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Biosynthesis of Ag and Cu nanoparticles and their antifungal potential against the dominant seed-borne mycoflora of rice (*Oryza sativa*)

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Abstract
The biosynthesis of nanoparticles was performed using Trichoderma as a reducing and stabilizing agent. Isolated nanoparticles were characterized by UV–VIS spectrophotometry and TEM. Antifungal potential of the nanoparticles studied on seed-borne pathogenic microflora of rice. Antifungal assays were performed. *In vitro*, by the well diffusion method using media supplemented with 100-500 ppm NP solution and amp, growth inhibition was calculated, and the degree of sensitivity was studied on the basis of the lethal concentration (LC) value and statistical analysis. Study shows that AgNp inhibits growth in *Fusarium fujikuroi*, *Aspergillus flavus*, and *Curvularia lunata* at concentrations ranging from 100 to 500 ppm. In Aspergillus, however, the percentage is 65.54%. LC 50 analysis shows that the LC 50 value is between concentrations of 100 ppm and 300 ppm, which is optimized up to 140 ppm. This study also concluded that silver nanoparticles are much more applicable as antifungal and antibacterial agents and are more diverse in function.

Keywords: Nanoparticles, AgNP, antifungal, seed borne mycoflora, biosynthesis

Introduction
Rice (*Oryza sativa*) is a staple food crop and is grown in almost the entire Indian subcontinent. Amusingly, in some regions of the subcontinent, rice is warmly ingrained in the culture, tradition, nutrition, and socioeconomic welfare of a large proportion of the population (Vijay & Roy, 2013) [35]. Despite being the origin of rice in India and having the largest area (42 million hectares) as well as having the second rank in production, a disheartening trend is that the productivity per hectare basis, the country has fifty-one rank and is continuously constant; however, no significant growth has been reported (Londo *et al*., 2006) [20]. Biotic factors are most crucial for this problem. Seed-borne diseases are known to contribute to the loss of crop yield (Asif *et al*, 2021) [5], and many of these diseases are caused by seed-borne mycoflora (Richards., 1953) [27] and have been considered potentially destructive to many varieties of rice crops grown in various locations in the country (Lowery *et al*., 2012) [21].
In the present study, we have an interdisciplinary approach to biochemical applications (Kartik KN *et al*., 2017) [16] and (Kumar A *et al*., 2020) [17] of nanotechnology, which focuses on nanoparticle synthesis with amended antimicrobial and antioxidant properties. Biosynthesis of nanoparticles using biogreen methods is advantageous over chemical and physical methods in the synthesis process due to the rapid, clean, simple, nontoxic, inexpensive and eco-friendly synthesis of nanoparticles(Tahakik R R, 2022) [28]. Biosynthesis of nanoparticles has been isolated from different organisms, such as plants, for example, *Calotropis procera* (Kareem *et al*., 2008) [36] euphorbia and *Nerium oleander*, which are nanofactories and have latent utility (Mussin *et al*., 2019). By the end of the past few years, nanoparticles of noble metals have received extensive attention owing to their applications in diverse fields and improved physical, chemical and biological properties (Mohamad Sukri *et al*., 2021) [22] By comparison, to other noble metals such as Au, Pt and Ag, Cu is a low-cost material; however, it is also accountable for oxidation, generally in nanoscale dimensions (Casagrande *et al*. 2019) [7]. Copper nanoparticles have various properties, such as antimicrobial, optical, catalytic, antifungal, anti-insect and anticancer properties (Li *et al*., 2022) [19]. In addition, we can conclude that the copper and silver nanoparticles show cogent antifungal activity against the phytopathogenic fungi *Aspergillus flavus*, *Curvularia lunate*, *Fusarium fujikuroi* and *Aspergillus niger* at low concentrations, which helps to use it in the future as one of the important applied factors in the agricultural field. (Hammood *et al*., 2022) [11].

