.39	0.1988
18	11	Axial	1	0	0	0	11.96	12.57	-0.6143
19	12	Axial	0	-1	0	0	13.94	14.51	-0.5699
4	13	Factorial	1	1	-1	-1	8.98	9.15	-0.1713
24	14	Axial	0	0	0	1	14.14	14.37	-0.2332
7	15	Factorial	-1	1	1	-1	2.83	3.24	-0.4079
12	16	Factorial	1	1	-1	1	11.24	10.93	0.3144
11	17	Factorial	-1	1	-1	1	3.1	3.44	-0.344
13	18	Factorial	-1	-1	1	1	2.91	2.9	0.0076
6	19	Factorial	1	-1	1	-1	10.78	10.6	0.1804
29	20	Center	0	0	0	0	14.62	14.39	0.2288
5	21	Factorial	-1	-1	1	-1	2.32	2.35	-0.0256
16	22	Factorial	1	1	1	1	11.53	12.12	-0.5913
30	23	Center	0	0	0	0	15.32	14.39	0.9288
22	24	Axial	0	0	1	0	13.01	13.68	-0.6677
28	25	Center	0	0	0	0	14.51	14.39	0.1188
17	26	Axial	-1	0	0	0	4.82	4.71	0.1135
14	27	Factorial	1	-1	1	1	12.87	12.54	0.3311
20	28	Axial	0	1	0	0	14.12	14.05	0.069
2	29	Factorial	1	-1	-1	-1	10.11	10.4	-0.2889
26	30	Center	0	0	0	0	14.47	14.39	0.0788
Variable						Code	-1	0	1
Selenium conc. (mM)						X ₁	5	15	25
Initial pH level						X ₂	7	8	9
Temperature (°C)						X ₃	26	28	30
Incubation time (days)						X ₄	4	7	10

In contrast, the minimum production was observed in Run 21, yielding just 2.32 mM under the combination of 5 mM sodium selenite, initial pH 7, 30 °C, and 4 days of incubation. The optimal pH of 8 recorded in this study is consistent with findings by Diko et al. [51], who similarly identified alkaline conditions as crucial for Se-NPs synthesis using *Trichoderma* sp. [51]. Furthermore, Tuyen et al. [52] and Iqbal et al. [53] report that moderately alkaline pH and

mid-range selenium precursor levels support efficient Se-NPs formation in comparable biological systems. Incubation time also showed a predictable pattern, where shorter durations yielded limited conversion and longer periods supported higher Se-NPs formation, a trend consistent with earlier green synthesis studies [54,55].

ANOVA and regression modeling

The ANOVA results in Table 3 confirm the statistical significance of the experimental design. The model yielded a high F -value of 156.01 and a P -value < 0.0001 , indicating that the quadratic model is statistically significant. Based on P -values < 0.05 , the linear terms (X_1 and X_4), interaction terms (X_1X_3 , X_1X_4 , and X_2X_3), and quadratic terms (X_1^2 and X_3^2) were identified as the most critical factors influencing the biosynthesis process. This hierarchy of effects aligns with recent RSM application [56,57,58], which similarly report that nanoparticle formation is jointly shaped by individual factors alongside their linear and quadratic interactions, rather than by any single factor acting independently. Conversely, the linear effects of pH (X_2) and temperature (X_3) were not significant within the tested ranges.

To validate the model's accuracy, the coefficient of determination (R^2) was analyzed. It is generally established that an $R^2 > 0.9$ reflects a robust correlation between predicted and actual data [59]. The obtained R^2 of 0.9932 exceeds this threshold, confirming the model's reliability. Additionally, the adjusted R^2 (0.9868) and predicted R^2 (0.9629) are consistent, with a difference of less than 0.2, which further supports the model's predictive precision.

The coefficient also clarified whether the Se-NPs yield was influenced in either a positive or negative direction. Synergism and antagonism, indicated by positive and negative coefficients respectively, represent the primary interaction types among variables [60]. A substantial coefficient assigned to an independent factor demonstrates its strong influence on the response, regardless of whether it is positive or negative. Generally, biosynthesis is enhanced at elevated levels of any variable displaying a positive coefficient [61].

Table 3 shows positive coefficients for sodium selenite (X_1), temperature (X_3) and incubation time (X_4), along with their interactions (X_1X_3 , X_1X_4 , X_2X_3). These positive values indicate that increasing these parameters generally improves Se-NPs production. Sodium selenite, in particular, had the strongest linear impact. However, this positive effect is countered by the large negative coefficient of its quadratic term (X_1^2). Consequently, the maximum nanoparticle yield was observed at intermediate rather than extreme precursor concentrations. Regarding data reliability, the low coefficient of variation (5.32%) reflects high experimental precision [61]. Similarly, the Adequate Precision value of 32.08 is well above the required threshold of

4, confirming strong model discrimination [62]. The model mean was 10.09 with a standard deviation of 0.54.

Table 3. Analysis of variance of FCCCD for Se-NPs biosynthesis using *T. longibrachiatum* as affected by Selenium conc. (mM), initial pH level, temperature (°C) and incubation period (days).

Source of variance		Coefficient estimate	Sum of Squares	Degrees of Freedom	Mean Square	F-value	P-value
Model	Intercept	14.39	627.95	14	44.85	156.01	< 0.0001
Linear effect	X ₁	3.93	278.56	1	278.56	968.88	< 0.0001
	X ₂	-0.23	0.95	1	0.95	3.30	0.0895
	X ₃	0.00	0.00	1	0.00	0.00	0.9828
	X ₄	0.58	6.11	1	6.11	21.26	0.0003
Interaction effect	X ₁ X ₂	-0.19	0.56	1	0.56	1.94	0.1836
	X ₁ X ₃	0.35	1.98	1	1.98	6.89	0.0191
	X ₁ X ₄	0.35	1.91	1	1.91	6.65	0.021
	X ₂ X ₃	0.35	1.94	1	1.94	6.74	0.0202
	X ₂ X ₄	-0.14	0.32	1	0.32	1.10	0.3108
	X ₃ X ₄	-0.10	0.16	1	0.16	0.55	0.4699
Quadratic effect	X ₁ ²	-5.75	85.69	1	85.69	298.03	< 0.0001
	X ₂ ²	-0.11	0.03	1	0.03	0.11	0.7441
	X ₃ ²	-0.71	1.31	1	1.31	4.55	0.0498
	X ₄ ²	-0.60	0.94	1	0.94	3.25	0.0914
Error effect	Lack of Fit		3.71	10	0.37	3.09	0.1126
	Pure Error		0.60	5	0.12		
R ²	0.9932	Std. Dev.	0.54				
Adj R ²	0.9868	Mean	10.09				
Pred R ²	0.9629	C.V. %	5.32				
Adeq Precision	32.08						

*C.V: Coefficient of variation, F: Fisher's variance ratio, P: Probability of significance.

