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Long term stability of *Trichoderma* mediated copper nanoparticles

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Abstract

In the present study, the stability of *T. asperellum* mediated copper nanoparticles (Tr-CuNPs) were investigated over a six-month period, focusing on their size distribution. The copper nanoparticles were synthesized using cell-free extracts of *T. asperellum*. Characterization was performed throughout the experiment using zeta potential and particle size analysis. The results revealed that the zeta potential of Tr-CuNPs remained stable for up to four months, with zeta potential values of -4.92, -6.43, -0.12, and -11.4 mV. However, after four months, the zeta potential became unstable, with values of -0.38 and 1.56 mV. Regarding particle size, it progressively increased starting from the second month, from 180 nm to 2395 nm by the fourth month. During the fifth and sixth months, the size continued to grow, reaching 3425 nm and 4959 nm, respectively. This increase in size after the fourth month resulted in the destabilization of the nanoparticles. Therefore, based on these findings, the copper nanoparticles remained stable for up to four months.

Keywords: *T. asperellum*, copper nanoparticles, zeta potential, particle size analysis

Introduction

Nanotechnology has emerged as a revolutionary field with widespread applications in various industries, including agriculture, medicine, and environmental remediation. Among the diverse nanomaterials, copper nanoparticles have gained significant attention due to their unique properties, such as high surface area, antimicrobial activity, and catalytic abilities. These characteristics make CuNPs ideal for applications in plant protection, particularly against soil-borne pathogens that pose significant challenges in agriculture. Traditionally, copper nanoparticles are synthesized through chemical methods, but these processes often involve the use of toxic reagents. As a result, there has been a growing interest in green synthesis methods, which utilize secondary metabolites of biological agents for nanoparticle production (Birari *et al.* 2025) [1]. One such promising approach involves using *Trichoderma asperellum*, a well-known biocontrol fungus capable of synthesizing CuNPs through the reduction of copper ions in the presence of its metabolites. The green synthesis of CuNPs using *T. asperellum* offers several advantages, including sustainability, eco-friendliness, cost-effectiveness, and low toxicity, making it an attractive alternative for agricultural applications.

While the biosynthesis of CuNPs using *T. asperellum* has shown promising results, the stability of these nanoparticles is a critical factor that affects their efficacy and practical application. Due to their large surface area, nanoparticles are highly reactive, and their stability can be influenced by various factors, such as pH, ionic strength, and the presence of stabilizing agents. Therefore, understanding the stability of CuNPs is essential for their effective use in agriculture. Nanoparticle stability not only impacts their antimicrobial activity but also governs their dispersion and interaction with target pathogens in soil environments.

To evaluate the stability of CuNPs, techniques such as zeta potential and particle size analysis are commonly employed. Zeta potential is a measure of the electrostatic potential at the surface of nanoparticles, which provides an indication of their stability in suspension. A high zeta potential (either positive or negative) typically correlates with stable nanoparticles, as it prevents aggregation through electrostatic repulsion. Particle size analysis, on the other hand, determines the size distribution of nanoparticles, directly impacting their surface

reactivity, biological activity, and stability. Smaller nanoparticles tend to have higher surface-to-volume ratios, making them more reactive; however, their stability may be compromised if they aggregate.

This study aims to investigate the stability of Tr-CuNPs using zeta potential and particle size analysis. By exploring the relationship between nanoparticle size, zeta potential, and stability, we seek to optimize the synthesis conditions for enhanced stability and biological efficacy. Understanding these parameters is crucial for advancing the use of CuNPs in agricultural practices, particularly in plant protection. The findings from this research could have significant implications for sustainable pest management and biocontrol strategies. By improving the stability of *T. asperellum*-mediated CuNPs, we can enhance their effectiveness in combating soil-borne pathogens, thereby promoting healthier crops and reducing the reliance on harmful chemical pesticides.

Materials and Methods

Collection of *Trichoderma asperellum*

The pure culture of *Trichoderma asperellum* was collected from Department of Plant Pathology, Post Graduate Institute, Dr. PDKV, Akola. The *T. asperellum* was further used for synthesis of copper nanoparticles.

Preparation of cell free extract of *Trichoderma asperellum*

The *Trichoderma asperellum* was inoculated in 100 ml of sterile potato dextrose broth and incubated in an orbital shaker for seven days at 28 °C for 150–200 rpm. The fully grown mycelial growth was removed and washed three times with sterilized distilled water. The washed mycelial growth was again resuspended in 100 ml of sterile distilled water and kept for five to six days for incubation at 28 °C temperature for 150-200 rpm. Following incubation, the cell free extract was centrifuged for ten minutes at 5000 rpm. The resulting supernatant was filtered and used as cell-free extract for the synthesis of CuNP's.