Material and Methodology

Mycosynthesis of silver and copper nanoparticles

For the synthesis of nanomaterials (AgN) and CuO, the culture of *Trichoderma asperllium* was grown in PDA supplemented with 0.01 mM $AgNO_3$ and 0.05 mM $CuSO_4$ separately at ambient light and temperature, and the culture of *Trichoderma spp*. was cultivated in Petri dishes for 72 h. Five grams of young mycelium from both cultures was isolated and transferred to 500 ml phosphate buffer supplemented with 200 ml potato extract and 0.5 gm D-glucose and kept under stirring conditions in an orbital shaker at 140 rpm for 7 days. After growth, 20 ml of culture was washed with D/W and separated using 'Whatsman filter paper' No. 1. The obtained extracts were physically characterized by UV–VIS spectrophotometry and monochromatic laser light and peroxidase activity (Table 1 and Fig 1).

Investigation of Seed Borne Mycoflora of Rice (*Oryza sativa*, L.)

For studying, mycoflora associated with rice were collected from various regions of eastern Uttar Pradesh Table 1, Sampling procedure specified by ISTA followed. Primary seed samples were collected and bulked into composite samples of 100 gm. obtained samples An improved method of testing seed mycoflora was carried out by the blotter method and agar plate method as recommended by ISTA ("The International Rules for Seed Testing," 2005) [9] Blotter paper method. A total of 100 seeds from each sample were collected in replication of 3 from each variety, and the test weight of 100 seeds of each variety was calculated (Butt AR, *et al*, 2011) [6]. Water-soaked filter paper of size 9-11 cm was placed at the bottom of a 9 cm well labelled plastic Petri dish and PDA supplemented for the agar plate method (Alsohali S, and Hasan B, 2018) [3]. The Petri dishes containing seeds were incubated at room temperature (25±2 °C) for 7 days under alternating cycles of light and darkness of 12 hours each (Lavare N. Barwant M., 2020) [18]. Seeds were examined at the end of the incubation period with a stereo binocular microscope for the presence of mycoflora (Fig 4).

Characterization and biological activity of silver and copper nanoparticles

The effects of biogenically synthetized AgN- on the mycelium growth of the plant fungal pathogens *A. flavus*, *A. niger*, *Curvularia lunata, and Fusarium spp*. isolated from the Culture Collection were evaluated. Thus, agar well diffusion (Prenz Christina, Pauli., 1990) [26] was carried out by placing a plug of 5 mm of young mycelium of each phytopathogen in the centre of a Petri plate containing potato-dextrose agar supplemented with 10 ppm of each nanoparticle and kept for 7 days at 24±2 °C under a 12 h L/D photoperiod (Janathul M, *et al*, 2015) [12]. Negative control cultures of fungal strains only grew in potato-dextrose agar. After that period, the diameter of the mycelium was measured. The growth effects of each type of synthesizer NP from the T. asperllium strain were already evaluated over their own growth. All assays were performed in triplicate(Ezeonu *et al*., 2022) [10].

Statistical Analysis

For the statistical analysis, all the observations related to seed mycoflora, seed germination and management of seed mycoflora were converted into sin values (Joshi V and Mukharjee KG, 1999) [13]. The final data from the evaluation of the seed mycoflora, seed germination, and seed vigour index were subjected to statistical methods such as analysis of variance (ANOVA) (Pearson K., 1900) [24] in SPSS as described by Velasquez *et al*. (2011) [32]. Standard error was calculated as per the above parameters to compare two treatment critical differences at a 5% level of significance (Singh, 2017) [31].

Table 1: UV–VIS Characterization of Copper and Silver Nanoparticles

Sr. No	Observation time in hrs	Wavelength	UV–VIS peaks	Ag N	CuO N
1	0	100-200	188	1.2	0.6
2	4	200-300	220	5.8	4.7
3	12	300-400	340	3.1	3.3
4	24	400-500	420	2.1	2.9
5	48	500-600	510	1.1	1.6
6	72	600-700	660	0.66	0.33