Finally, the summary of model fit statistics (Table 4) justifies the selection of the quadratic model over the Linear and 2FI models. The lack of fit of the quadratic model proved statistically insignificant ($F = 3.09$, $p = 0.1126$), meaning it accurately represents the data. Conversely, the other models exhibited significant lack of fit.

This quadratic model also resulted in the lowest standard deviation (SD = 0.54) alongside a PRESS statistic (23.48), demonstrating superior predictive capability compared to the Linear (PRESS = 483.36) and 2FI (PRESS = 1193.63) models.

To determine the maximum potential yield, the second-order polynomial model was applied (Equation 5). This mathematical function correlates the Se-NPs biosynthesis level (Y) with the four tested variables (sodium selenite, pH, temperature, and incubation time) as follows:

$$Y = 14.39 + 3.93X_1 - 0.23X_2 + 0.00X_3 + 0.58X_4 - 0.19X_1X_2 + 0.35X_1X_3 + 0.35X_1X_4 + 0.35X_2X_3 - 0.14X_2X_4 - 0.10X_3X_4 - 5.75X_1^2 - 0.11X_2^2 - 0.71X_3^2 - 0.60X_4^2. \quad \text{Equation (5)}$$

Table 4. Fit summary of FCCCD for Se-NPs biosynthesis using *T. longibrachiatum*

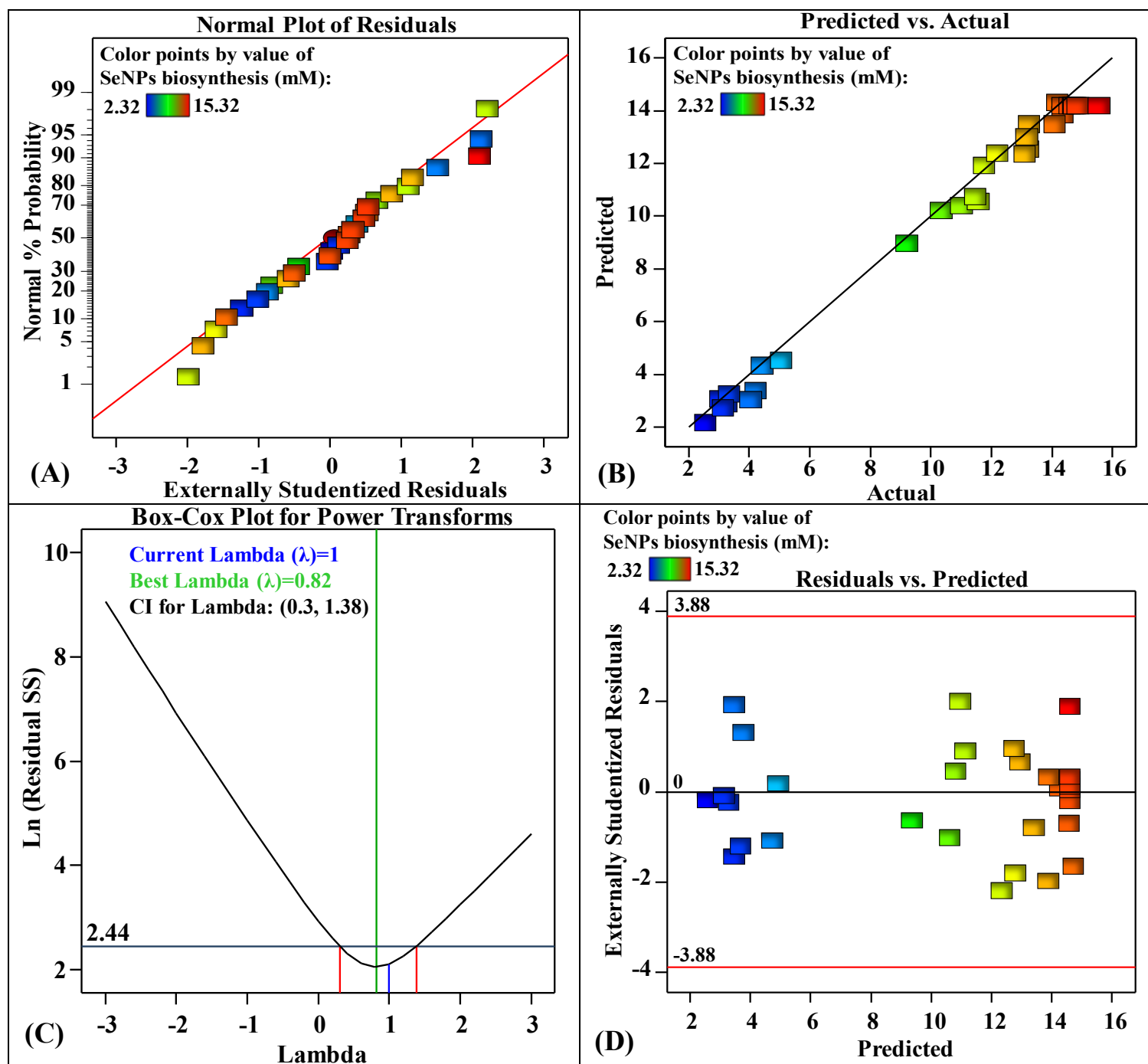
Lack of Fit Tests					
Source	Sum of Squares	Df	Mean Square	F-value	P-value Prob >F
Linear	346.04	20	17.3	143.92	< 0.0001
2FI	339.17	14	24.23	201.53	< 0.0001
Quadratic	3.71	10	0.3712	3.09	0.1126
Model Summary Statistics					
Source	Standard deviation	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS
Linear	3.72	0.4517	0.364	0.2355	483.36
2FI	4.23	0.4626	0.1798	-0.8879	1193.63
Quadratic	0.54	0.9932	0.9868	0.9629	23.48

* Significant values, *df*: degree of freedom, PRESS: sum of squares of prediction error, two factors interaction: 2FI

Model adequacy and diagnostic evaluation

Statistically, the normal probability plot (NPP) is used to check if the model's residuals follow a normal distribution [63]. Since residuals represent the deviation between the predictions and the actual data [64], smaller deviations indicate higher accuracy. As shown in Fig. 4A, the residual points are aligned along the diagonal straight line. This alignment confirms that the residuals follow a normal distribution, validating the statistical assumptions of the experimental design [65].

Figure 4B illustrates the correlation between the predicted and experimental values. The data points lie close to the regression line, showing strong agreement between the model predictions and the actual laboratory results [66].