Biosynthesis of copper nanoparticles

The copper nanoparticles were synthesized using cell free extract of *Trichoderma asperellum* with some modified procedure of Maheo *et al.*, (2022) [11]. Firstly, 60 ml 0.05M CuSO₄·5H₂O solution was kept in a beaker for stirring. Then, 20 ml cell free extract of *Trichoderma asperellum* was added dropwise to the solution and stirred continuously on a magnetic stirrer for 30 min at 45 °C. Then, 20 ml of 0.6M NaOH was added drop wise to enhance the synthesis of copper nanoparticles. Finally, this mixture was stirred and heated continuously for three hours till the color change in the solution was observed from blue to dark green and ultimately brownish red. The final precipitate settled at the bottom was centrifuged for three times using distilled water

at 5000 rpm for ten minutes. Then the brown powder obtained was dried properly and used for further studies.

Stability of Tr-CuNP's

The characterization of copper nanoparticles was done by using zeta potential and particle size analyzer to check the stability of copper nanoparticles up to six months. The zeta potential and particle size analysis were done at Sophisticated Analytical Instrument Facility (SAIF), Shivaji University, Kolhapur.

Results and Discussion

Biosynthesis of copper nanoparticles

The formation of copper nanoparticles was characterized by colour change from light blue to dark reddish-brown precipitate on addition of microbial extract in presence of sodium hydroxide through visual observation means, indicating the reduction of copper ions into their nanoparticles. (Sawake *et al.* (2022) [16] and Manoj GM *et al.* 2024) [10].

Similar results were obtained by many scientists on the bio synthesis of CuNPs, from *Trichoderma* species cell free extracts including Dorjee *et al.* (2024) [2] and Saravanakumar *et al.* 2019 [15]. Also, Nassar *et al.* (2023) [13], Tsilo *et al.* (2023) [18], Hassan *et al.* (2023) [5] and Kashyap *et al.* (2023) [8] reported the bio synthesis of copper nanoparticles.

Stability of Tr-CuNP's

In Table 1, results for stability of Tr-CuNP's are given. The stability of Tr-CuNP's was checked using zeta potential and for size, particle size analysis was used. As the duration of copper nanoparticles is increased, the size of nanoparticles goes on increasing, ultimately affecting the stability of nanoparticles.

The stability of *T. asperellum*-mediated copper nanoparticles (Tr-CuNPs) was evaluated over a 150-day period based on zeta potential and particle size analysis. Initially, the zeta potential was -4.92 mV, indicating moderate stability, and improved to -6.43 mV after 30 days, suggesting enhanced electrostatic repulsion. However, by 60 days, the zeta potential dropped to -0.12 mV, signalling reduced stability, and -11.4 mV at 90th day. The zeta potential -0.38 mV and 1.56 mV at 120th day and 150th day resulted in unstable nanoparticles. In terms of particle size, the nanoparticles began with a size range of 70-90 nm at initial day i.e. immediate after synthesis and increased progressively over time, reaching 2395 nm by 90th day. The particle size 3425 nm by 120th day, and 4959 nm by 150th day, indicated significant aggregation which resulted in unstable nanoparticles. These findings suggest that the stability of Tr-CuNPs was maintained up to four months, after which the nanoparticles became unstable due to aggregation and changes in the zeta potential, leading to formation of larger particle sizes (Fig 1).

Table 1: Stability of Tr-CuNP's after synthesis

Sr. no.	Days after synthesis	Zeta Potential (mV)	Particle Size (nm)
1	0	-4.92	70-90
2	30	-6.43	180.1
3	60	-0.12	561.4
4	90	-11.4	2395
5	120	-0.38	3425
6	150	1.56	4959

