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Mycogenic Synthesis of Copper Nano-particles by Bio-controlling Fungi (*Aspergillus niger* and *Trichoderma viride*) and its Antifungal activity on Plant Pathogens

***Naveen Babu Duvvi*[1], *Suneetha N. N.*[1], *Anil Kumar N*[2], *Nagadesi P. K.*[3] *and V. N. D. Rao Yenumula*[4]**

[1]*Department of Biochemistry, R & D Wing, St. Joseph Dental College, Duggirala, Eluru, (Andhra Pradesh), India.*
[2]*S.V.K.P & Dr. K.S. Raju Arts & Science College, Penugonda, West Godhavari, India.*
[3]*Department of Botany, P.G. Block, Andhra Loyola College, Near Gunadala, Vijayawada-8, (Andhra Pradesh), India.*
[4]*Department of Physics, Anurag Engineering College, Kodada-508206, (Telangana), India.*

(Corresponding author: Nagadesi P.K.)
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ABSTRACT: Nano-particle synthesis by biological method has large biomedical applications worldwide. So the fungal mediated synthesis of copper nano-particle was studied in this paper. The fungal samples were isolated from a soil sample of Andhra Loyola College, Vijayawada by Pore plate and spread plate methods. The highest number of isolates was obtained by the spread plate when compared with the pour plate method. For selection of suitable synthetic medium to synthesize copper nano-particles different isolated fungi were grown on Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Yeat extract Malt extract Dextrose (YMD), Yeast extract Malt extract (YM), Malt extract Dextrose (MD) broth mediums. The best medium for synthesis of copper nano-particles is PDA broth when compared to all medium used in the test. The bio-control agents like *Aspergillus niger* and *Trichoderma viride* shown better growth and biomass production in PDA broth when compared to all mediums. The fungal filtrates and live-cell extract exhibited a characteristic change in colour i.e. gradual change from blue to dark green colour, which indicating the formation of CuNPs by extracellularly and intracellularly. The UV- Visible spectroscopy analysis of the fungal filtrate and live-cell extract shown characteristic Surface Plasmon Resonance (SPR) spectra with an absorbance peak at 610 nm which further indicates the formation of Copper nano-particles. The best solvent for preparation of *A. niger* and *T. viride* extracts was methanol. The Copper nano-particles showing best antifungal activity against the phytopathogenic fungi like *Rhizopus artocarpi, Penicillium citrinum, Fusarium roseae, Alternaria alternata, Fusarium oryzae,* and *Cladosporium cladosporoides,* which were 100% inhibited by 20% and 25% concentration.

Keywords: Biocontrol agent, Copper nano-particles, Mycochemicals, Antifungal, Phytopathogens.

I. INTRODUCTION

Transition metal materials have prospective applications in optics, electronics, and medicine and in manufacturing of lubrication, nano-fluids, conductive films, and antimicrobial agents [1-3]. Among transition metal materials, copper is the most important material. The unique catalytic and optical properties show that copper nano-particles (CuNPs) are potential candidates for the reaction of H_2O dissociation [4]. Synthesis of transition metal nano-particles like CuNPs has been demonstrated by many physical and chemical methods [5]. The physical, and chemical methods for synthesise of CuNPs shown low productivity, non eco-friendly, capital intensive and toxic. So, there is an urgent need to develop an eco-friendly, non-toxic, biological method for copper nano-particle synthesis. The extracellular and intracellular synthesis of copper nano-particles by using higher plants and microbes have gained importance because of rapid, clean, simple, low cost, availability and eco-friendly process [6-8]. Biological based nano-particle synthesis was reported by Ahmadi *et al.*, [9], Theivasanthi and Alagar [10]. Biosynthesis of stable AgNPs by fungi has been reported by Vigneshwaran *et al.*, [11], Dura *et al.*, [12]. The biosynthesis of CuNPs by extracellular and intracellular mean was still an open challenge for bio-nanotechnologists. In the present paper, the mycogenic synthesis of copper nano-particles by bio-control agent like *A. niger* and *T. viride* was tested. The synthesized CuNPs are characterized by UV-Visible spectrophotometry and the antifungal/bio-controlling nature on phytopathogenic fungi was tested.

