

Article

Trichoderma* as a Biological Nanofactory: Metabolic Control of Silver Nanoparticle Biosynthesis and Activity Against the Grapevine Trunk Pathogen *Neofusicoccum parvum

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Abstract

Grapevine trunk diseases, particularly those caused by *Neofusicoccum parvum*, represent a major threat to vineyard productivity and are increasingly difficult to control with conventional fungicides. Green synthesis of silver nanoparticles (AgNPs) using biocontrol fungi offers a promising alternative, but the factors governing the efficiency and bioactivity of biogenic nanoparticles remain poorly understood. Here, three *Trichoderma* species (*T. harzianum*, *T. asperellum* and *T. virens*) were evaluated as biological nanofactories for AgNP production. Cell-free fungal filtrates were used to synthesize AgNPs, which were characterized by UV-visible spectrophotometry, Dynamic Light Scattering (DLS) and transmission electron microscopy, while fungal redox metabolism was assessed using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assays and HPLC profiling of extracellular metabolites. AgNPs were tested against two isolates of *N. parvum* in vitro. The *Trichoderma* strains differed markedly in nanoparticle yield, size and antifungal activity, with *T. harzianum* T0 producing the highest amounts of small, well-dispersed AgNPs that exerted a strong fungistatic effect on *N. parvum*. Nanoparticle production correlated with antioxidant capacity and the abundance of redox-active metabolites. Integration of these parameters into a Fungal Nanofactory Efficiency Index (FNEI) revealed that nanoparticle bioactivity depends on both dose and biological origin. These results demonstrate that fungal metabolism is a key determinant of biogenic nanoparticle performance and identify *Trichoderma* as a platform for sustainable nanotechnology-based control of grapevine trunk pathogens.

Keywords: *Trichoderma*; silver nanoparticles; green synthesis; fungal redox metabolism; biocontrol; grapevine trunk diseases; *Neofusicoccum parvum*; nanobiotechnology



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1. Introduction

Viticulture underpins global wine production; however, grapevine trunk diseases (GTDs), particularly those associated with Esca and Botryosphaeriaceae complexes, severely compromise both yield and fruit quality. Field studies have reported yield losses of 34–36% associated with rugose wood and Grapevine leafroll-associated virus [1], while surveys in major European wine-producing regions indicate that Esca-related pathogens affect

8–19% of vines in Italy, while France shows increasing and region-dependent incidence levels [2]. Even low proportions of Esca-affected fruit (~5%) are sufficient to impair wine sensory quality, while Esca infection is associated with reduced catechin, epicatechin and anthocyanin contents and decreased proanthocyanidin polymerization in grape skins [3]. Infected grapes also show altered sugar, acidity and nitrogen levels, together with delayed ripening and impaired berry maturation [1,4,5].

The economic consequences of GTDs are equally severe. Costs associated with vine replacement and disease management are high, and regional surveys have documented substantial infection rates in commercial vineyards [4,6,7]. Among the most damaging GTD pathogens, *Neofusicoccum parvum* stands out for its aggressive colonization of woody tissues and its central role in Botryosphaeria dieback, making it a major target for sustainable disease control strategies.

Current management of GTDs relies largely on chemical fungicides, including triazoles, strobilurins and copper compounds. These products are widely detected in vineyard soils and aquatic ecosystems [8], and copper concentrations in vineyard soils often exceed European Union limits [9]. Moreover, many fungicides persist in the environment for extended periods (median dissipation time $\approx$ 54 days) and have documented toxic effects on non-target organisms such as pollinators, soil microbiota, fish and invertebrates. Repeated fungicide applications also promote the rapid emergence of resistant pathogen populations through target site mutations and efflux-based mechanisms.

As a result, there is growing interest in sustainable alternatives for GTD management. Biocontrol agents, particularly *Trichoderma* species, as well as antimicrobial peptides and botanical fungicides, have shown promising efficacy with a reduced environmental footprint [10]. More recently, nano-enabled approaches, including nanoparticle formulations and RNA- or peptide-based technologies, have emerged as innovative tools for plant disease control, although their ecological behavior and long-term safety remain under investigation.

One particularly promising strategy is the green synthesis of silver nanoparticles (AgNPs), which harnesses the antimicrobial properties of silver through biological reduction processes rather than toxic chemical reagents. In this approach, extracellular metabolites secreted by *Trichoderma* (notably phenolics, flavonoids and other redox-active compounds) reduce silver ions (Ag^+) from silver nitrate (AgNO_3) to elemental silver (Ag^0), generating stable AgNPs [5]. These biogenic nanoparticles are known to inhibit a broad range of phytopathogens, but their biological activity varies widely depending on the organism and conditions used for synthesis.

Several studies have reported the biosynthesis of silver nanoparticles (AgNPs) using fungal systems, including species of *Trichoderma*. Reviews describing fungal-mediated nanoparticle synthesis indicate that multiple *Trichoderma* species, such as *T. asperellum*, *T. reesei*, *T. harzianum*, *T. viride*, *T. longibrachiatum* and *T. virens*, are capable of extracellularly reducing Ag^+ ions into metallic Ag^0 nanoparticles through redox-active metabolites and enzymatic systems, including NADH-dependent nitrate reductases [11,12]. The resulting nanoparticles are typically spherical, with sizes ranging from approximately 5 to 50 nm, and exhibit strong antimicrobial activity against several plant pathogenic fungi such as *Fusarium*, *Botrytis*, *Rhizoctonia* and *Sclerotinia* [11]. These biologically synthesized nanoparticles have been proposed as environmentally friendly alternatives to chemically synthesized nanomaterials due to their lower toxicity, reduced production costs and potential compatibility with existing *Trichoderma*-based biocontrol strategies. However, despite these promising findings, the mechanistic understanding of fungal nanoparticle biosynthesis and the reproducibility of biological nanofactories remain incomplete, highlighting the need for further studies linking fungal metabolism, nanoparticle formation and biological performance.

In this context, the present study explores the ability of *Trichoderma* strains with contrasting biological characteristics to function as fungal nanofactories for AgNP biosynthesis and evaluates how metabolic differences influence nanoparticle production and antifungal activity.

Despite the growing literature on green AgNPs, most studies remain descriptive, reporting antimicrobial activity without addressing how the metabolic state of the producing organism determines nanoparticle formation and function. Understanding this relationship is essential for moving toward rational and predictive use of nanomaterials in plant disease control.

The objective of this study was to evaluate different *Trichoderma* strains with contrasting biocontrol phenotypes as biological nanofactories for the green synthesis of silver nanoparticles (AgNPs). Specifically, we aimed to determine how differences in extracellular redox metabolism and metabolite composition influence nanoparticle biosynthesis and antifungal activity against the grapevine trunk pathogen *Neofusicoccum parvum*. By integrating biochemical, nanomaterial, and biological data, we sought to establish a framework linking fungal metabolic traits with the efficiency and bioactivity of biogenic nanoparticles.