1 **Figure 4.** Diagnostic evaluation for the biogenic synthesis of SeNPs by *T. longibrachiatum*:
2 (A) Normal probability distribution for the externally-studentized type of residuals, (B)
3 Correlation between actual and predicted outputs, (C) Box-Cox curve for power
4 transformations, and (D) The distribution of such studentized residuals across predicted values.
5 To further assess the need for data transformation, the Box-Cox plot was analyzed (Fig. 4C).
6 The vertical red lines mark the 95% confidence interval (C.I.) limits of 0.3 and 1.38. The
7 current lambda value of 1, representing the untransformed data, is marked by the blue line,
8 whereas the green line pinpoints the optimal lambda estimate of 0.82. Since the blue line is

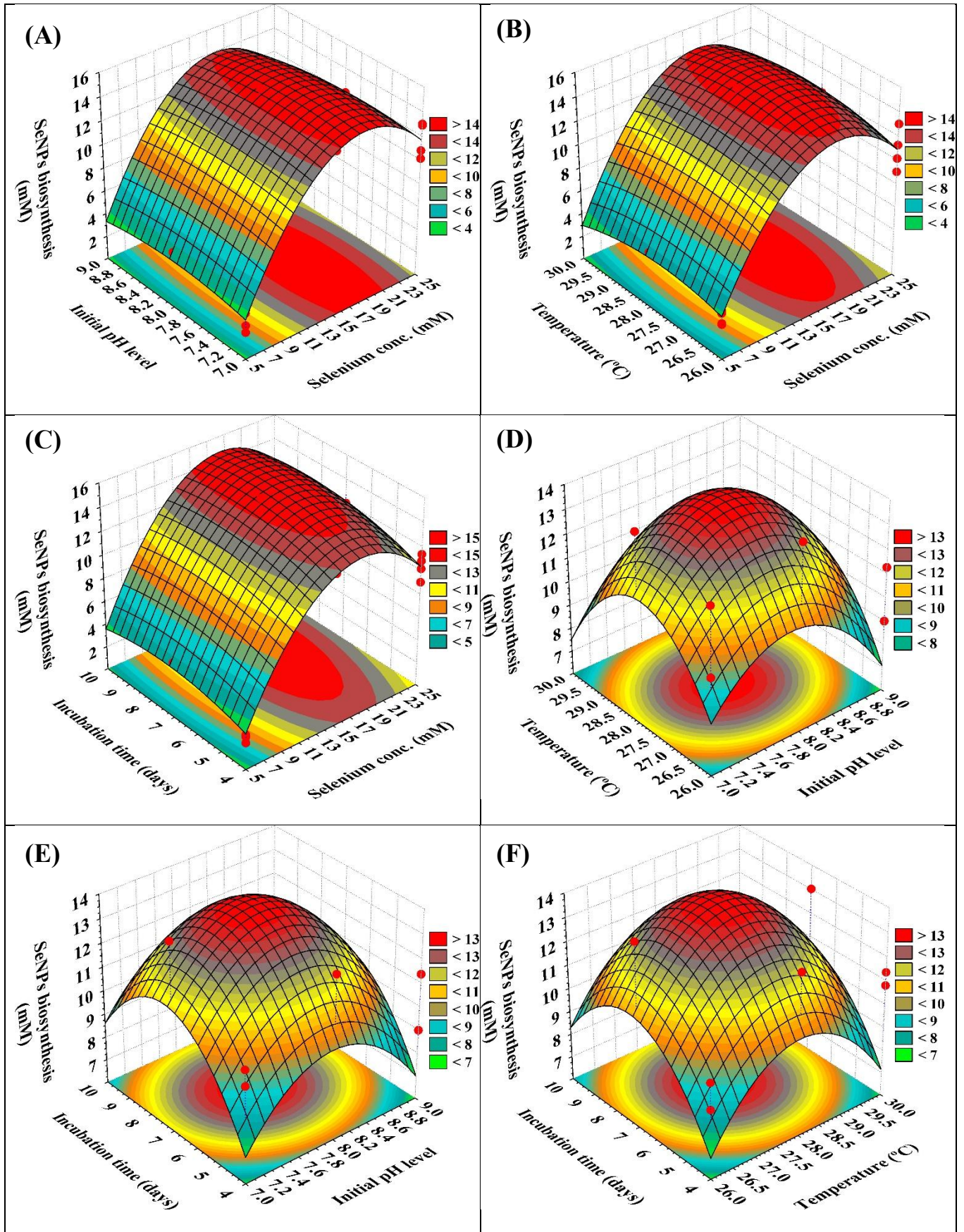
located within the optimal zone bounded by the red lines, no data transformation is needed for this model [67]. Figure 4D presents the plot of externally studentized residuals versus the predicted values. The data points are scattered freely around the zero line without following any specific order or shape. This random distribution is a clear indicator of constant variance, proving that the variation is consistent across the predicted range [65].

Three-dimensional (3D) surface plots interpretation

The 3D response surface plots (Fig. 5A–F) were generated to visualize the interactions between variables and define the boundaries for optimal Se-NPs yield. In these graphical representations, the vertical axis (Z) displays the Se-NPs yield as a function of two varying parameters, while the remaining factors are maintained strictly at their central values. It is well-established that the efficiency of fungal-mediated-Se-NPs biosynthesis affected severely on optimizing conditions, particularly precursor concentration, pH, and temperature [51,68].

Figure 5A illustrates how the interaction of sodium selenite (X_1) alongside initial pH (X_2) influences Se-NPs biosynthesis, while maintaining the zero-coded levels for temperature ($X_3 = 28\text{ }^{\circ}\text{C}$) and incubation time ($X_4 = 7\text{ days}$). The yield of Se-NPs exhibited a gradual increase until reaching an optimal pH of approximately 8; shifting the pH above or below this specific point caused a subsequent decline in the yield. This observation is consistent with Diko et al. [51], who reported that while slightly alkaline conditions favor extracellular synthesis, extreme alkalinity leads to the inhibition of fungal growth and disruption of the enzymatic machinery required for selenite reduction. Similar studies have shown that alkaline conditions often favor the stability and formation of nanostructures [53,52]. Comparable results were recorded for *Streptomyces bikiniensis*, which confirmed that a slightly alkaline environment (pH 7–8) maximizes the selenite reduction process [69]. Also, increasing selenite increased the yield up to the middle range (15 mM), but adding more caused a drop. The drop in yield at high concentrations can be attributed to the toxicity of selenium, which damages the cellular proteins responsible for Se-NPs synthesis [70].

Figure 5B demonstrates the interactive effect of sodium selenite (X_1) alongside temperature (X_3) on the Se-NPs yield, while the initial pH (X_2) and time (X_4) are held constant at their zero-coded levels. The measured yield exhibited a steady increase along with rising temperature and precursor concentration, ultimately reaching a maximum near their respective midpoints. However, the production dropped when these factors were increased further. This decline suggests that the reducing agents degrade under excess temperature [57].