II. METHODS

A. Isolation and identification of fungi

A survey was conducted from 2017 to 2019 for isolation of fungi from soil samples collected in Andhra Loyola College, Vijayawada-8. The soil samples were sieved to remove small stones and used for isolation of fungi by Dilution agar plate technique [13]. The Potato Dextrose Agar medium plates were inoculated by pore plate and spread plate methods. The isolated fungi was stained with lactophenol cotton blue to observe morphological and cultural characters. The macroscopic and microscopic character of isolated fungi was observed and studied by using Zheng *et al.,* [14]. They were identified using standard manuals [15, 16]. The identified fungi were confirmed by microbial expert. The percentage occurrence of each fungal species was calculated using the following formula:

Percentage of fungal species

$$= \frac{\%\,\text{Occurrence of individual species}}{\%\,\text{Occurrence of total species}} \times 100$$

B. Selection of medium for synthesis of CuNPs

The isolated fungi were grown on Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) Yeast extract, Malt extract, Dextrose (YMD) Yeast extract, Malt extract (YM) and Malt extract Dextrose (MD) broth in 250 ml conical flasks at 27℃ for 7 days. After incubation, the

mycelium mat was harvested on previously dried and weighed Whatman filter paper No. 1 and washed with distilled water to remove any components of the medium. The filter paper is finally weighed to calculate the growth of bio-controlling fungi by the following formula:

Percentage biomass

$$= \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

III. MYCOGENIC SYNTHESIS OF CUNPS

A. Intracellular

For the synthesis of Copper nano-particles, the PDA broth was prepared by supplementing copper sulphate with 0.1 mg to 0.4 mg salt and without salt as control. Fresh colonies of bio-controling agents like *A. niger* and *T. viride* was inoculated in 150 ml flasks containing PDA broth medium and incubated at 27℃ for 7 days. After completion of the incubation period, the flasks were filtered with pre-weighed Whatman filter paper No 1 to separate the mycelium of a fungal isolate. The filter papers were dried in oven at 90℃ for 24 hrs the dry weight of fungal isolate was calculated by following formula mentioned above. The formation of copper nano-particle was observed by the change in colour in test flasks compared control and inoculated with fungal isolates. To obtain the mycelia water extract having CuNPs, a weight of 20 gm mycelia biomass was added into a flask containing 100 ml of distilled water and mixed well for 2 hrs at room temperature. The mycelia free water extract having copper nano-particles was obtained by filtration and centrifugation at 16000 rpm for 15 min. The obtained product was dried at 80℃ for 2 hrs and then subjected to characterization of CuNPs by using UV–Visible spectroscopy.

B. Extracellular

The collected biomass was washed with sterilized distilled water to remove any media components and suspended in 100 ml distilled water for 72 hrs at 30 ± 2℃. The biomass filtrate obtained by passing it through Whatman filter paper No. 1. Finally, filtrate is then collected and ready to use for synthesis of copper nano-particles. 20 mM ($CuSO_4.5H_2O$) was added to 100 ml biomass filtrate and kept for 6 hrs at 35℃ in dark condition.

C. UV-visible Spectroscopy Analysis

Change in colour of the fungal filtrate incubated with copper sulphate solution was visually observed over some time. Absorption measurements of the filtrate were carried out after 24 hrs using UV-visible spectrophotometer (Shimadzu UV-2550) from 550 to 650 nm, at a resolution of 1 nm. The spectral analysis of several weeks old samples was also carried out to check the stability of synthesized CuNPs [17, 18].

D. Mycochemical composition

The screening of mycochemicals in fresh mycelium mat of *A. niger* and *T. viride* was tested by using standard methods followed by Evans and Trease (1989) [19], Gokhale *et al*., [20], Trease, and Evans (1997) [21], Harborne [22], Shanmugam *et al.,* (2010) [23]. The antifungal activity of bicontrolling agents like *A. niger* and *T. viride* was tested by using method followed by Nagadesi and Arya [24].

IV. RESULTS AND DISCUSSION

A. Isolation and identification of fungi

The isolated fungi from soil samples in Andhra Loyola College, Vijayawada-8, Krishna District, Andhra Pradesh, India were identified and shown in Table 1. The highest number of isolates was obtained by spread plate when compared to pour plate method. The highest number of isolates obtained by pour plate was *A. terreus* and in spread plate method was *F. moniliformae*.