2. Materials and Methods

2.1. Fungal Strains and Culture Conditions

Four *Trichoderma* isolates were used in this study and are referred to throughout the manuscript by their original laboratory codes (T0, T2, T3 and T5). Species identity was established by ITS sequencing and GenBank comparison as reported in Gómez-Garay et al. [13], with the following assignments: *Trichoderma harzianum* (T0: HQ149776.1; T2: KC819133.1), *Trichoderma asperellum* (T3: JX677934.1) and *Trichoderma virens* (T5: KF150223.1). This nomenclature is maintained to ensure consistency with the nanomaterial, biochemical and metabolomic datasets, while molecular identification provides full taxonomic traceability.

Strains of *Trichoderma harzianum*, *T. asperellum* and *T. virens* were selected for nanoparticle biosynthesis based on their contrasted biocontrol phenotypes against *Neofusicoccum parvum* reported in Gomez-Garay et al. [13], which identified *T. harzianum* as the most effective suppressor of trunk disease symptoms, *T. asperellum* as moderately active, and *T. virens* as highly antagonistic in vitro but phytotoxic in planta.

Two isolates of the grapevine trunk pathogen *Neofusicoccum parvum* (JL396 and JL445) from the IRTA (Barcelona, Spain) microbial collection were used in this study. Both isolates were originally obtained from symptomatic *Vitis vinifera* wood and have been previously characterized for phytotoxic metabolite production and pathogenicity by Martos et al. [14].

All strains were maintained on Potato Dextrose Agar (PDA; Condalab, Madrid, Spain) at 22 ± 2 °C in the dark and subcultured every two weeks. For liquid cultures, five 5 mm mycelial plugs from 5-day-old PDA cultures were inoculated into 100 mL Potato Dextrose Broth (PDB) and incubated at 25 °C with shaking at 120 rpm for 7 days.

2.2. Preparation of *Trichoderma* Cell-Free Filtrates

After incubation, fungal biomass was removed by filtration through sterile Miracloth followed by centrifugation at $\sim 10,000 \times g$ for 15 min at 4 °C. Supernatants were filtered through 0.22 µm membrane filters to obtain sterile cell-free filtrates, which were used for nanoparticle biosynthesis and biochemical assays.

2.3. Green Synthesis of Silver Nanoparticles

Silver nanoparticles (AgNPs) were synthesized by mixing equal volumes of *Trichoderma* cell-free filtrates and 1 mM aqueous AgNO₃ (Sigma-Aldrich, St. Louis, MO, USA). Reactions were incubated at room temperature (22–25 °C) in the dark to prevent photo-

chemical reduction of silver ions. Nanoparticle formation was monitored visually and by UV–Vis spectroscopy. Controls lacking either AgNO₃ or fungal filtrate were included.

2.4. Characterization of Silver Nanoparticles

AgNP formation was confirmed by recording UV–visible absorbance spectra between 300 and 700 nm. The characteristic surface plasmon resonance of silver nanoparticles was monitored, and absorbance at 400 nm (A_{400}) was used as a proxy for nanoparticle yield.

The hydrodynamic diameter of AgNPs was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Measurements were performed at 25 °C in triplicate for each *Trichoderma*-derived nanoparticle suspension.

AgNP morphology was examined by transmission electron microscopy (TEM). Nanoparticle suspensions were diluted in ultrapure water, deposited onto carbon-coated copper grids and air-dried. Transmission electron microscopy (TEM) analysis was performed using a JEOL JEM-2100 microscope (JEOL Ltd., Tokyo, Japan) at the Centro Nacional de Microscopía Electrónica (Madrid, Spain).

2.5. HPLC Profiling of *Trichoderma* Extracellular Metabolites

Extracellular metabolites from *Trichoderma* cell-free filtrates were analyzed by high-performance liquid chromatography (HPLC) at the CAI Mass Spectrometry Unit (Universidad Complutense de Madrid). Samples were separated by reverse-phase chromatography using a C18 column and a water–acetonitrile gradient. Compounds were detected by UV absorbance, and chromatographic profiles were recorded as retention time and peak area. Major peaks were identified based on their UV spectra and relative retention times and used for comparative profiling of redox-active metabolite families, including phenolic acids, flavonoid-like aromatics and quinone-related compounds.

2.6. Antioxidant (Reducing) Capacity

The antioxidant capacity of *Trichoderma* cell-free filtrates was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay as described by Brand-Williams et al. [15]. Briefly, aliquots of fungal filtrates were mixed with a freshly prepared DPPH solution and incubated in the dark at room temperature. After 30 min, absorbance was measured at 517 nm using a UV–visible spectrophotometer.

DPPH radical scavenging activity was calculated according to the following equation:

$$\text{DPPH scavenging (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where A_{control} is the absorbance of the DPPH solution without fungal extract and A_{sample} is the absorbance in the presence of the *Trichoderma* filtrate.

2.7. Fungal Nanofactory Efficiency Index (FNEI)

To integrate the biochemical and nanomaterial outputs of each *Trichoderma* strain into a single quantitative descriptor, a Fungal Nanofactory Efficiency Index (FNEI) was defined. The index combines nanoparticle yield, reducing capacity and particle size according to the following equation:

$$\text{FNEI} = \frac{A_{400} \times \text{DPPH}}{D_{\text{mean}}}$$

where A_{400} is the absorbance at 400 nm from UV–visible spectroscopy (proxy for AgNP yield), DPPH is the radical scavenging activity (%) of the fungal filtrate, and D_{mean} is the mean hydrodynamic diameter (nm) of the AgNPs determined by DLS.

This index provides an integrated measure of the efficiency with which each *Trichoderma* strain converts its redox-active metabolome into functional antimicrobial nanoparticles.

2.8. Antifungal Activity of AgNPs

The antifungal activity of *Trichoderma*-derived AgNPs was evaluated against *Neofusicoccum parvum* isolates JL396 and JL445 on PDA supplemented with AgNPs at 0, 0.05, 0.125, 0.5 and 1 mg mL^{−1}. Plates were inoculated with 5-mm-diameter mycelial plugs taken from the margin of actively growing colonies and incubated at 25 °C. Radial mycelial growth was measured, and percentage inhibition was calculated relative to untreated controls.

Percentage inhibition of radial growth (PIRG) was calculated as:

$$\text{PIRG (\%)} = \frac{R_{\text{control}} - R_{\text{treated}}}{R_{\text{control}}} \times 100$$

where R_{control} is the radial growth of the pathogen on control plates and R_{treated} is the radial growth in the presence of AgNPs.

2.9. Statistical and Multivariate Analyses

All experiments were performed with three biological replicates, and data are presented as mean ± standard deviation. Basic statistical analyses were performed using Microsoft Excel 2021. Differences among treatments were evaluated by one-way analysis of variance (ANOVA), and mean separation was performed using post hoc multiple comparison tests (Tukey's HSD and Duncan's test) at a significance level of $p < 0.05$.

Correlation analyses were conducted to examine relationships among antioxidant capacity (DPPH), AgNP yield (A_{400}), particle size (DLS), Fungal Nanofactory Efficiency Index (FNEI) and antifungal activity. Correlation coefficients were calculated using Pearson's correlation analysis ($n = 4$ strains).

