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Green Synthesis of CuNPs as Bio-Controlling Agents Against *Sclerotium rolfsii* Causing Stem Rot sickness of *Arachis hypogaea L.*

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Abstract

Groundnut stem rot caused by *Sclerotium rolfsii* Sacc causing stem rot on more than 500 plant species of agricultural and horticultural crops throughout the world. Most of the first symptom associated with *S. rolfsii* are usually yellowing and wilting of leaves following stem rot infections. Biological management of the disease through antagonists is an eco-friendly approach apart from better alternative to the use of chemicals. In the present study, the thirty antagonistic microorganisms were evaluated by dual culture technique for their antagonistic effect against *S. rolfsii* under in-vitro conditions. Maximum inhibition of mycelial growth (92.78%) was noticed in *Hypocrea koningii* (Gunthakal field isolate) which was followed by T. Viride (Kadiri field) (63.33%). Least inhibition was observed in *T. harzianum* RDGFI isolate (31.67%). The results indicated that the application of these micro-organisms successfully decreases the stem rot incidence and also increases the growth of the groundnut plants. As the *Hypocrea koningii* has several benefits to crop plants. The efficient usage of antagonist was needful. To utilize the antagonist effectively copper Nanoparticles are biosynthesized by reducing copper nitrate. The size and surface Plasmon of Cu NP are compatible to enhance the efficacy of antagonist. However, CuNPs talc-based powder formulation was developed and commercialized. For this These *Hypocrea koningii* copper Nanoparticles were characterized by using the techniques such as X-ray Diffractometry (XRD), Fourier transform infrared spectrophotometry (FT-IR), UV–Vis spectrophotometry, Dynamic Light Scattering (Particle size), Zeta Potential and Transmission Electron Microscopy (TEM) with EDX.

Keywords

Groundnut; stem rot; *Sclerotium rolfsii*; Biological control; *Trichoderma; Hypocrea koningii* CuNPs

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1. INTRODUCTION

Peanut crop is prone to many diseases viz., tikka or leaf spot disease by *Cercospora*, collar rot by *Aspergillus*, seed rot and stem rot by *Sclerotium rolfsii* etc. Among these, stem rot caused by *Sclerotium rolfsii* which is gaining importance. *S. rolfsii* is an economically important pathogen on numerous crops worldwide (Punja, 1985). Moist weather is conductive to sclerotial germination and mycelial growth. Consequently, the diseases caused by the fungus are more serious in tropical and subtropical regions than in temperate regions. The

huge number of sclerotia produced by *S. rolfsii* and their ability to persist in the soil for several years, as well as the profuse growth rate of the fungus make it well suited facultative parasite and a pathogen of major importance throughout the world. The first confirmed report of losses due to the pathogen in USA was made by Rolfs in 1892 on tomato (*Lycopesicon esculentum* Miller) in Florida (Aycock, 1966).

The fungus survives in soil mainly as sclerotia which represent the main source of inoculums and it remains viable in soil for several months (Higgins, 1927). Most of the first symptom associated with *S. rolfsii* are usually yellowing and wilting of leaves following stem rot infections. Natural management of the disease through antagonists is an eco-friendly approach apart from better alternative to the use of chemicals. Trichoderma sp. has been reported to be potential antagonists and these gained considerable success for the control of plant diseases (Upadhay, 1986).

Nanobiotechnology, a branch of nanoscience has been playing a decisive role in 21st century in deciphering diverse tribulations particularly in the fields of farming, medicine, and electronics. Nanoscience poses a basic scientific challenge as it requires a control over the connections between atoms. All physiochemical methods of nanoparticles synthesis are having inherent limitations up to a certain extent which impose an important hurdle in the maturation of this science. The possibility of utilizing biological materials for nanoparticles synthesis has appeared as the most efficient and greener approach (Supraja et al. 2015) Nanomaterials exhibit unique and considerably changed physical, chemical, and biological properties compared to their bulk counterparts (Singh et al. 2011) Although physical and chemical methods (Prasad et al. 2012) are more popular for nanoparticle synthesis, the use of toxic compounds limits their applications (Prabha et al. 2014)

The thought of a sustainable agricultural practice and environmental defense is enhancing the need of biocontrol as an alternative technique to keep away from chemical hazards on both human beings and beneficial soil microorganisms (Campbell, 1989). The application of bio-control agents is the key elements for sustainable agriculture. Therefore, the adoption of a sustainable agricultural practice, using strategies that are environmentally friendly, less dependent on agricultural chemicals is gaining worldwide recognition.

In view of the above findings the present study was carried out by some of the beneficial bio agents collected from different farmer's fields and tested against the *S. rolfsii* to determine their antagonistic potential in vitro by using copper nanoparticles.

2. MATERIALS AND MEASUREMENTS METHODS

2.1 Isolation and identification of the pathogen

The infected specimens were cut into small bits and washed in running water. These bits were surface sterilized with 1% mercuric chloride solution and then aseptically transferred to Petri plates containing sterilized PDA medium. The plates were incubated at 27±1°C for three days. The fungal growth on fourth day, which arise through the infected tissue was taken by inoculation loop and transferred aseptically to the PDA slants. The pure culture of the fungus was obtained by further growing the culture and following hyphal tip culture method under aseptic conditions (Rangaswami, 1988).