B. Selection of medium for synthesis of CuNPs

For selection of suitable medium for synthesis of copper nano-particles different isolated fungi were grown on synthetic mediums of PDA, MEA YMD, YM, MD broth. The best mycelium mat production was observed in PDA broth is *A. niger*, in MEA, YMD, MY broth is *T. viride*, in MD broth is *A. flavus*. When compared to all mediums, the fungi showing least mycelium mat production in *P. chrysogenum*. The best medium used for the synthesis of copper nano-particles is PDA broth when compared to all medium used in the test (Table 2).

Table 1: Different fungal isolates obtained from soil sample of ALC, Vijayawada.

S. No	Fungal isolate	Pore plate Method				Spread plate method			
		2017	A	2018	A	2017	A	2018	A
1.	*Absidia*	6	2.2	4	1.3	—	—	—	—
2.	*Alternaria alternata*	12	4.5	15	5.0	13	3.8	10	3.2
3.	*Aspergillus flavus*	8	3.0	10	3.3	14	4.1	13	4.2
4.	*Aspergillus fumigatus*	10	3.8	12	4.0	17	5.0	11	3.5
5.	*Aspergillus niger*	8	3.0	6	2.0	25	7.4	27	8.7
6.	*Aspergillus terreus*	45	17.1	66	22.2	22	6.5	20	6.5
7.	*Chaetomium globosum*	13	4.9	15	5.0	15	4.4	16	5.2
8.	*Cladosporium herbarum*	16	6.1	14	4.7	—	—	—	—
9.	*Curvularia lunata*	—	—	—	—	11	3.2	14	4.5
10.	*Fusarium oxysporum*	12	4.5	9	3.0	22	6.5	18	5.8
11.	*Fusarium moniliformae*	8	3.0	3	1.0	37	11.0	31	10.0
12.	*Fusarium roseae*	6	2.2	4	1.3	8	2.3	10	3.2
13.	*Fusarium solani*	—	—	—	—	14	4.1	17	5.5
14.	*Mucor racemosus*	18	6.8	14	4.7	18	5.3	15	4.8
15.	*Nigrospora sphaerica*	—	—	—	—	14	4.1	10	3.2
16.	*Penicillium chrysogenum*	9	3.4	15	5.0	13	3.8	11	3.5
17.	*Phoma glomerata*	8	3.0	12	4.0	—	—	—	—
18.	*Rhizopus stolonifer*	20	7.6	18	6.0	24	7.1	21	6.8
19.	*Trichoderma harzianum*	14	5.3	16	5.3	23	6.8	20	6.5
20.	*Trichoderma viride*	8	3.0	5	1.6	2	0.5	5	1.6
21.	Sterile black mycelium	23	8.7	34	11.4	27	8.0	25	8.1
22.	Sterile white mycelium	18	6.8	25	8.4	15	4.4	13	4.2
	Total	**262**		**297**		**334**		**307**	

A: % Occurrence, --: not isolated

Table 2: Different isolates of fungi showing growth in different synthetic mediums.

S. No	Fungal isolate	% wet weight (mg)				
		PDA	MEA	YMD	MY	MD
1.	*Aspergillus niger*	59900	43450	47400	42800	35040
2.	*Aspergillus flavus*	37500	22500	43400	48200	49450
3.	*Aspergillus fumigatus*	50200	33200	45300	35600	42300
4.	*Fusarium oxysporum*	29300	28300	25000	32400	43600
5.	*Mucor racemosus*	25600	39600	34500	21500	22500
6.	*Penicillium chrysogenum*	27900	46900	42300	28600	19800
7.	*Rhizopus stolonifer*	26600	28600	38000	29400	25400
8.	*Trichoderma viride*	55700	46230	54000	52000	46540
9.	*Trichoderma harzianum*	50500	39500	28000	32600	34200
PDA: Potao Dextrose Agar, MEA: Malt Extract Agar, YMD: Yeast Extract Malt Extract Dextrose, MY: Mat Extract Yeast Extract, MD: Malt Extract Dextrose						

Table 3: Intracellular synthesis of Copper nano-particles in PDA Broth.