1 **Figure 5.** Interactive influence of the examined variables on Se-NPs biosynthesis by *T.*
2 *longibrachiatum*, visualized via 3D surface graphs.

Figure 5C illustrates the interaction between the production of Se-NPs and the interaction of between X_1 (Sodium Selenite) and X_4 (Incubation Time), while X_2 and X_3 were kept constant. The biosynthesis of Se-NPs rose rapidly alongside increasing concentration and time, continuing this rise until reaching their midpoints. After that point, the yield decreased. According to Gharieb et al. [68], the decline in production at high concentrations is attributed to the toxicity of selenium to the cellular biomolecules implicated in the reduction process. Moreover, incubation time also plays a critical role in the nanoparticles production. As noted by Bužková et al. [71], while the reaction needs time to complete the reduction, prolonged incubation at high concentrations can induce particle aggregation, which in the end lowers the yield.

Figure 5D presents the interaction of initial pH (X_2) and temperature (X_3), where both X_1 and X_4 are held at zero levels. Se-NPs production increased as temperature and pH rose toward the center values. The yield then decreased as these factors went higher. As reported in fungal studies, extreme pH or temperature alters the structure of reductase enzymes and disrupts membrane permeability, thereby suppressing nanoparticle synthesis [72].

Figure 5E illustrates the interaction between incubation time (X_4) and initial pH (X_2). The 3D plot shows that yield increases as both factors approach their center points. However, maintaining the reaction for extended periods at extreme pH levels leads to a noticeable decline in production. This reduction occurs because prolonged exposure to unfavourable pH conditions progressively alters the structure of reductase enzymes.

The interactive effect of temperature (X_3) alongside incubation time (X_4) on the biosynthesis of Se-NPs is illustrated in Fig. 5F. The Se-NPs yield increased gradually as both variables increased toward their central values. However, the production decreased with any subsequent increase in temperature or time. This reduction is likely because prolonged exposure to high temperature may damage the fungal enzymes required for the reduction process. This observation aligns with Ibrahiem et al. [73], who observed that optimizing the incubation period and temperature is crucial for maximizing the biosynthesis of selenium nanomaterials.

Desirability-based optimization

Determining the best operating conditions for a specific response is standardly achieved through desirability function analysis (DFA) [74,75]. DFA was applied using design-expert software (version 12), as shown in Fig. 6. The model calculated a theoretical maximum Se-NPs yield of 15.40 mM at the following settings: 18.14 mM sodium selenite, pH 7.16, 27.64 °C, and 7.75 days. The desirability value was 1. To validate this prediction, a confirmatory experiment was conducted using these specific conditions. The actual experimental yield

obtained was 15.53 mM. Comparing the predicted value (15.40 mM) with the experimental result (15.53 mM) showed a high accuracy with a negligible error (0.84%), ensuring the model's reliability. The desirability value was 1, confirming a well-defined optimum and supporting the accuracy and robustness of the developed model [76].

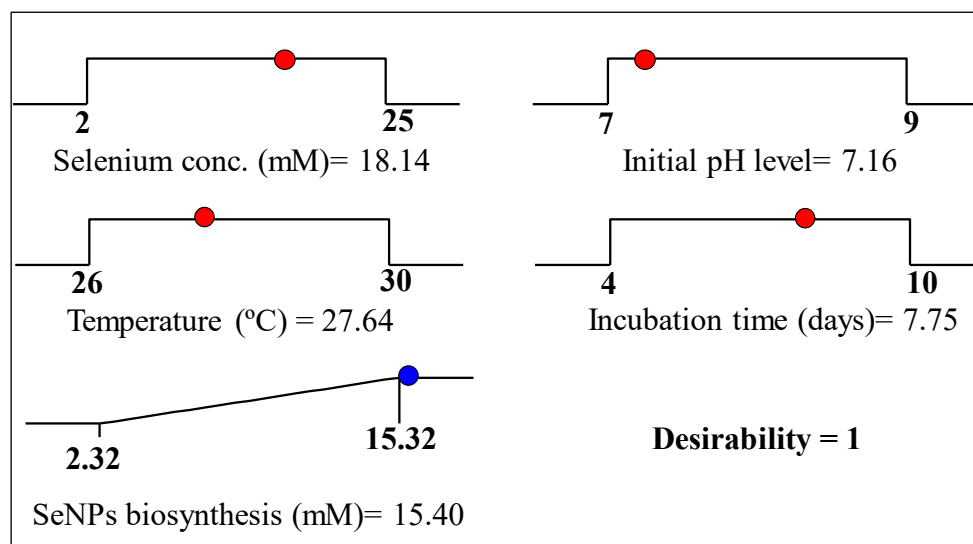


Figure. 6. DFA results illustrating the predicted conditions for maximizing Se-NPs biosynthesis from *T. longibrachiatum*.

Ultrastructural characterization of the synthesized Se-NPs

Microscopic profiling of Se-NPs mycosynthesized through *T. longibrachiatum* filtrate was investigated using TEM and SEM with EDX. SEM provided an overview of the surface morphology of the biogenic Se-NPs, confirming the prevalence of predominantly spherical particles with irregular aggregated clusters (Fig. 7A). Particle diameters observed in the SEM field ranged between approximately 30 and 67 nm. Similar spherical morphologies have been widely reported for green-synthesized Se-NPs with an average size of approximately 74.43 nm [77] and 41 to 44 nm [78]. The presence of limited aggregation is common in biologically produced nanoparticles and is typically attributed to uneven distribution of organic capping layers on their surfaces. To confirm the structural details observed by SEM, TEM was used for a more detailed characterization of SeNP. The resulting images highlighted the formation of distinct, spherical Se-NPs, with diameters falling within the range of 8.99–43.42 nm (Fig. 7B and C). The measurement of 268 particles yielded a mean size (mean $\pm$ SD) of 20.16 ± 6.08 nm, indicating a relatively narrow log-normal distribution typical of green-synthesized Se-NPs. These findings confirm the nanoscale dimensions of the biosynthesized Se-NPs and are consistent with the size ranges reported for biogenic Se-NPs in previous studies. These values fall within the TEM size ranges reported for biosynthesized Se-NPs, such as and 23 to 35 nm

[79]. Similarly, structural characterization via TEM revealed that green synthesised Se-NPs showed spherical particles in a range of 20 to 60 nm, which could impact the biological efficacy of Se-NPs [80].