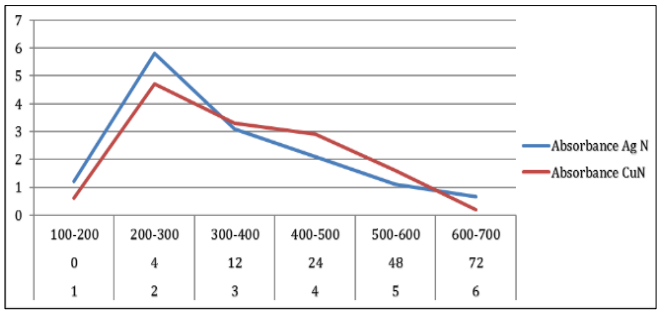

Fig 1: UV–VIS Spectrophotometric Analysis of Copper and Silver Nanoparticles

Results and Discussion

Biosynthesis of silver and copper nanoparticles

From aqueous cell-free extracts of *Trichoderma hamatum*, silver and copper oxide NPs were successfully synthesized in separate experiments after adding 1–2 mM silver nitrate, copper sulfate or zinc sulfate. The formation of metallic NPs was evidenced by changing the cell-free extract color suspension after their respective salt addition under continuous stirring at 45 °C in dark conditions. Although the pH reaction from 6 to 12 was evaluated, rapid precipitation of NPs was observed within 10 min when the initial CFCF at pH 6 was alkalinized with NaOH to pH 12. The reduction of Ag+ to Ag0 by the cell-free extract was immediately noticed after the addition of AgNO3, changing from pale yellow–brown to colloidal dark brown due to the formation of AgNPs. Meanwhile, CuO NP formation occurred after the reduction of sulfate ions from Cu+2 to Cu0, as observed by an immediate change of the blue CFCF solution to a pale yellow–brown solution. After the same conditions mentioned above. Hence, this is the first work in which a sole *T. harzianum* cell-free extract is able to synthesize two types of metallic NPs under the same conditions. For the biosynthesis and characterization of silver and copper nanoparticles, (Pilley *et al*., 2021) [25] utilized *Magnetospirillum magnetotactic*, a bacterium isolated from silver mines, using MACS column chromatographic separation techniques to produce spherical nanoparticles of 3-25 nm in range. Various studies have shown that different types of microorganisms, such as *Pseudomonas stutzeri* (Hammood *et al*., 2022) [11].

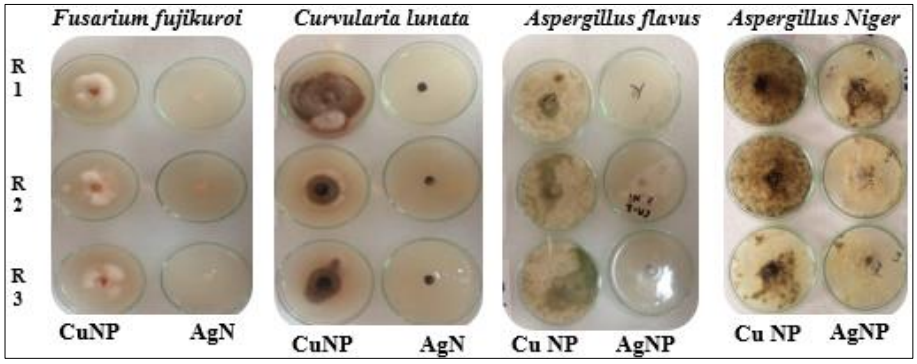

Fig 2: Antagonistic Effect of Copper & Silver Nanoparticle Agents

Table 2: Effect of silver and copper nanoparticles against the dominant seed mycoflora of rice

Mycoflora	CN MG (mm)	AgNP MGI (mm)	MGI (in %)	CN MG (mm)	CuO NP MGI (mm)	(MGI) (in %)
Aspergillus flavus	45.67	11.33	75	45	25	45
Aspergillus niger	43.33	17.67	59	44	25	44
Curvularia lunata	43	6	86	46	21	54
Fusarium fujikuroi	47	15.33	67	42	21	51
C.D.	9.47	5.57	13.4	45	6.89	13.27
SE(m)	2.01	1.71	4.18	1.50	2.10	4.07

Note: C- Control, AgNP- Silver Nanoparticles, CuO Copper Nanoparticles, MG – Mycelial Growth, MGI- Mycelial Growth Inhibition