Data visualization and multivariate representations were generated using Microsoft Excel (Microsoft Corp., Redmond, WA, USA), Plotly (<https://plotly.com/>) and Flourish (<https://flourish.studio/>) (accessed on 10 December 2025). Bubble plots were used to visualize nanofactory efficiency landscapes, and heatmaps were generated from normalized HPLC peak areas and biological activity data.

Three-dimensional response surface visualizations were generated using Plotly to illustrate trends in *Neofusicoccum parvum* growth inhibition across the experimental FNEI and AgNP dose space. Surfaces were constructed by interpolation between experimentally measured inhibition values obtained at discrete FNEI levels and AgNP concentrations. No formal response surface methodology (RSM) optimization or polynomial model fitting was performed; the surfaces are intended as descriptive visualizations rather than predictive statistical models.

3. Results

3.1. *Trichoderma* Strains Differ Markedly in Their Capacity to Generate Silver Nanoparticles

The four selected *Trichoderma* strains (*T. harzianum* T0 and T2, *T. asperellum* T3, and *T. virens* T5) were all able to reduce Ag⁺ ions and generate silver nanoparticles, as evidenced by the rapid development of a characteristic brown coloration in the reaction mixtures (Figure 1). This color change, which intensified with increasing AgNO₃ concentration, is consistent with the formation of colloidal AgNPs through surface plasmon resonance and was observed within the first hour of incubation for the most active strains.

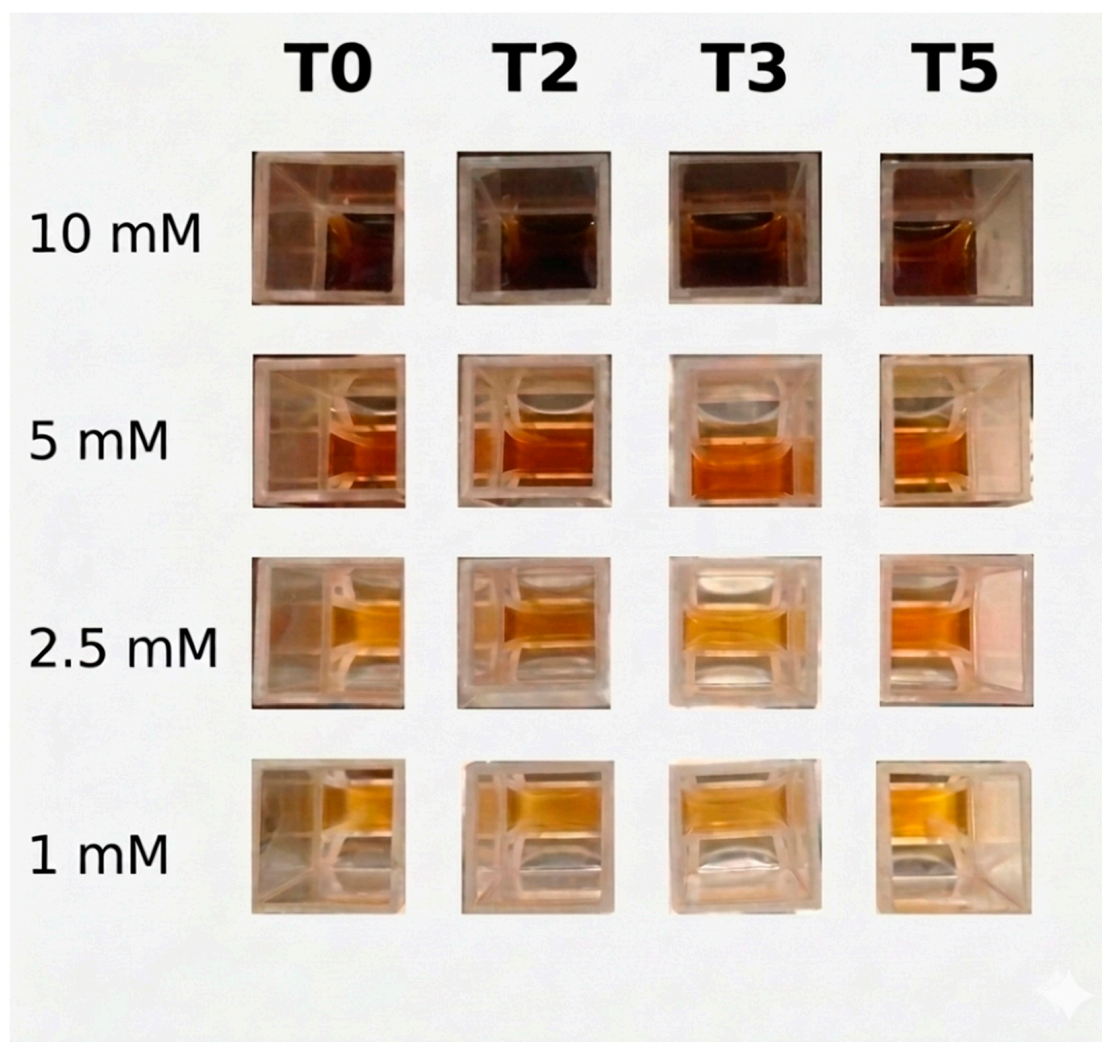


Figure 1. Biosynthesis and characterization of silver nanoparticles (AgNPs) produced by *Trichoderma* strains. Representative photograph of the reaction mixtures corresponding to four *Trichoderma* strains (T0, T2, T3, T5) incubated with increasing concentrations of AgNO_3 (1–10 mM). The progressive development of a brown coloration indicates the reduction of Ag^+ ions and the formation of AgNPs, with intensity varying according to both the fungal strain and the AgNO_3 concentration.

Quantitative monitoring by UV–visible spectrophotometry revealed pronounced strain-dependent differences in nanoparticle formation kinetics (Figure 2). In all strains, increasing AgNO_3 concentration resulted in higher absorbance at 400 nm (A_{400}), but the magnitude and rate of this increase varied markedly among isolates. T0 (*T. harzianum*) showed the steepest increases in A_{400} over time, particularly at 10 mM AgNO_3 , indicating rapid and sustained nanoparticle formation. In contrast, T3 displayed intermediate kinetics, whereas T5 showed only modest increases in A_{400} , even at the highest silver concentration. These differences were confirmed by comparison of final A_{400} values after 5 h of incubation (Figure 3). T0 produced the highest AgNP yield, followed by T2 and T3, while T5 consistently generated significantly lower nanoparticle levels ($p < 0.05$).