Fourier-transform infrared (FTIR) analysis

The functional groups associated with the surface chemistry of the biogenic Se-NPs were characterized through FTIR analysis. The comparative spectra of the *T. longibrachiatum* filtrate and the Se-NPs, and the prominent spectral shifts are detailed in Fig. 7D and Table 5. The initial spectrum of the fungal filtrate showed a broad O–H and N–H stretching band at 3262.02 cm⁻¹. Upon Se-NPs synthesis, this band shifted to 3272.65 cm⁻¹, indicating the active involvement of proteinaceous or hydroxyl-containing biomolecules in nanoparticle stabilization [81]. Aliphatic C–H stretching vibrations observed at 2932.25 and 2882.16 cm⁻¹ in the filtrate shifted to 2922.83 and 2850.73 cm⁻¹, pointing to lipidic components. This perfectly aligns with the metabolic profile typically identified in fungal extracts [42].

Table 5. FTIR peak assignments and corresponding spectral shifts for the *T. longibrachiatum* filtrate and the biosynthesized Se-NPs.

Filtrate peak (cm ⁻¹)	Se-NPs peak (cm ⁻¹)	Shift (Δ cm ⁻¹)	Vibrational assignment	Associated biomolecules
3262.02	3272.65	10.63+	O–H / N–H stretching	Proteins, Polysaccharides, Phenolics
2932.25	2922.83	9.42-	C–H stretching (asymmetric)	Aliphatic chains, fatty components
2882.16	2850.73	31.43-	C–H stretching (symmetric)	Aliphatic chains, fatty components
1662.49	1654.16	8.33-	Amide I (C=O stretching)	Proteins (Peptide bonds)
1583.86	1542.28	41.58-	Amide II (N–H bending)	Proteins (Peptide bonds)
1461.67	1451.94	9.73-	C–H bending (scissoring)	Aliphatic methyl/methylene groups
1026.55	/ 1081.49 1026.94	Split	C–O / C–O–C stretching	Polysaccharides, Ethers/Alcohols
–	779.71	New peak	Se–O vibrations	Biogenic Se-NPs core

Protein-related absorptions dynamically dominated the mid-region. The Amide I (peptide C=O stretching) and Amide II (N–H bending) bands, originally located at 1662.49 and 1583.86 cm⁻¹ in the filtrate, exhibited marked downshifts to 1654.16 and 1542.28 cm⁻¹, respectively. These significant modifications support the involvement of protein residues in nanoparticle capping [81]. Additionally, the C–O and C–O–C stretching vibrations, which reflect contributions from polysaccharides in the fungal matrix [68], shifted from 1026.55 cm⁻¹ to split peaks at 1081.49 and 1026.94 cm⁻¹. Furthermore, minor signals observed in the lower frequency region (e.g., 779.71 cm⁻¹ in Se-NPs) fall within the typical selenium–oxygen (Se–O) vibrational ranges

reported for biogenic selenium nanoparticles [82]. Overall, the FTIR findings, together with the SEM–EDX elemental analysis, support the association of lipid-, polysaccharide-, and protein-related functional groups with the biogenic Se-NPs, which may contribute to particle capping and stabilization.

Analysis of X-ray Diffraction (XRD)

The XRD pattern of the biosynthesized Se-NPs showed four main reflections at $2\theta = 23.52^\circ$, 29.72° , 43.68° , and 45.41° , which match the characteristic planes of crystalline trigonal selenium (Fig. 7E; Table 6). A broad hump between 20° and 35° is also present. This feature is typical of biogenic Se-NPs and reflects the coexistence of nanocrystalline t-Se domains with an organic capping layer, as commonly reported for green-synthesized Se-NPs [83,84]. Crystallite size was estimated using the Scherrer equation based on the FWHM of the four main peaks. The calculated values ranged from 13.1 to 20.9 nm, with an average of 17.2 nm. Because instrumental broadening was not removed, these values represent lower-bound estimates in accordance with recommended practice for nano powders [85]. The peak positions and overall pattern agree with the data indexed by the International Centre for Diffraction Data (ICDD) as well as Joint Committee on Powder Diffraction Standards (JCPDS) for trigonal selenium and with recent reports on biologically synthesized Se-NPs, including the assignment of these reflections to the (100), (101), (102), and (111) planes [83,84].

Table 6. XRD peaks and crystallite size of Se-NPs synthesized by *T. longibrachiatum*

2 θ (deg)	FWHM (deg)	Crystallite size D (nm)
23.52	0.4836	16.8
29.72	0.4553	18.1
43.68	0.6521	13.1
45.41	0.4126	20.9

Dynamic light scattering (DLS) and Zeta-potential determination of Se-NPs

Dynamic light scattering was used to characterize the size and dispersion behaviour of the *T. longibrachiatum*-mediated Se-NPs. The analysis revealed a diameter (Z-average) of 120 nm with a PDI of 0.35 (Fig. 7F). Furthermore, the measured zeta potential was -25.8 mV, reflecting an 8.2 mV deviation alongside a conductivity of 3.04 mS/cm, as shown in Fig. 7G. This potential value is consistent with electrostatic stabilization in aqueous media and suggests the presence of negatively charged functional groups associated with the fungal metabolites bound to the SeNPs surface.