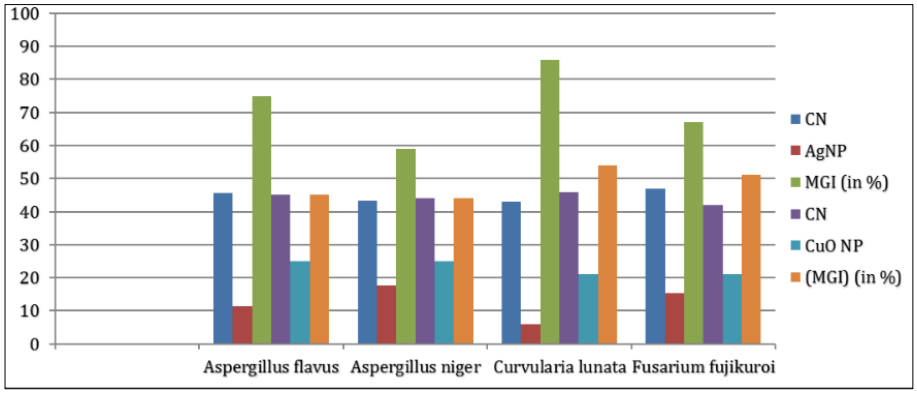

Fig 3: Mean performance of CuN and Agn against the seed mycoflora of rice

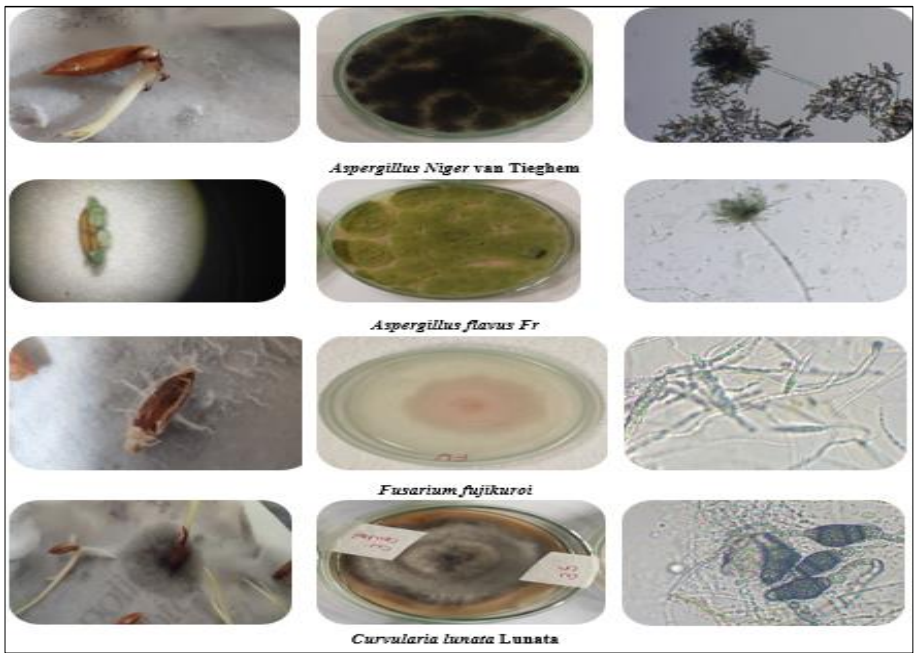

Fig 4: The Dominant Seed Mycoflora of Rice Selected for Further Studies

Investigation of the seed-borne mycoflora of rice

In the present investigation, the important pathogens that could also be detected included *Fusarium fujikuroi*, *Bipolaris oryzae*, *Alternaria padwickii* and *Curvularia lunata* (Table 1 & fig 1-3). Scientists from West Bengal (Mujumdar & Chattopadhyay, 1975) [23] reported similar findings about the association of *Helminthosporium oryzae and Fusarium fujikuroi* in rice seeds. Significant findings were also reported by (Mohamad Sukri *et al*., 2021) [22], who concluded that seed mycoflora isolated from kymore regions of central India shows the association of *Bipolaris oryzae*, followed by *Curvularia lunata and Alternaria padwickii i*n all three replications and 11 varieties. Another study (Singh *et al*., 2017) [31] & Kabir A, *et al*., 2011) [14] showed potential disease-causing organisms in northern Indian rice varieties. In total, they reported 7 pathogenic organisms (*Acrocylindrium oryzae*, *Alternaria padwickii*, *Bipolaris oryzae*, *Curvularia spp. Fusarium moniliforme*, *Pyriculria* oryzae and *Tilletia barclayana*). As observed in the present study, most of the reports confirmed that *Fusarium fujikuroi*, *Bipolaris oryzae*, *Alternaria padwickii*, *Aspergillus niger* and *Curvularia lunata* were more common in occurrence on rice seeds of all 4 varieties represented in Fig. 1. However, along with these fungal species, saprophytes such as Penicillium notatum and *Rhizoctonia solani* were also detected in certain varieties. The results were compared with (Ansari H, 2010) [4]. The agar plate method was found to be a more efficient method in detecting different fungal species and the total mycoflora recovered compared to the blotter method, as reported by some (Singh *et al*., 2017) [34] (Sudhini & Adithya, 2016) [32].

Mean performance of copper and silver nanoparticles on the dominant pathogenic fungus of the rice seed mycoflora

The potential of Ag and CuO to control *Aspergillus niger*, *Aspergillus flavus*, *Curvularia lunata*, and *Fusarium fujikuroi* was assayed by plating fungal culture media supplemented with 10 ppm of each type of NP, and the diameter of mycelium growth was measured after 7 days. Both Ag and CuO NPs caused a significant reduction in the mycelia development of *Aspergillus niger*, *Aspergillus