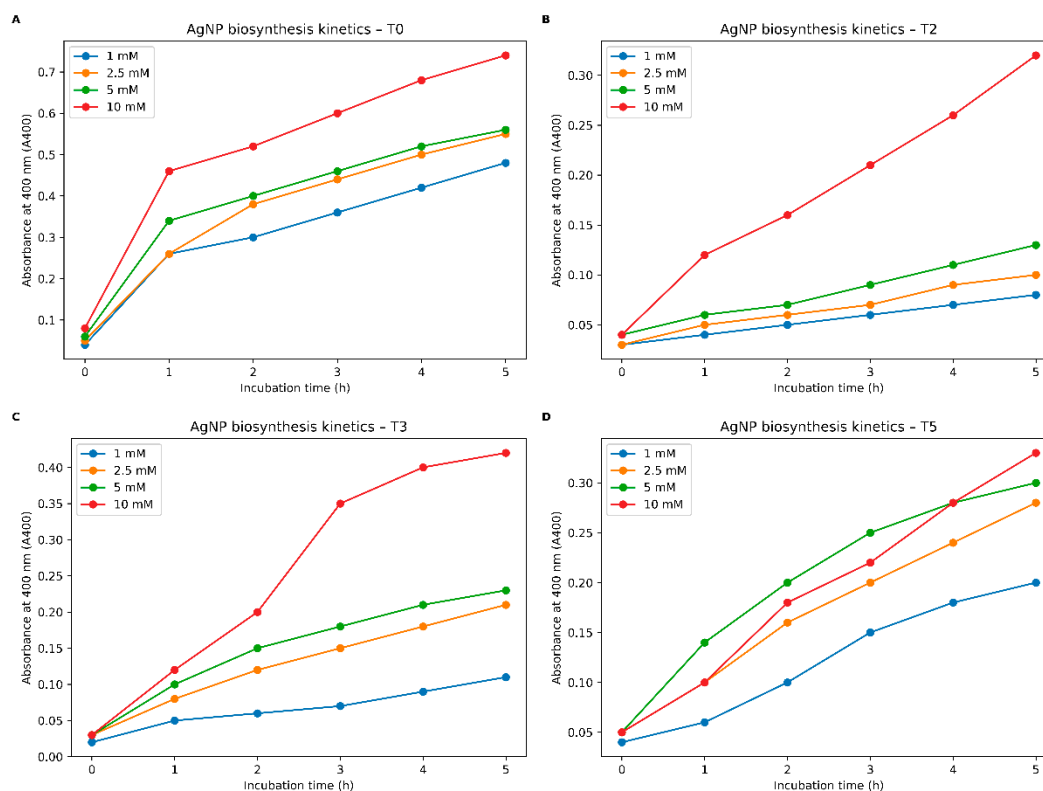


Figure 2. Time-course of silver nanoparticle (AgNP) biosynthesis by *Trichoderma* strains monitored by UV-visible spectrophotometry. Absorbance at 400 nm (OD_{400}), indicative of AgNP formation, is plotted as a function of incubation time (hours). Each panel corresponds to a different *Trichoderma* strain (A: T0; B: T2; C: T3 and D: T5). For each strain, AgNP synthesis was evaluated at increasing silver nitrate ($AgNO_3$) concentrations (1, 2.5, 5, and 10 mM), as indicated in the legend. The x -axis represents incubation time (h), and the y -axis represents absorbance at 400 nm (OD_{400}).

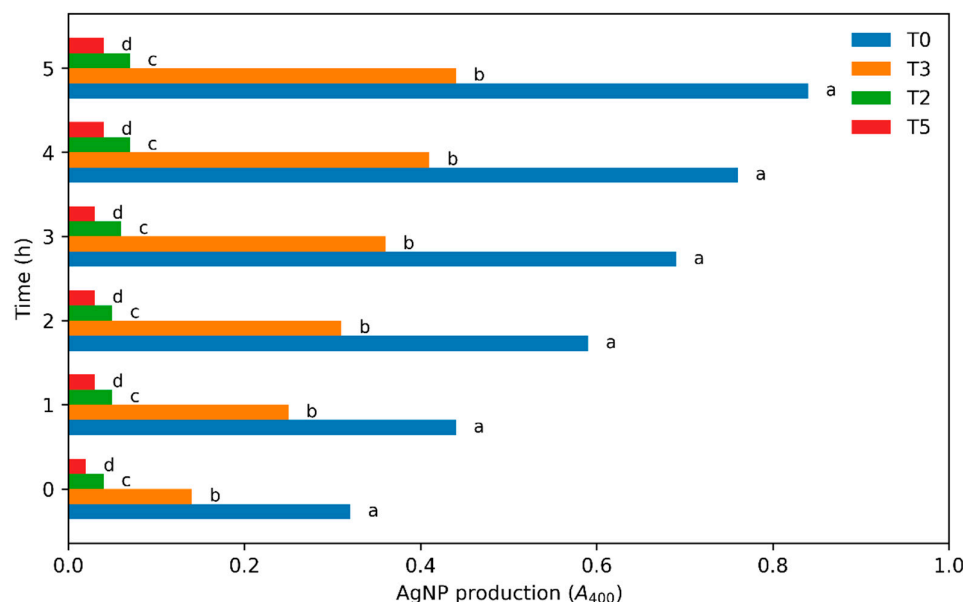


Figure 3. Quantitative comparison of silver nanoparticle (AgNP) production by *Trichoderma* strains based on UV-visible spectrophotometry. AgNP production is expressed as absorbance at 400 nm (A_{400}) and measured at different incubation times (0–5 h). Bars represent mean A_{400} values for each *Trichoderma* strain (T0, T2, T3, and T5), as indicated in the legend. The x -axis represents AgNP production (A_{400}), and the y -axis represents incubation time (h). Different letters indicate statistically significant differences among strains at the same incubation time ($p < 0.05$).

Transmission electron microscopy further validated these differences at the nanostructural level (Figure 4). AgNPs synthesized by T0 were predominantly spherical, well dispersed, and within the nanometric size range, whereas nanoparticles produced by T3 and T5 appeared more heterogeneous and aggregated, consistent with their lower A_{400} values.

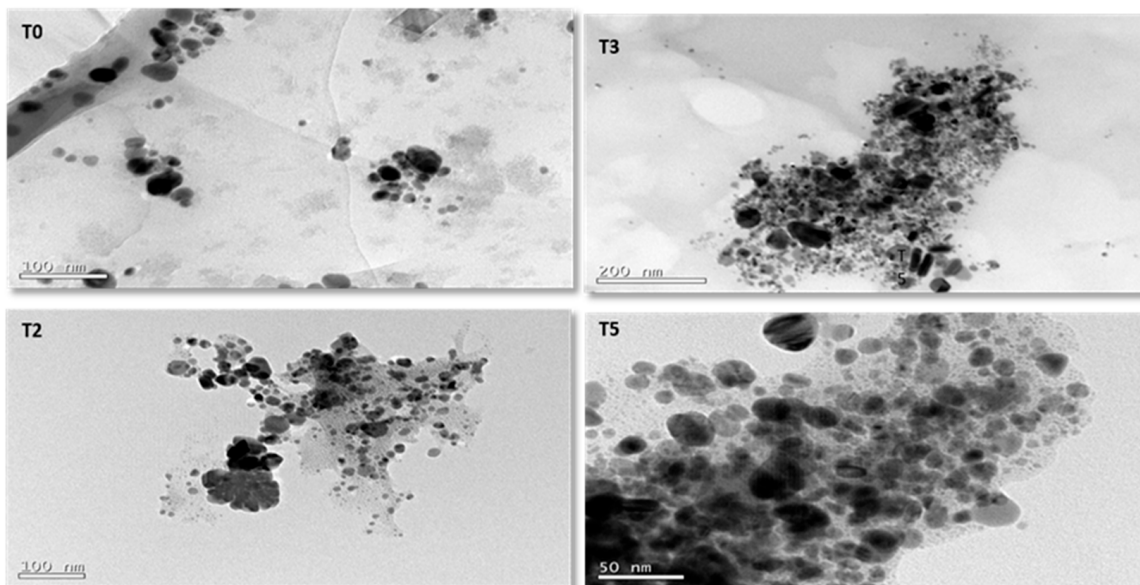


Figure 4. Biosynthesis and characterization of silver nanoparticles (AgNPs) produced by *Trichoderma* strains. Transmission electron microscopy (TEM) images of AgNPs synthesized by the different strains (T0, T2, T3, and T5), showing strain-dependent differences in nanoparticle size, morphology, and aggregation. Scale bars are indicated in each micrograph.