S. No	Medium	Fungal isolate	% dry weight (mg)	CuNPs
1.	PDA broth (control)	*Aspergillus niger*	31300	- -
2.	0.1 mg $CuSO_4$	*Aspergillus niger*	10300	+
3.	0.2 mg $CuSO_4$	*Aspergillus niger*	41500	+++
4.	0.3 mg $CuSO_4$	*Aspergillus niger*	89000	+++
5.	0.4 mg $CuSO_4$	*Aspergillus niger*	96000	++++
6.	PDA broth (control)	*Trichoderma viride*	14200	--
7.	0.1 mg $CuSO_4$	*Trichoderma viride*	32000	++
8.	0.2 mg $CuSO_4$	*Trichoderma viride*	35000	++
9.	0.3 mg $CuSO_4$	*Trichoderma viride*	36950	+++
10.	0.4 mg $CuSO_4$	*Trichoderma viride*	42050	+++++

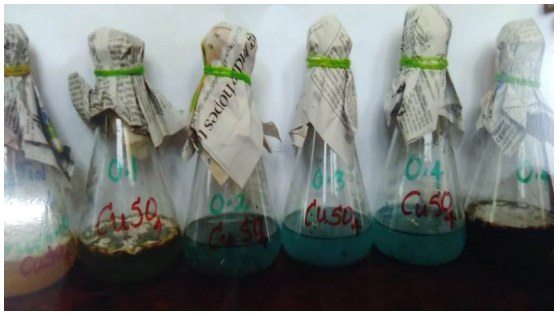
C. Mycogenic synthesis of CuNPs

As the concentration of copper salt increases in the PDA broth containing flasks, the growth and biomass production was increased for *A. niger* and *T. viride*. The fungal species *A. niger* and *T. viride* were found to reduce copper salt into copper nano-particles by visual observation of the fungal filtrates. These two fungal filtrates exhibited a gradual change to dark green/brown colour, clearly indicating the formation of CuNPs (Table 3).

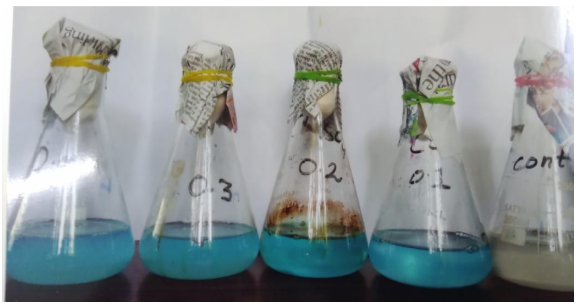
The *A. niger* and *T. viride* exhibited the most intense dark green/brown colour in 0.4mg copper sulphate solution containing PDA broth when compared to 0.1, 0.2, 0.3 mg copper sulphate solution containing PDA broth. The biocontrol agents like *A. niger* and *T. viride* have chitin as fungal cell wall so the chitin/chitosan obtained from live-cell fungal extract was responsible for the extracellular and intracellular mycogenic synthesis of CuNPs [Plate I Fig. (a), (b), (c), Plate II Fig. (a), (b)].

The colour changes observed can be attributed to the surface plasmon resonance of deposited CuNPs [25, 26]. In the present paper also colour change was observed for both biocontrolling agents. The preparation of copper nano-particles using *Aspergillus* species [27], *A. niger* [28], *P. vaksmanii, P. aurantiogriseum* and *P. citrinum*, isolated from soil, have been used for the synthesis of copper nano-particles [29]. In the present paper the mycogenic synthesis of the copper nano-particle by bio-controling fungi isolated from soil samples of ALC, Vijayawada was reported. Chitosan offers good stabilization compared to starch owing to its ability to form chemical bonds with metals. So the use of chitosan as a stabilizing agent increases the stability of metal nano-particles [30]. Copper nano-particles are stabilized by protonated chitosan that prevented coagulation [31]. In the present paper, the Bio-controling agents like *A. niger* and *T. viride* have chitin as fungal cell wall material so the chitin/chitosan obtained from live-cell fungal extract was responsible for the synthesis of CuNPs.

(a) The unincolulated Copper sulphate containing PDA broth.