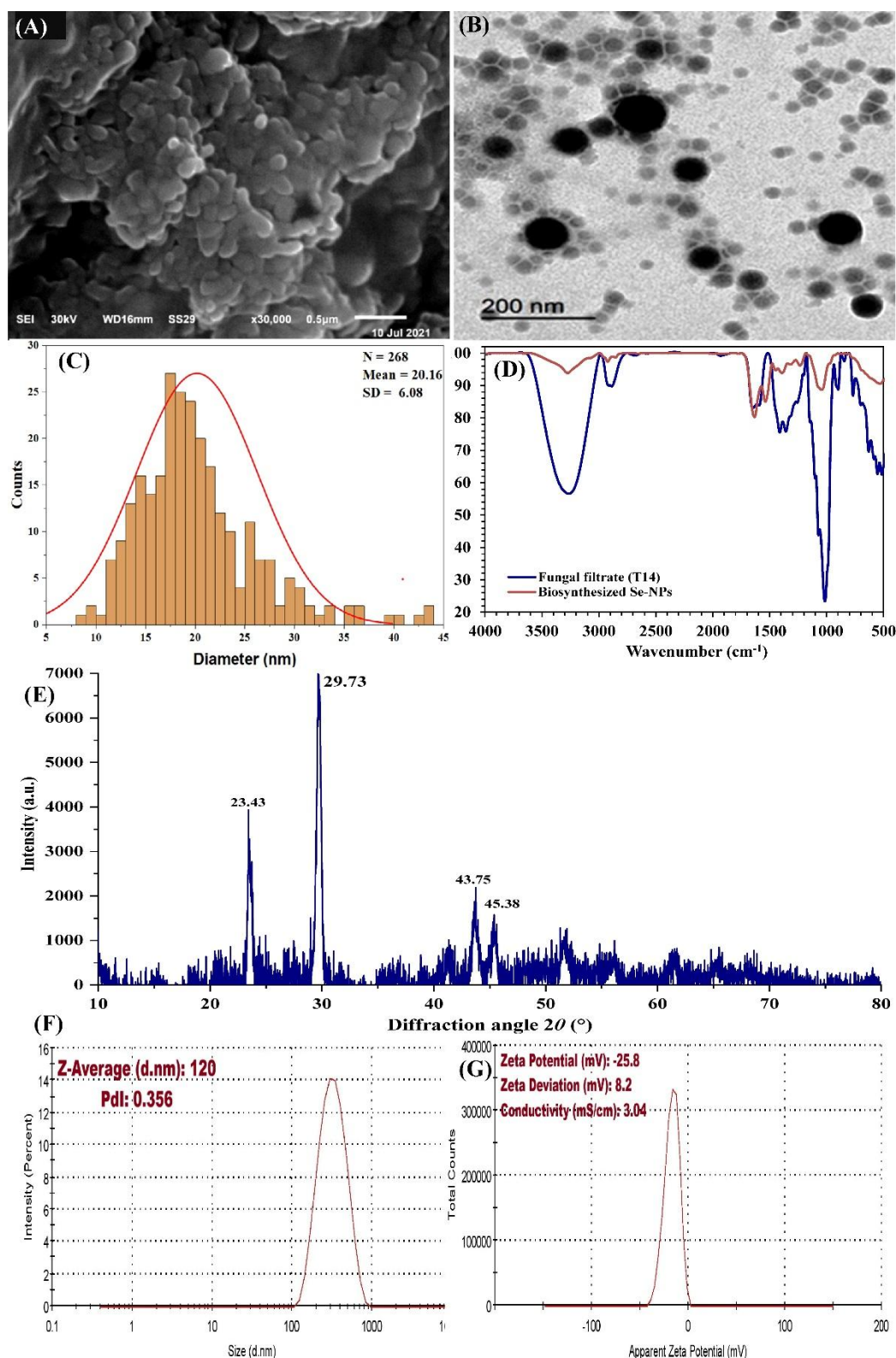


Figure. 7. Comprehensive structural and physicochemical characterization of the *T. longibrachiatum*-mediated Se-NPs. (A) SEM imaging depicting surface morphology; (B) TEM micrograph showing ultrastructural details; (C) Particle size distribution statistics derived from TEM analysis; (D) Comparative FTIR spectra of the fungal filtrate and synthesized Se-NPs;

(E) XRD diffractogram confirming the crystalline nature; (F) Hydrodynamic size distribution profile via DLS; and (G) Zeta potential distribution.

The larger hydrodynamic diameter measured by DLS compared with the TEM size is a well-established phenomenon, as DLS measures the hydrodynamic size of Se-NPs in aqueous media, which may include contributions from a hydrated biomolecular corona, whereas TEM analysis under high vacuum reflects the dimensions of the dried particles after dehydration of the surface-associated layer [86]. Comparable values have been recorded for other mycogenic Se-NPs, with literature citing Z-averages of 153 nm, PDI values of 0.27, and zeta potentials around -10.5 mV, further supporting the reproducibility of fungal synthesis [87]. Not only mycogenic but also cyanobacteria-synthesized Se-NPs have been reported to possess a negative charge around -17 mV, which indicates the high dispersity and stability of Se-NPs [88]. These physical properties align with the chemical profile found in FTIR and GC-MS. The combined results support the association of fungal biomolecules with the Se-NP surface, which may contribute to the observed particle characteristics and negative surface charge.

EDX elemental mapping of the biosynthesized Se-NPs

The elemental composition and spatial distribution of the biosynthesized Se-NPs were evaluated using EDX and elemental mapping. The EDX spectrum showed the characteristic X-ray emission peaks for selenium at approximately 1.37 keV (Se $L\alpha$) and 11.22 keV (Se $K\alpha$), confirming the presence of selenium in the synthesized nanoparticles [89]. Regarding the EDX elemental mapping analysis (Fig. 8), it clearly revealed a homogeneous distribution of selenium that spatially overlapped with biogenic elements, specifically carbon (C), oxygen (O), phosphorus (P), and other biological trace elements (e.g., K, Ca, Na, Cl). The simultaneous detection of these elements alongside selenium suggests the presence of surface-associated biological components that may contribute to the capping and stabilization of the Se-NPs [90,91]. Taken together with the FTIR findings and previous reports, the elemental profile is consistent with the association of fungal-derived biomolecules with the Se-NP preparations, which may contribute to surface capping and stabilization. Furthermore, the EDX analysis indicated a selenium content of 8.17 wt%, which was of a comparable magnitude to values previously reported for biogenic Se-NPs. For instance, Cremonini et al. [90] reported comparable selenium contents (9.26 – 11.01 wt%) for microbially synthesized Se-NPs heavily enveloped by biological macromolecules, supporting the elemental composition observed for the biogenic Se-NPs in the present study.