3.2. Redox-Active Metabolites and Antioxidant Capacity Underpin Strain-Dependent AgNP Biosynthesis

Marked differences in the chemical reducing capacity of the *Trichoderma* strains were revealed by the DPPH radical scavenging assay (Figure 5A). T0 exhibited the highest antioxidant activity (89.3%), followed by T3 (75.0%), whereas T2 showed a substantially lower value (39.3%). In contrast, T5 displayed virtually no detectable radical scavenging activity. This pattern closely mirrored the strain-dependent differences observed in AgNP production, suggesting a direct link between fungal redox metabolism and nanoparticle biosynthesis.

A quantitative correlation analysis revealed a strong positive relationship between antioxidant capacity and AgNP production ($r = 0.79$), indicating that differences in fungal redox activity largely account for the observed variability in nanoparticle yield.

Chromatographic profiling of extracellular filtrates further supported this relationship (Figure 5B). A total of 75 metabolites were tentatively detected across the four *Trichoderma* strains. These compounds included alcohols, organic acids and esters, aromatic compounds and terpenoid derivatives. Based on their chemical features, several metabolites were grouped into redox-active families, including phenolic acids, flavonoid-like aromatic compounds and quinone-related molecules, which are known to participate in electron transfer reactions involved in Ag^+ reduction.

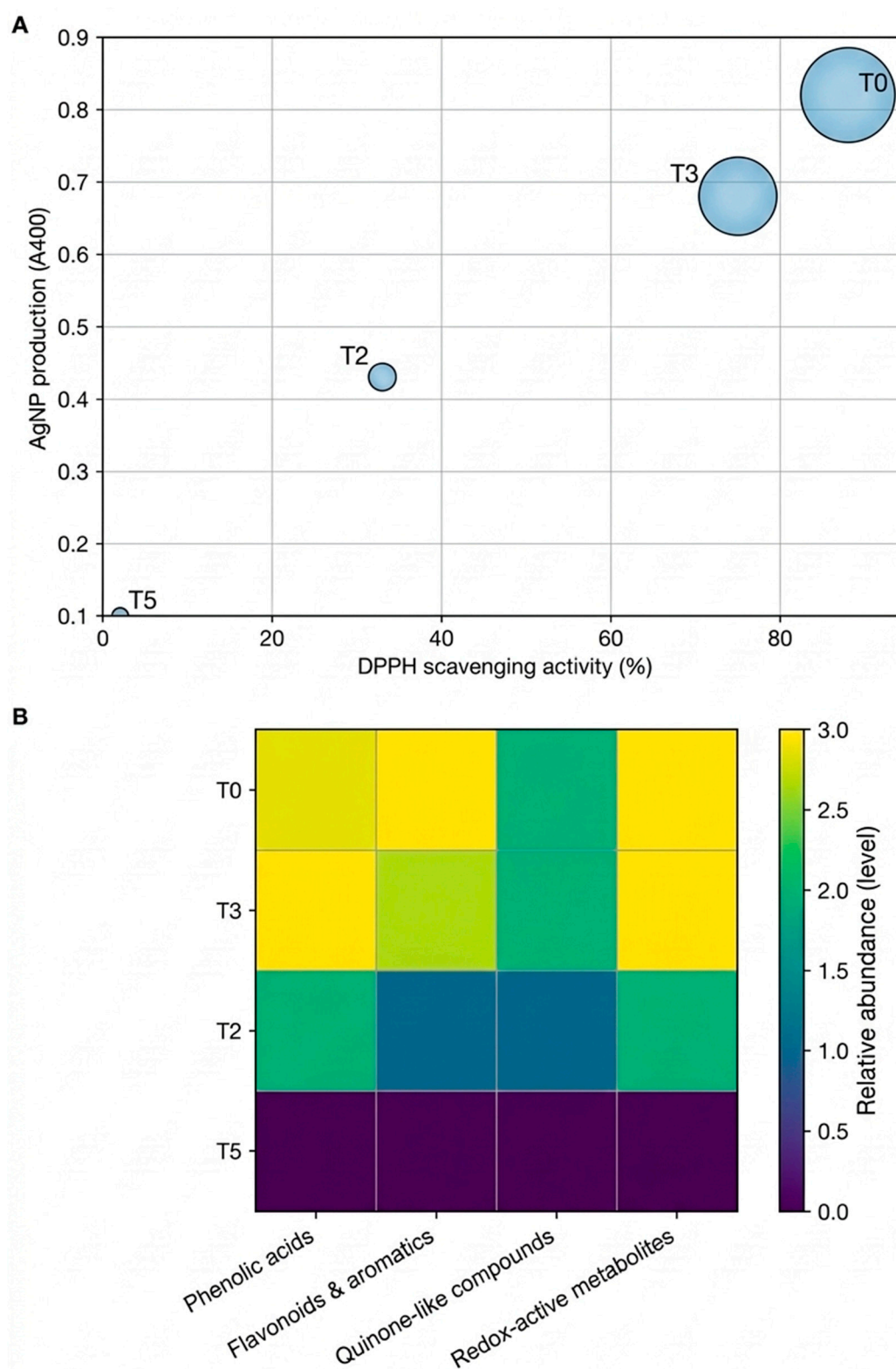


Figure 5. Redox capacity of *Trichoderma* strains (T0, T2, T3, and T5) and their relationship with silver nanoparticle (AgNP) biosynthesis. **(A)** Antioxidant activity of extracellular filtrates determined by the DPPH radical scavenging assay correlation analysis. Bubble size represents FNEI (nanofactory efficiency index). **(B)** Heatmap summarizing the relative abundance of redox-active carbon fractions in extracellular metabolites, highlighting enrichment in strains with higher reducing activity. Color intensity reflects relative signal levels (low 0 to high 3).

Clear strain-dependent differences were observed in the distribution of these metabolite classes. T0 and T3 were strongly enriched in redox-active metabolite families, whereas T2 showed intermediate levels. In contrast, the extracellular filtrate of T5 was largely depleted in these compounds and was mainly composed of terpenoid and hydrocarbon-like metabolites, which generally lack strong electron-donating functional groups. This compositional pattern is consistent with the negligible antioxidant activity detected for T5 and its limited ability to generate AgNPs.