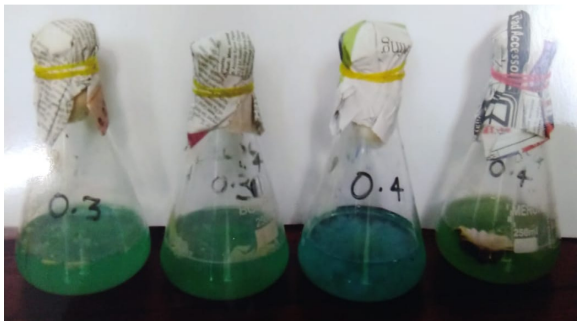
(b) Inoculated Copper sulphate containing medium by *T. viride*.



(c) Uninoculated Copper sulphate containing medium.

Plate-I

(a) Inoculated copper sulphate containing medium by *A. niger.*



(b) Inoculated copper sulphate containing medium by *A. niger.*

Plate-II

D. UV- Visible spectroscopy analysis

The UV-Visible spectroscopy analysis of the filtrate and live-cell extract of fungi shown characteristic surface plasmon resonance (spr) spectra with an absorbance peak at 610 nm which indicates the formation of Copper nano-particles (Fig. 1, 2). Ultraviolet-visible spectroscopy is employed because the absorption peak positions are dependent upon particle size and shape [4]. UV visible spectrum showed absorption of CuNPs synthesized via *A. niger* filtrate and it also showed wide absorption range from 250 to 400 nm [28]. The biosynthesis of copper or copper oxide nano-particles by the mycelium-free extract of *S. hirsutum* in presence of 5 mM of different copper salts ($CuCl_2$, $Cu(NO_3)_2$ or $CuSO_4$) and different pH values (5.0, 7.9, and 9.0) showed surface-plasmon resonance band between 620 to 710 nm with the three copper salts tested [33]. The change in the characteristic peak maximum mentioned above is because of change in size and shape of CuNPs synthesized by *A. niger* and *S. hirsutum*. The 5 mM copper sulphate solution containing flask with *Morganella* sp shown the characteristic peak maximum at 610 nm which indicates the formation of Cu nano-particles [18]. In the present papers also the peak maximum at 610 nm was observed when tested with a bio-controlling agent.

E. Mycochemical composition

Mycochemical compounds screening of water, methanol, ethanol and 50% Hydro-methanol extracts of *A. niger* and *T. viride* is prepared by using Maceration and the results of the solvent having mycochemical compounds are presented in Table 4. The best solvent for preparation of *A. niger* and *T. viride* extraction is methanol because all tests for mycochemical composition shown the excellent concentration of mycochemicals. The mycochemical amino acids are absent in water and hydromethanolic extracts of both fungal cell filtrates (Table 4). For the first time the bio active compounds like Phenols, Alkaloid and Anthocyanins were reported from *G. lucidum* and *G. applanatum* [32]. In the present papers the biocontroling agents also shown different bioactive compounds in copper nanoparticles synthesized filterate.