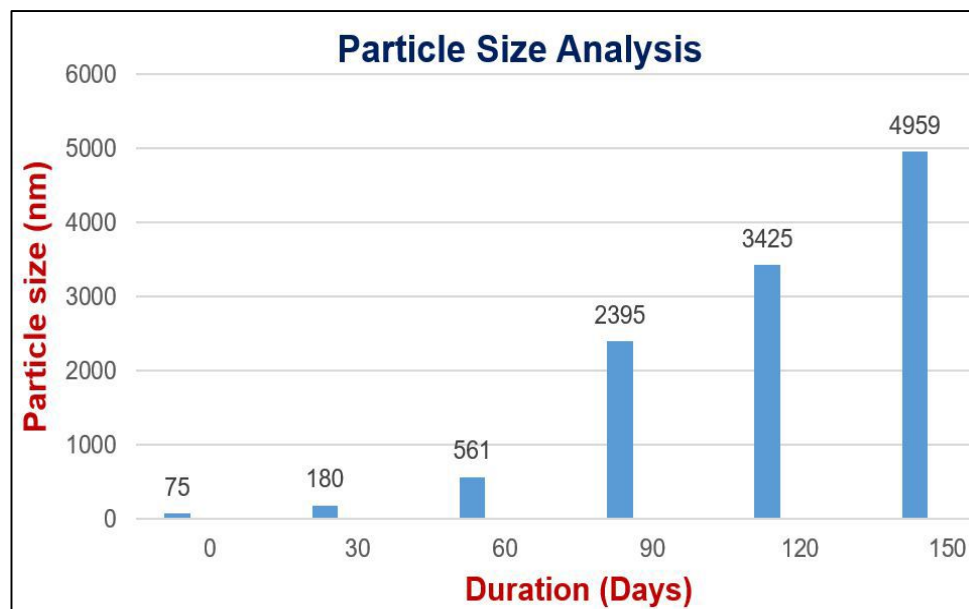


Fig 1: Particle size analysis of Tr-CuNP's

The results obtained in the present study align with those of Ntasiou *et al.* (2021) ^[12], who investigated the stability of copper nanoparticles using different stabilizers through zeta potential and particle size analysis. Their study revealed that nanoparticles synthesized with ionic polymers exhibited a small particle size (4.90 nm) and a high zeta potential (-16.44 mV), indicating good stability. In contrast, nanoparticles synthesized using animal proteins had a slightly larger particle size (5.23 nm) and a lower zeta potential (-12.23 mV). Non-ionic polymer-synthesized nanoparticles displayed the largest particle size (10.41 nm) and the lowest zeta potential (-4.64 mV), indicating reduced stability. Furthermore, Roy *et al.* (2016) ^[14] investigated the stability and gradual formation of CuNPs using UV-visible spectroscopy at regular intervals. Their results showed that the maximum absorbance occurred near 550 nm, confirming the synthesis of CuNPs. The absorbance spectra revealed a gradual increase in absorbance over time, which peaked after 12 hours of incubation, and eventually declined after 24 hours, indicating that the rate of nanoparticle formation began to stabilize. These findings corroborate the results of the current study, highlighting the importance of monitoring both particle size and zeta potential to assess the stability of CuNPs.

Similarly, Han *et al.* (2023) ^[4] investigated the stability and dispersion mechanisms of copper nano powders using various stabilizing agents. Their study highlighted that the addition of polyvinyl pyrrolidone (PVP) as a stabilizer during nanoparticle synthesis significantly enhanced stability due to the steric hindrance provided by PVP. This resulted in the formation of a thick hydration layer around the nanoparticles, which effectively prevented particle growth and agglomeration, particularly when optimized concentrations, dispersion time, and solid-liquid ratios were used. In another study, Tokarek *et al.* (2013) ^[17] evaluated the stability of CuNPs synthesized with different stabilizers, including chitosan, ascorbic acid, and sodium phosphinate monohydrate. They found that chitosan-stabilized nanoparticles exhibited superior stability compared to those stabilized with ascorbic acid or sodium phosphinate monohydrate, as demonstrated by their more uniform size distribution. These findings further emphasize the crucial

role of stabilizers in enhancing the stability and preventing the agglomeration of copper nanoparticles, supporting the results observed in this study.

It has been observed that increasing the concentration of capping or stabilizing agents generally leads to a decrease in nanoparticle size. In this regard, Jain *et al.* (2015) ^[6] documented that as the concentration of L-ascorbic acid increased from 0.08 to 0.10 mol/L, the size distribution of copper nanoparticles decreased from 28 nm to 12 nm, indicating the formation of more stable nanoparticles. Similarly, studies by Mandava *et al.* (2017) ^[9], Maximino *et al.* (2021) ^[7], Umer *et al.* (2014) ^[17], and Granata *et al.* (2016) ^[3] also reported the stability of CuNPs using various stabilizing agents. These studies collectively concluded that the stability of copper nanoparticles is largely dependent on the concentration of stabilizers used, as it directly or indirectly influences the size distribution and overall stability of the nanoparticles. These findings further corroborate the notion that the appropriate choice and concentration of stabilizing agents are crucial factors in controlling the size and stability of CuNPs.

Conclusion

The above study concluded that *T. asperellum*-mediated copper nanoparticles remained stable up to four months. After this period, the size of the copper nanoparticles continued to increase, leading to form unstable nanoparticles, as evidenced by the zeta potential values of -0.38 mV and 1.56 mV. Based on this data, it can be concluded that the copper nanoparticles remains stable for a period of up to four months, after which the increased particle size and changes in the zeta potential contributed to their instability.

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