To integrate chemical, nanomaterial and biological data into a single functional framework, a Fungal Nanofactory Efficiency Index (FNEI) was constructed for each *Trichoderma* strain by combining three key variables: (i) extracellular redox capacity (DPPH scavenging activity), (ii) abundance of redox-active metabolite families, and (iii) AgNP yield (A_{400}). This multidimensional integration allowed a direct comparison of the ability of each strain to convert its metabolic potential into functional antimicrobial nanomaterials.

Projection of the four strains in the FNEI space revealed a clear stratification of nanofactory performance (Figure 5A). T0 occupied the upper-right region of the plot, characterized by high antioxidant capacity, high abundance of redox-active metabolites and high AgNP production, identifying it as the most efficient biological nanofactory. T3 also clustered in the high-redox, intermediate-yield region, indicating strong reducing power but a lower capacity to translate this into nanoparticle formation. In contrast, T2 displayed intermediate values for all parameters, whereas T5 clustered near the origin, reflecting its negligible redox activity and poor nanoparticle biosynthesis.

Importantly, this integrated representation resolved inconsistencies that were not apparent when individual variables were considered in isolation. For example, although T2 exhibited moderate AgNP production, its relatively low redox capacity and metabolite abundance resulted in a substantially lower FNEI than T0 or T3. Conversely, the high redox metabolome of T3 was not fully translated into AgNP yield, highlighting qualitative differences in metabolite composition and nanoparticle nucleation efficiency.

3.3. Biogenic AgNPs Exert a Fungistatic Effect Against *Neofusicoccum parvum*

The biological activity of AgNPs synthesized by the most efficient nanofactory strain, T0 (*T. harzianum*), was evaluated against two virulent isolates of *Neofusicoccum parvum* (JL396 and JL445). In both cases, the presence of AgNPs in PDA medium led to a clear alteration of fungal growth dynamics and colony morphology (Figure 6).

In untreated controls, both *N. parvum* isolates rapidly colonized the available well area, forming dense and radially expanding mycelial colonies. In contrast, colonies grown in the presence of T0-derived AgNPs exhibited a markedly reduced expansion rate and developed a more compact, less aerial mycelium, indicative of growth stress. Although mycelial coverage was not completely abolished, the overall architecture of the colonies was profoundly modified, demonstrating a strong inhibitory effect on fungal development.

This pattern was consistently observed for both pathogen isolates, indicating that the response to AgNPs was not isolate-specific but rather a general trait of *N. parvum* sensitivity to biogenic silver nanoparticles. The absence of complete growth arrest, together with the pronounced reduction in radial expansion and altered colony structure, indicates that T0-derived AgNPs act primarily in a fungistatic rather than fungicidal manner.

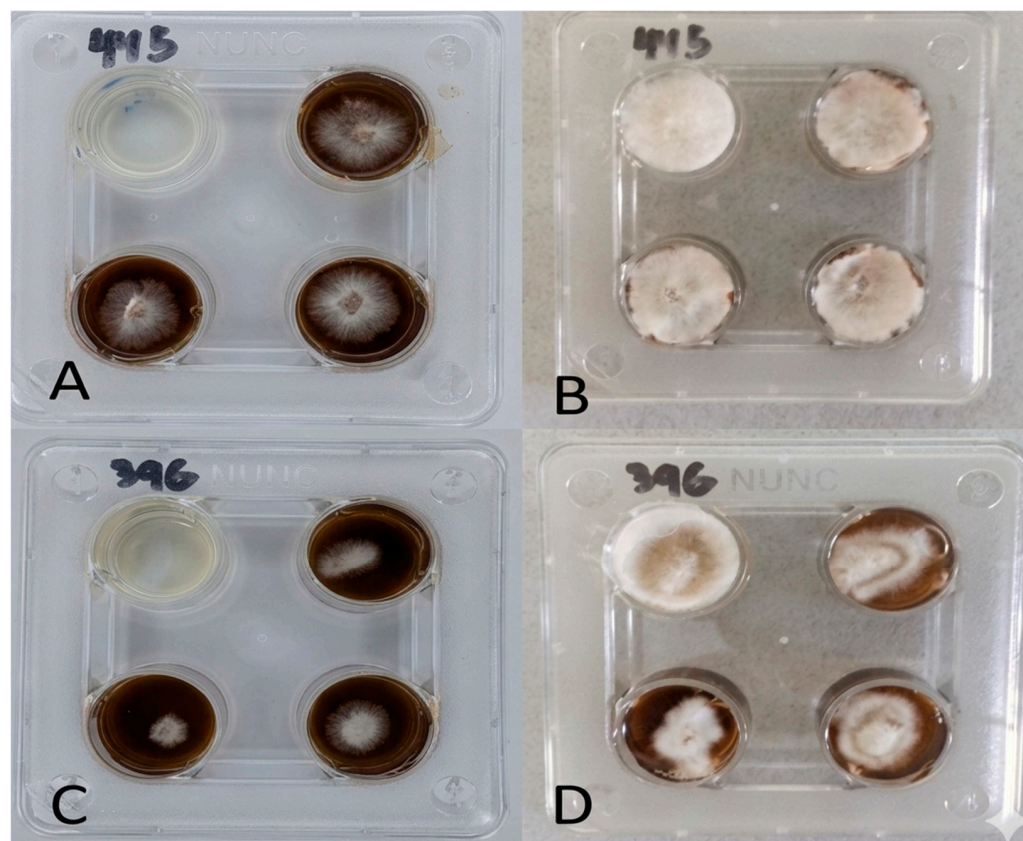


Figure 6. Antifungal effect of silver nanoparticles on the growth of the phytopathogenic fungus *Neofusicoccum parvum*. Growth of *N. parvum* strains 445 (A,B) and 396 (C,D) on PDA medium supplemented with silver nanoparticles is shown at 2 days (A,C) and 4 days (B,D) post-inoculation. In each plate, the well located in the upper left corner corresponds to the control (PDA without nanoparticles), whereas the remaining wells contain PDA supplemented with silver nanoparticles (1 mg mL^{-1}).

3.4. FNEI–Dose Response Surface Illustrates the Antifungal Performance of Biogenic AgNPs

To integrate nanofactory efficiency with nanoparticle application rate, a three-dimensional response surface visualization was generated using the FNEI values of the producing strains and the applied AgNP concentration as independent variables, and *Neofusicoccum parvum* growth inhibition as the response (Figure 7). This representation allows visualization of how antifungal activity varies across the experimental FNEI and AgNP dose space.

Across both *N. parvum* isolates, growth inhibition increased with AgNP concentration and FNEI. At low FNEI values, corresponding to strains with reduced redox metabolic capacity and low nanoparticle yield (e.g., T5), increasing AgNP concentration resulted in only limited inhibition of mycelial growth. In contrast, strains with high FNEI values, particularly T0, produced AgNPs that caused markedly higher growth inhibition, even at comparatively low nanoparticle doses.