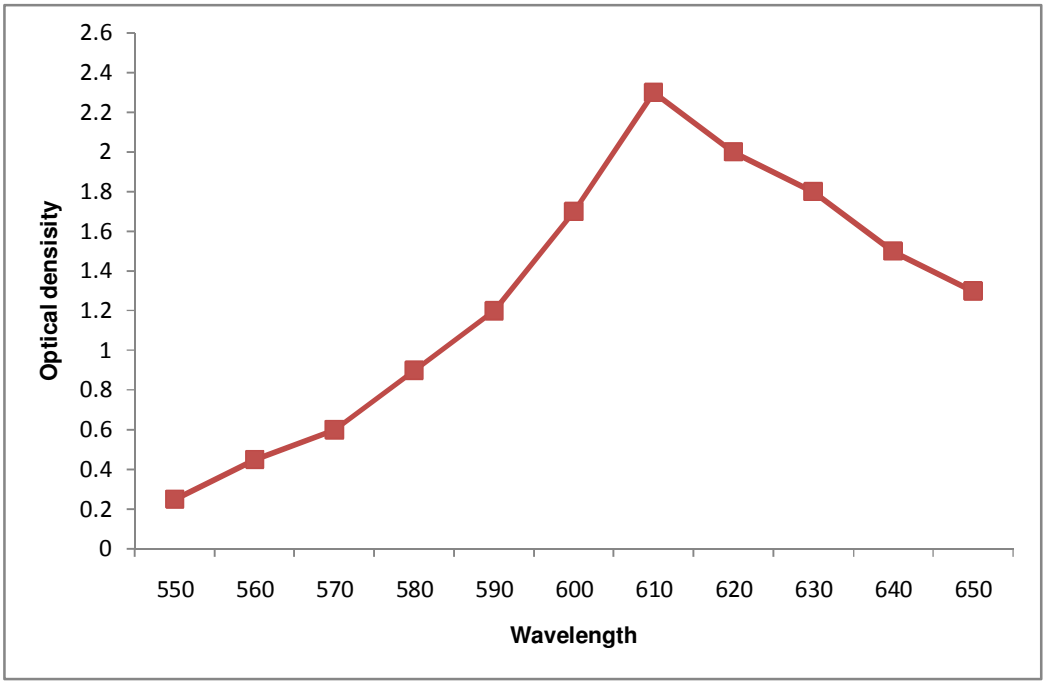


Fig. 1. Formation of CuNPs by *Aaspergillus niger*.

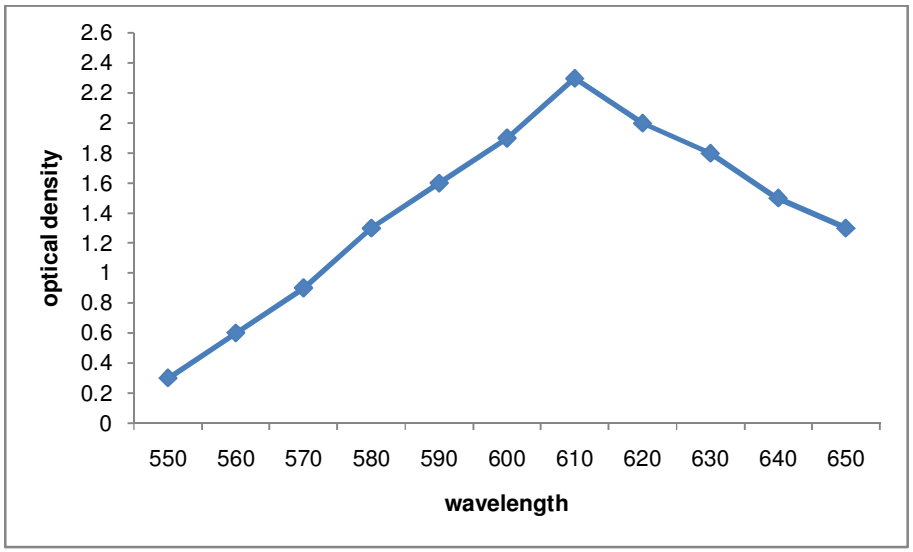


Fig. 2. Formation of CuNPs by *Trichoderma viride*.

Table 4: Mycochemicals produced by CuNPs Synthesizing Biocontrol agents like A. niger, T. Viride.

Aspergillus niger										
Extraction by maceration	A	B	C	D	E	F	G	H	I	J
Water	+	++	+	-	++	+	++	+	+	+
Methanol	+++	++	+	+	++++	++++	+++	+++	+++	+++
Ethanol	++	++	+	+	++++	++++	+++	+++	+++	+++
50% methanol	+++	+++	+	-	+++	+++	+++	+++	++	++
Trichoderma viride										
Water	++	+	+	-	+++	++	++	++	++	++
Methanol	+++	+	+	+	++++	+++	+++	++++	+++	+++
Ethanol	++	+++	+	+	++++	+++	+++	++++	+++	+++
50% methanol	++++	++	+	-	++	++	+++	++++	+++	+++
+= present, ++ (or) +++= moderately present, ++++ (or) ++++= Excellent, A: Alkaloid, B: Carbohydrates, C: Proteins, D Amino Acids, E: Tannins, F: Flavonoids, G: Phenols, H: Terpenoids I: Di Terpenoids, J: Anthocyanins										

F. Antifungal test

The live-cell filtrates of *T. viride* producing CuNPs shown better controlling on plant pathogenic fungi tested when compared to live-cell filtrates of *A. niger*. The 20% and 25% concentrations of a bio-controlling agent like *A. niger* and *T. viride* filtrates showed 100% inhibition of plant pathogenic fungi.