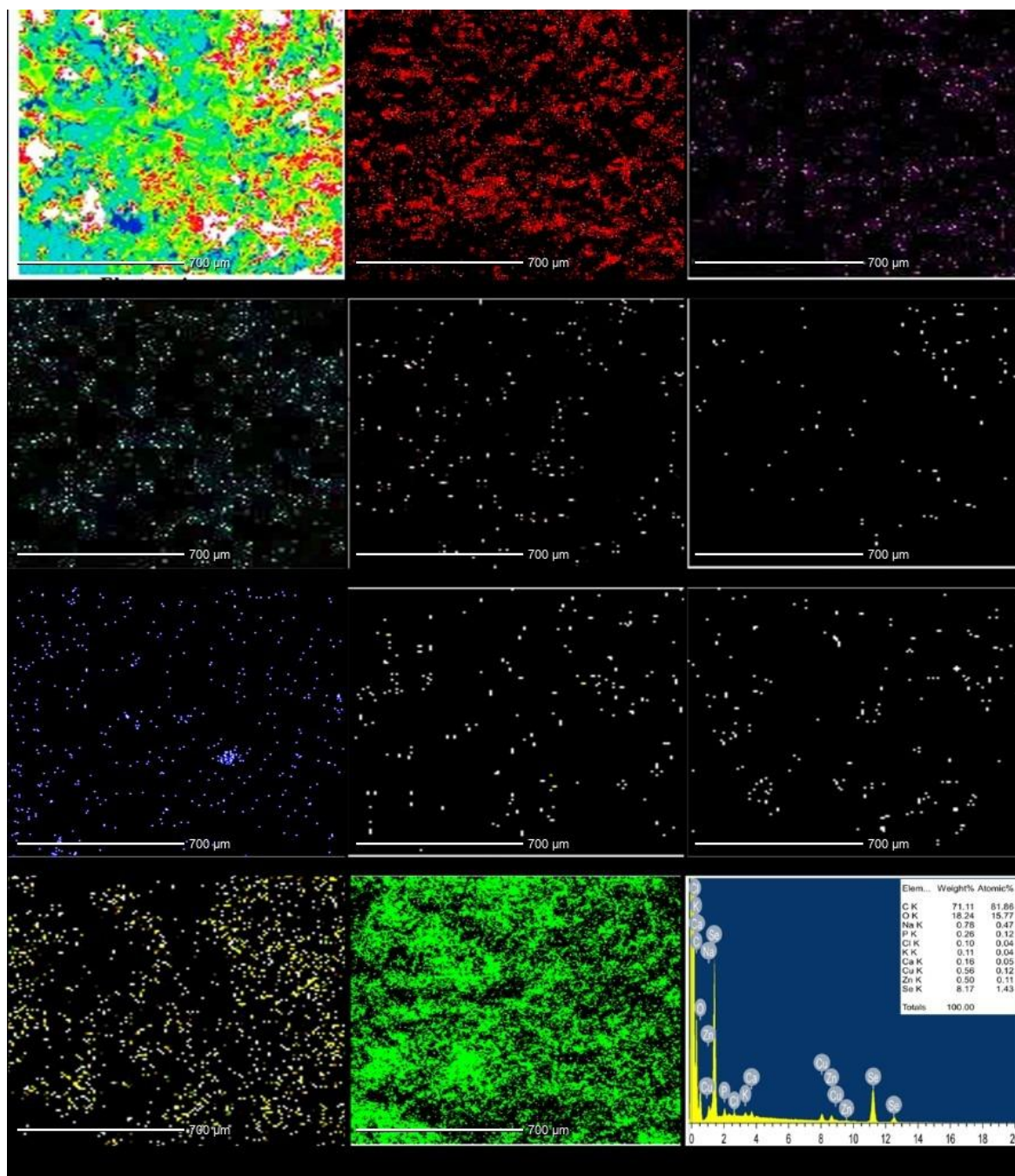


Figure 8. EDX elemental mapping of *T. longibrachiatum*-mediated Se-NPs, illustrating spatial localization and elemental composition (Scale bars = 700 μ m).

***In-vitro* cytotoxicity of biogenic Se-NPs**

In vitro cytotoxicity models are indispensable tools in cancer research, serving as cost-effective screening platforms for evaluating potential therapeutic agents [92]. To assess cytotoxicity, the MTT assay is a standard and widely used method. This assay relies on the metabolic state of viable cells, specifically the activity of mitochondrial enzymes. Therefore, the measured mitochondrial activity correlates linearly with the viable cell count [93]. In the current study, the cytotoxicity of biogenic Se-NPs was profiled against human malignant models representing

breast (MCF-7), hepatic (HepG2), and colon (HCT-116) carcinomas, in addition to non-malignant WI-38 fibroblasts. As illustrated in the concentration–response profiles (Fig. 9), Se-NPs (1.56–100 µg/mL) induced a graded, dose-dependent reduction in cell viability. The calculated IC₅₀ values (Table 7) revealed a distinct selectivity pattern. MCF-7 cells showed the highest sensitivity to Se-NPs (IC₅₀ = 8.42 ± 0.6 µg/mL), followed by HepG2 cells (IC₅₀ = 14.34 ± 1.2 µg/mL), whereas HCT-116 cells showed comparatively lower sensitivity (IC₅₀ = 31.79 ± 2.1 µg/mL). One-way ANOVA followed by Tukey’s HSD test showed significant cytotoxicity relative to the untreated control from 1.56 µg/mL in all three cancer cell lines, whereas significance in WI-38 fibroblasts was observed from 3.125 µg/mL (Table 8). In contrast, non-malignant WI-38 fibroblasts showed lower sensitivity to Se-NPs (IC₅₀ = 81.95 ± 4.3 µg/mL) than the three cancer cell lines.

This differential cytotoxicity profile, characterized by greater sensitivity of the cancer cell lines and lower sensitivity of WI-38 fibroblasts, is consistent with previous studies on biogenic Se-NPs reporting comparatively lower cytotoxic effects in non-cancerous models than in breast and liver cancer cell lines [43,94,95]. Comparison with the reference drug doxorubicin (DOX) further highlighted the differential cytotoxicity profile of Se-NPs. Although DOX showed strong cytotoxic activity across the cancer cell lines (IC₅₀ = 3.04–7.44 µg/mL), it also exhibited substantial cytotoxicity toward non-malignant WI-38 fibroblasts (IC₅₀ = 8.35 ± 0.7 µg/mL), resulting in a narrower differential between malignant and non-malignant cells. This contrast was further reflected in the Selectivity Index (SI).

Se-NPs achieved SI values of 9.7, 5.7, and 2.6 for MCF-7, HepG2, and HCT-116, respectively, demonstrating differential *in vitro* selectivity across the tested cancer cell lines. The observed differential cytotoxicity may be related to previously reported Se-NP mechanisms involving ROS generation, mitochondrial dysfunction, and apoptosis, which have been associated with differential responses between malignant and non-malignant cells [21].

Table 7. IC₅₀ values (µg/mL) of *T. longibrachiatum*-mediated Se-NPs across the tested malignant and non-malignant cell lines after 24 h of exposure, with doxorubicin as the reference standard.

Component	<i>In vitro</i> cytotoxicity IC ₅₀ (µg/mL)			
	WI-38	HCT-116	HepG2	MCF-7
DOX	8.35±0.7	7.44±0.5	6.14±0.4	3.04±0.2
SeNP	81.95±4.3	31.79±2.1	14.34±1.2	8.42±0.6
SI (SeNP)	-	2.58	5.72	9.74

* SI = IC₅₀(WI-38)/IC₅₀(cancer); higher SI indicates greater selectivity toward the corresponding cancer cell line relative to WI-38 fibroblasts.

Table 8. One-way ANOVA and Tukey-adjusted significance vs control by cell line for DOX and biogenic Se-NPs (24 h MTT).

Cell line	DOX		Se-NPs	
	ANOVA F	Significant vs 0 $\mu\text{g/mL}$ (Tukey)	ANOVA F	Significant vs 0 $\mu\text{g/mL}$ (Tukey)
WI-38	4212.2	$\geq 1.56 \mu\text{g/mL}$	2899.8	$\geq 3.125 \mu\text{g/mL}$
HCT-116	12861.9	$\geq 1.56 \mu\text{g/mL}$	10728.4	$\geq 1.56 \mu\text{g/mL}$
HepG2	26414.9	$\geq 1.56 \mu\text{g/mL}$	6559.0	$\geq 1.56 \mu\text{g/mL}$
MCF-7	7300.4	$\geq 1.56 \mu\text{g/mL}$	10623.2	$\geq 1.56 \mu\text{g/mL}$

Significance vs control assessed by one-way ANOVA with Tukey's HSD (family-wise $\alpha = 0.05$); all ANOVAs $P < 0.0001$; Bartlett's test met. The lower bound of each significant range is the minimum effective dose (MED).