The response surface visualization further highlights that the increase in inhibition with AgNP concentration is more pronounced for high-FNEI strains than for low-FNEI strains. While increasing nanoparticle concentration enhanced inhibition across all strains, low-FNEI strains did not reach comparable levels of growth suppression even at the highest tested concentrations. These patterns indicate that antifungal performance depends on the combined influence of nanoparticle dose and the biological origin of the nanoparticles, as reflected by the FNEI.

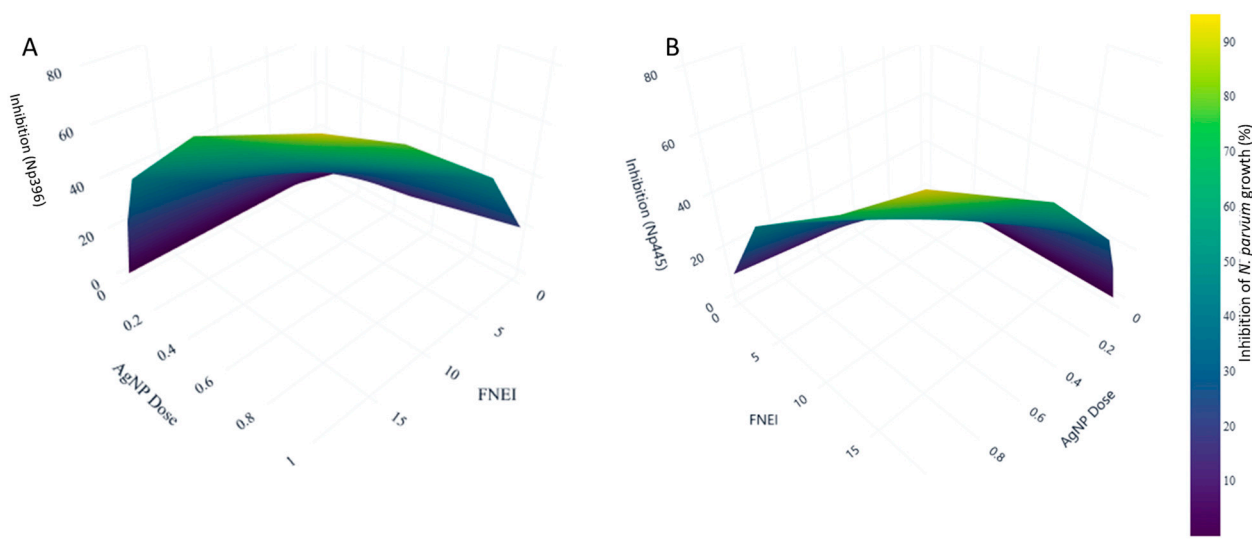


Figure 7. Three-dimensional response surface visualizations showing the combined effects of AgNP dose and redox metabolic efficiency (FNEI) of the producing strains on growth inhibition of *Neofusicoccum parvum*. Panel (A) corresponds to isolate Np396 and panel (B) to isolate Np445. Color gradients represent the magnitude of growth inhibition.

4. Discussion

This study establishes a new conceptual and applied framework for the sustainable management of grapevine trunk diseases by demonstrating that *Trichoderma* can act simultaneously as a biological control agent and as a biological nanofactory producing antifungal silver nanoparticles active against *Neofusicoccum parvum*, one of the most aggressive Botryosphaeriaceae associated with grapevine dieback. By integrating fungal metabolism, nanoparticle biosynthesis and pathogen inhibition, the results link microbial ecology with nanotechnology in a way that is directly relevant for vineyard disease control.

A first key outcome of this work is the pronounced strain-to-strain variability in AgNP biosynthesis observed within *Trichoderma*. Previously, Alves and Murray [16] emphasized that not all fungi are equally competent nanofactories, underscoring the importance of strain selection. The strong differences detected in nanoparticle yield among strains indicate that the ability to function as an efficient nanofactory is not a generic species trait, but rather a strain-specific phenotype. Although *T. harzianum* and *T. asperellum* have both been widely studied, direct comparisons of their extracellular redox and secondary metabolomes under identical conditions are still scarce [17]. Most available information derives from heterogeneous experimental contexts, such as responses to chemical stress or plant–fungus interactions [18], which makes it difficult to distinguish intrinsic metabolic differences from environmentally induced effects.

Within this standardized system, strains T0 (*T. harzianum*) and T3 (*T. asperellum*), which showed the highest AgNP yields, were characterized by extracellular metabolomes enriched in phenolic acids, flavonoids and quinone-like compounds. These compositional differences provide a biochemical basis for the contrasting reducing capacities and nanoparticle yields observed among strains. Such metabolites are well known to function both as electron donors for Ag^+ reduction and as capping agents during nanoparticle nucleation and growth, as phenolic and flavonoid compounds have been shown to act as major reducing agents converting AgNO_3 into AgNPs [19]. Furthermore, the capping process not only stabilizes the nanoparticles but also modulates their antimicrobial activity [20]. In this context, functional groups such as hydroxyl moieties supply primary reducing equivalents, whereas carboxyl and carbonyl groups contribute to secondary reduction processes and surface

passivation, thereby shaping nanoparticle size, dispersity and stability, as demonstrated by FTIR and redox analyses of phenolic antioxidants acting as both reducing and capping agents [21].

Although not exclusive, the close association observed here indicates that the extracellular redox metabolome is a major determinant of *Trichoderma* nanofactory performance. Accordingly, strains rich in phenolic, flavonoid and quinone-like metabolites generate a more reducing extracellular environment, which favors the efficient conversion of Ag^+ into AgNPs. This interpretation is supported by the strong correlation between DPPH radical-scavenging activity and AgNP yield. Similar relationships between antioxidant capacity and nanoparticle biosynthesis have been reported by Mistry et al. [22] and Naik and Mallikarjun [23]. The resulting FNEI–dose response surface thus offers a descriptive framework for visualizing how strain selection and application rate jointly influence antifungal performance.

Beyond nanoparticle production, the biological relevance of this strain-dependent nanofactory performance becomes evident in the antifungal assays. AgNPs synthesized by T0 strongly inhibited the growth of *N. parvum* isolates JL396 and JL445, producing compact, stressed colonies characteristic of fungistatic activity. This behavior is consistent with previous reports showing that biogenic AgNPs are intrinsically active against *N. parvum*, including up to 84% inhibition at 40 ppm with plant-derived AgNPs [24] and comparable effects with pollen-synthesized nanoparticles [25].

A fungistatic mode of action is particularly relevant for the management of grapevine trunk diseases, where even partial growth suppression can substantially limit lesion development and pathogen spread. In this context, Štůšková et al. [26] reported 54–68% inhibition of several trunk pathogens by biogenic AgNPs, while Tariq et al. [27] highlighted their potential as sustainable alternatives to chemical fungicides. The ability of biogenic AgNPs to remain effective against fungicide-resistant isolates [28] further supports their integration into resistance-management strategies. At the mechanistic level, Kumari et al. [29] showed that biogenic AgNPs induce higher superoxide production and more effectively disrupt fungal antioxidant defenses than chemically synthesized formulations, providing a plausible explanation for their broad-spectrum and resistance-robust activity.