Table 5: Antifungal activity of live cell filtrates of CuNPs synthesized by *A. niger* and *T. viride*.

Aspergillus niger					
Fungi	**5%**	**10%**	**15%**	**20%**	**25%**
Alternaria alternata	75	89	90	100	100
Cladosporium cladosporoides	80	85	92	100	100
Fusarium oryzae	89	86	98	100	100
Fusarium roseae	82	88	95	99	100
Pencillium citrinum	73	92	96	100	100
Rhizopus artocarpi	88	98	100	100	100
Trichoderma viride					
Fungi	**5%**	**10%**	**15%**	**20%**	**25%**
Alternaria alternata	91	95	100	100	100
Cladosporium cladosporoides	93	97	100	100	100
Fusarium oryzae	89	97	98	100	100
Fusarium roseae	86	99	100	100	100
Pencillium citrinum	83	91	99	100	100
Rhizopus artocarpi	82	93	99	100	100

The *A. niger* live-cell filtrate showing least control on plant pathogenic fungi like *P. citrinum*. The *T. viride* live-cell filtrate showing least control on *R. artocarpi* in 5% concentration (Table 5). The synthesized CuNPs shown the highest effectiveness against plant pathogenic fungi like *F. oxysporum, Phoma destructive, C. lunata* and *A. alternata* with a diameter of inhibition 24, 22, 21 and 18 mm respectively [34]. In the present paper also the phytopathogenic fungi like *R. artocarpi, P. citrinum, F. roseae, A. alternata, F. oryzae* and *C. cladosporoides* was 100% inhibited by 20% and 25% concentration containing CuNPs. Ouda [35] studied about antifungal activity of silver and copper nano-particles on two plant pathogens *A. alternata* and *B. cinerea*. In the present paper also the *A. alternata* obtained from infected Brinjal was controlled by CuNPs containing 20% and 25% concentration.

V. CONCLUSIONS

In the present world, Nano-particle synthesized by biological methods has very important biomedical applications. So the Mycogenic synthesis of copper nano-particle was tested. Different fungal isolates were isolated from a soil sample of ALC, Vijayawada by two methods i.e. Pore plate and spread plate methods. The highest number of isolates was obtained by spread plate when compared with pore plate method. Within two years, 641 isolates were obtained by spread plate method whereas only 559 isolates was obtained by pore plated method. Different synthetic mediums like PDA, MEA, YMD, YM, MD broth mediums was used to synthesize copper nano-particles by fungal isolates. The best medium for synthesis of copper nano-particles is PDA broth when compared to all medium used in the

test. The bio-control agents like *A. niger* and *T. viride* shown better growth and biomass production in PDA broth when compared to all mediums. For identification of CuNPs synthesis in the fungal filtrates and live-cell extracts exhibited a characteristic change in colour i.e. gradual change from blue to dark green colour, which confirms the formation of CuNPs by extracellularly and intracellularly respectively. The synthesized CuNPs was characterized by using UV- Visible spectroscopy analysis, which reveals that fungal filtrate and live-cell extract shown characteristic Surface Plasmon Resonance (SPR) spectra with an absorbance peak at 610 nm which further indicates the formation of Copper nano-particles. Different solvents was used to prepare filtrates to test the presence of different mycochemical composition in it. The best solvent for preparation of *A. niger* and *T. viride* extracts was methanol which shown the excellent concentration of mycochemicals tested. The CuNPs shown better bio-controlling on different Phytopathogenic fungi like *R. artocarpi, P. citrinum, F. roseae, A. alternate, F. oryzae,* and *C. cladosporoides*. These phytopathogenic fungi were 100% inhibited by 20% and 25% concentration of CuNPs filtrate. The mycogenic synthesis of copper nano-particles by bio-controlling agents have a wide scope in nanotechnology, conductive films, Antimicrobial, and Antifungal agents

Conflict of Interest. No conflict of intrest.

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