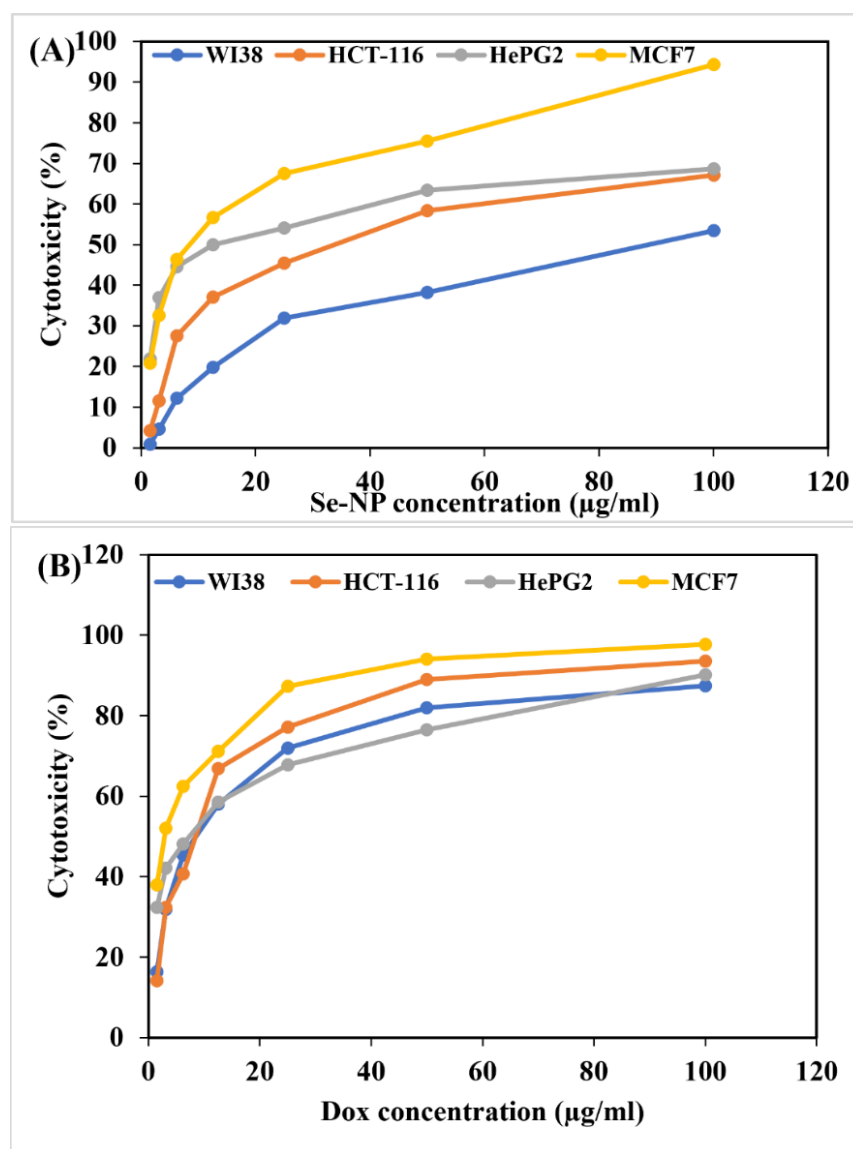


Figure 9. Dose-response cytotoxicity curves of (A) the biosynthesized Se-NPs and (B) doxorubicin (DOX) against non-malignant (WI-38) and cancer (HCT-116, HepG2, and MCF-7) cell lines after 24 h of treatment (MTT assay). DOX was used as the reference chemotherapeutic standard.

Conclusions

This study highlights the biogenic synthesis of selenium nanoparticles (Se-NPs) using *Trichoderma longibrachiatum* filtrate as an eco-friendly platform. Through FCCCD-based statistical optimization, the production yield was successfully maximized to 15.53 mM. Comprehensive physicochemical characterization revealed predominantly spherical Se-NPs (~20 nm) with a crystalline trigonal phase and a zeta potential of −25.8 mV, indicating moderate colloidal stability. *In vitro* biological evaluations revealed that these biogenic Se-NPs possess selective cytotoxicity against malignant cell lines, exhibiting the strongest efficacy against breast (MCF-7; IC₅₀ = 8.42 µg/mL) and hepatic (HepG2; IC₅₀ = 14.34 µg/mL) cells. The cytotoxicity profile showed differential selectivity among the tested cell lines, with the highest selectivity index observed for MCF-7, followed by HepG2 and HCT-116. Ultimately, this *Trichoderma*-mediated approach offers a sustainable pathway for producing functionalized nanomaterials, and the resulting Se-NPs warrant further investigation as potential anticancer agents.

List of abbreviations

ANOVA: Analysis of variance
ATCC: American Type Culture Collection
C.I.: Confidence interval
DFA: Desirability function analysis
DLS: Dynamic light scattering
DMSO: Dimethyl sulfoxide
DOX: Doxorubicin
EDX: Energy-dispersive X-ray spectroscopy
FCCCD: Face-centered central composite design
FTIR: Fourier-transform infrared spectroscopy
FWHM: Full width at half maximum
GC-MS: Gas chromatography-mass spectrometry
GM: Geometric mean
GSD: Geometric standard deviation
HCT-116: Human colorectal carcinoma cell line
HepG2: Human hepatocellular carcinoma cell line
HSD: Honestly significant difference
IC50: Half-maximal inhibitory concentration
ICDD: International Centre for Diffraction Data

JCPDS: Joint Committee on Powder Diffraction Standards
MCF-7: Human breast adenocarcinoma cell line
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS: Phosphate-buffered saline
PCA: Plate count agar
PDA: Potato dextrose agar
PDB: Potato dextrose broth
PDI: Polydispersity index
PRESS: Predicted residual error sum of squares
ROS: Reactive oxygen species
RSM: Response surface methodology
SD: Standard deviation
SEM: Scanning electron microscopy
Se-NPs: Selenium nanoparticles
SI: Selectivity index
SPR: Surface plasmon resonance
SRTA-City: City of Scientific Research and Technological Applications
TEM: Transmission electron microscopy
TIC: Total ion chromatogram
UV-Vis: Ultraviolet–visible
WI-38: Human non-malignant diploid fibroblast cell line
XRD: X-ray diffraction

Declarations
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materials. K.A.H., H.M.S., K.M.G., and Y.M.S. were responsible for data curation, investigation, and validation. All authors read and approved the final manuscript.

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