Importantly, antifungal performance cannot be attributed to silver dose alone. Instead, it depends strongly on nanoparticle quality, including size, dispersity and surface chemistry, all of which are controlled by the biological nanofactory. Considerable variability in fungal AgNPs has been reported, ranging from 3–17 nm particles with 25–95% dispersity [16] to much larger 80–100 nm nanoparticles produced by *Aspergillus* species [30]. These physicochemical differences translate directly into biological activity [30,31].

Several studies have reported the biosynthesis of silver nanoparticles using *Trichoderma* species, with characteristics comparable to those observed in the present work. Devi et al. [32] screened 75 *Trichoderma* isolates and reported nanoparticles ranging from 8 to 60 nm, highlighting strong strain-dependent variability, a pattern also observed in this study, where the four strains showed marked differences in nanoparticle production and performance. Similarly, Tomah et al. [33] described AgNPs synthesized by *T. virens* (5–50 nm) that completely inhibited hyphal growth and sclerotia formation of *Sclerotinia sclerotiorum*, whereas in our study, *T. harzianum* T0 produced small and well-dispersed nanoparticles that exerted a strong fungistatic effect against *Neofusicoccum parvum*. Comparable antifungal activity has also been reported for *T. harzianum*-derived nanoparticles averaging 31 nm that inhibited *S. sclerotiorum* growth by 87.8% [34], as well as smaller particles of 6–15 nm effective against pathogens such as *Fusarium fujikuroi*, *Rhizoctonia solani* and *Macrophomina phaseolina* [35]. In contrast, our results further show that not all *Trichoderma* strains behave as equally efficient nanofactories, since strain T5 generated

fewer and more heterogeneous nanoparticles and displayed markedly lower biological activity. These findings suggest that differences in extracellular metabolism, rather than species identity alone, strongly influence the efficiency of nanoparticle biosynthesis and antifungal performance.

Consistent with this framework, TEM analysis showed that the *Trichoderma* strains studied here differed not only in the quantity but also in the quality of the AgNPs they generated. AgNPs produced by T0 were predominantly spherical, well dispersed and within a narrow nanometric size range (corroborated by DLS analysis), whereas those synthesized by T3 and T5 were more heterogeneous and more prone to aggregation. This is biologically significant, as smaller AgNPs (10–20 nm) are known to exhibit much higher antimicrobial activity than larger ones due to enhanced ROS generation, cellular uptake and intracellular Ag⁺ release [36,37]. Indeed, Kong et al. [38] reported that small AgNPs can be up to 4.3-fold more toxic than larger particles. Polydispersity further modulates bioactivity [16,39,40], as even a small fraction of highly active nanoparticles can dominate the overall biological response. Together, these relationships show that antifungal activity emerges from an integrated process in which fungal metabolism controls nanoparticle quantity and quality, which in turn determines biological performance.

Within this conceptual framework, the Fungal Nanofactory Efficiency Index (FNEI) integrates nanoparticle yield, size distribution and redox metabolism, explaining why strains producing smaller and better-capped AgNPs exhibit disproportionately higher antifungal activity at equivalent silver concentrations. The FNEI therefore provides a quantitative and mechanistic link between fungal secondary metabolism, nanomaterial biosynthesis and biological efficacy, enabling rational selection of *Trichoderma* strains with superior nanofactory performance.

The differences observed among the four *Trichoderma* strains also highlight the importance of strain-specific metabolic traits in determining nanofactory performance. Strains T0 and T3, which exhibited the highest FNEI values, were characterized by extracellular filtrates enriched in redox-active metabolites and higher antioxidant capacity, which likely facilitated efficient Ag⁺ reduction and nanoparticle nucleation. In contrast, strain T5 showed negligible antioxidant activity and a metabolite profile largely dominated by terpenoid and hydrocarbon-like compounds, which generally lack strong electron-donating functional groups. Consequently, this strain produced fewer and more heterogeneous nanoparticles and exhibited the lowest FNEI value. These observations suggest that differences in extracellular metabolite composition strongly influence the ability of fungal strains to convert metabolic reducing power into functional nanomaterials.

Taken together, these results demonstrate that the antifungal activity of biogenic AgNPs cannot be predicted from nanoparticle dose alone. This is consistent with metabolic and pharmacological evidence showing that multicomponent indices provide more reliable predictions of bioactivity than single-variable approaches, because biological effects emerge from compositional balance and synergistic interactions [41,42]. In this system, antifungal performance arises from the interaction between fungal metabolic capacity and nanomaterial quality, as captured by the FNEI. The resulting FNEI–dose response surface thus offers a conceptual framework to guide strain selection and inform application rate in the sustainable control of grapevine trunk pathogens.

From an applied perspective, this is particularly relevant for *N. parvum*, one of the most damaging grapevine trunk pathogens, for which chemical control options are increasingly constrained. The dual role of *Trichoderma* as both a biocontrol agent [13] and a biological nanofactory, as demonstrated here, offers a novel integrated strategy: AgNPs generated in association with *Trichoderma* could enhance suppression of *N. parvum* while preserving the ecological benefits of biological control. By linking fungal metabolism, nanoparticle quality

and pathogen inhibition, the FNEI shifts grapevine disease management from empirical screening toward predictive nanobiocontrol.

Although *in vitro* assays often show limited ability to predict biocontrol efficacy *in planta*, because host tissues and associated microbiota strongly modulate outcomes [43–45], they remain indispensable for mechanistic dissection. Controlled and standardized systems are required to isolate intrinsic microbial traits, such as redox metabolism and nanoparticle biosynthesis, from the confounding effects of the host environment. By resolving, in a standardized system, how *Trichoderma* metabolism governs nanoparticle yield, size and antifungal activity, this study establishes the nanobiological baseline required to design interpretable greenhouse or vineyard trials. Without such prior characterization, *in planta* testing would remain largely empirical and difficult to rationalize.

5. Conclusions

This study demonstrates that different *Trichoderma* strains differ markedly in their ability to function as biological nanofactories for the synthesis of silver nanoparticles. Among the strains evaluated, *T. harzianum* T0 produced the highest yield of AgNPs, generating small and well-dispersed nanoparticles that exerted a strong fungistatic effect on *Neofusicoccum parvum*.

Nanoparticle production was closely associated with extracellular antioxidant capacity and the abundance of redox-active metabolites, indicating that fungal metabolic traits play a key role in determining AgNP biosynthesis. The Fungal Nanofactory Efficiency Index (FNEI) provided an integrated framework linking fungal metabolism, nanoparticle yield and antifungal performance.

These findings highlight the importance of strain selection when using *Trichoderma* as a biological nanofactory for nanoparticle-based disease control strategies.

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Abbreviations

The following abbreviations are used in this manuscript:

AgNPs	Silver nanoparticles
DLS	Dynamic light scattering
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FNEI	Fungal Nanofactory Efficiency Index
GTDs	Grapevine trunk diseases

HPLC	High-performance liquid chromatography
ITS	Internal transcribed spacer
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PIRG	Percentage inhibition of radial growth
RSM	Response surface methodology
ROS	Reactive oxygen species
TEM	Transmission electron microscopy
UV–Vis	Ultraviolet–visible spectrophotometry

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