flavus*, *Curvularia and Fusarium fujikuroi* against control as shown in, Results are shown (Table 2 Fig 2 and 3) the revealed that treatment the mycelial diameter for was reduced mean of all three replication was approximately 75% for *Aspergillus flavus*, 60% *Aspergillus niger*, 86% *Curvularia lunata* and 67% in the *Fusarium fujikuroi* in at 10 similarly 10 ppm concentration of Copper Nanoparticles shows the reduction in the mycelial growth 45% for *Aspergillus flavus*, 44% *Aspergillus niger*, 42% *Curvularia lunata* and 51% in the *Fusarium fujikuroi*, Finally, it shows *Aspergillus niger* significant with *Aspergillus flavus* while both *Aspergillus flavus* and *Aspergillus niger* are significantly at par with the *Fusarium oxysporium* and *Curvulara lunata*, while both are significant with each other's. Similar results were reported in all three replications. All the results are significant in the statistical analysis. In recent years, some researchers have focused on the development of modified or novel synthetic strategies for silver nanoparticles, in contrast to the use of conventional methods, which are strongly associated with toxic environmental footprints. (Abraham & Silambarasan, 2011) [1]. Some scientists have shown multidrug resistance against pathogens of plant species by utilizing nanoparticles conjugated with *P*. grandiflora tuber extracts (Alnaimat A, & Aljamaeen, 2020) [2]. Green rapid syntheses of spherically shaped silver nanoparticles with dimensions of 50-70 (Li *et al*., 2022) [19].

LC_{50} Analysis

Higher concentrations of AgNPs affect the growth of mycoflora; however, lower concentrations do not show any kind of change compared to the control. The results show that 0.05 mg/L promotes fungicidal potential.

Conclusion

Silver and copper nanoparticles were successfully mycosynthesized using *Trichoderma asperllium* and characterized using a UV–VIS spectrophotometer and polarimeter. Cell viability assays using the agar plate method revealed that silver and copper nanoparticles inhibit the biological activity and significantly inhibit the mycelial growth of seed-borne mycoflora, *such as Aspergillus flavus*, *Aspergillius niger*, *Curvularia lunata*, *and Fusarium fujikuroi.* This study also concluded that due to the physical, chemical and optical properties of silver nanoparticles, they are applicable in the agriculture industry as antifungal and antibacterial agents and are more diverse in their functions.

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