2.2 Proving the Pathogenicity.

Sterilized soil was taken in earthen pots of size 45x30 cm. Thirty days old culture grown on sorghum grains was mixed thoroughly with soil to get sick soil. Then apparently healthy groundnut seeds (K-6 variety) were planted in pots filled with sick soil groundnut seeds sown in pots without inoculums served as control. Soil moisture was maintained at 25% moisture holding capacity of soil by adding water on weight basis throughout the period. Re-isolation was made from such affected portion of the plant tissue and compared with that of original culture.

2.3 Evaluation of bio-agents

In vitro evaluation was carried out with Ten bio-agents were collected from different farmer fields against GNATP Sr. (groundnut Ananthapuramu (dy)/dx *S. rolfsii*) isolate of *S. rolfsii* through dual culture technique.

2.4 Dual culture technique

Twenty ml of sterilized and cooled potato dextrose agar was poured into sterile petriplate and allowed to solidify. For evaluation of fungal biocontrol agents, mycelial discs of test fungus were inoculated at one end of the petriplate and antagonistic fungus was placed opposite to it on the other end. The plates were incubated at 27±1°C and zone of inhibition was recorded by measuring the clear distance between the margin of the test fungus and antagonistic organism. The colony diameter of pathogen in control plate was also recorded. The per cent inhibition of growth of the pathogen was calculated by using the formula suggested by Vincent (Vincent, 1947).

I= (C-T)/CX100

Where,

I = per cent inhibition

C = growth in control

T = growth in treatment

2.5 Bio synthesis of Cu Nanoparticles

Copper Nanoparticles are biosynthesized by reducing copper nitrate. The size and surface Plasmon of Cu NP are compatible to enhance the efficacy of antagonist. However, CuNPs talc-based powder formulation was developed and commercialized.

2.6 Preparation of aqueous extract (AE)

The collected *Hypocrea koningii* was inoculated in Potato dextrose broth and incubated for 3days after the formation of mycelium the fungal extract was filtered by using Whatman No. 1 filter paper and collected in plastic bottle and stored at 4±C for further characterization and experimentation.

2.7 Preparation of Hypocrea koningii mediated copper Nanoparticles

Copper nitrate (>99% pure) was purchased from Sigma- Aldrich, India. Nutrient broth, nutrient agar plate, was supplied by Hi-Media, India. To prepare the CuNPs, a 90-mL aqueous solution of $1.0x10^{-3}$M copper nitrate was mixed with a 10-mL of 5% aqueous solution of *Hypocrea koningii* extract. The *Hypocrea koningii* Cu solution was blue in color and the solution was stirred repeatedly for an hour, and it was observed that the color of the solution has been changed to dark bluish green which visually confirms the formation of Nanoparticles. These *Hypocrea koningii* copper Nanoparticles were characterized by using the techniques such as UV–Vis spectrophotometry, Fourier transform infrared spectrophotometry (FT-IR), X-ray Diffractometry (XRD), Dynamic light scattering (Particle size), zeta potential, Transmission electron microscopy (TEM) and Energy dispersion X-ray Spectrum (EDX).

2.8 Characterization of Nanoparticles

2.8.1 UV–visible spectrum for synthesized Nanoparticles

The bio-reluctant Nanoparticles were monitored by UV– visible (UV–Vis) spectrum at various time intervals. The UV–Vis spectra of this solution were recorded in spectra 2450, SHIMADZU Spectrophotometer, from 400 to 800 nm.

2.8.2 FT-IR analysis for synthesized nanoparticles

The FTIR spectrum was taken in the mid-IR region of 400–4,000cm-1. The spectrum was recorded using ATR (attenuated total reflectance) technique. The dried sample was mixed with the KBr (1:200) crystal, and the spectrum was recorded in the transmittance mode.

2.8.3 XRD analysis for synthesized nanoparticles

The nanoparticles were harvested and characterized by XRD and TEM. The XRD pattern was recorded using computer controlled XRD-system, JEOL, and Model: JPX-8030 with CuK radiation (Ni filtered= 13418 A^{o}) at the range of 40kV, 20A.

2.8.4 Particle size and zeta potential analyzer for synthesized Nanoparticles

The aqueous suspension of the synthesized Nanoparticles was filtered through a 0.22µm syringe-driven filter unit, and the size and distribution of the Nanoparticles were measured using dynamic light scattering technique (Nanopartica, HORIBA, SZ-100).

2.8.5 Transmission Electron Microscopy (TEM) and EDX

The morphology of the nanoparticles was characterization by Transmission electron microscopy was performed on JEOL (JEM-1010) instrument, with an accelerating voltage of 80kv after drying of a drop of aqueous nanoparticles on the carbon-coated copper TEM grids Samples were dried and kept under vacuum in desiccators before loading them onto a specimen holder. The particle size and surface morphology of nanoparticles were evaluated using ImageJ 1.45s software equipped with an energy dispersive X-ray spectrum (EDX)

3. RESULTS AND DISCUSSION

The Pathogenicity test of *S. rolfsii* to groundnut was proved by soil inoculation method, carried out under glasshouse conditions and for this mass multiplication of the pathogen was done (**Fig.1a to e**) as per the procedure described in 'Material and Methods'. Control was maintained with sterilized soil. The pathogen infected first at stem region. Leaves of such infected plants became pale green followed by yellowing. During advanced stage of infection, the white mycelium grew around the stem region and completely covered it. The plant gradually dried and toppled. The sclerotial bodies were formed on infected parts. The fungus was re-isolated from affected plant tissue and compared with the original culture (Fig.1c). The nine antagonistic micro-organisms were evaluated by dual culture technique for their antagonistic effect against *S. rolfsii* under in-vitro conditions. Inhibition zone in mm was recorded and the per cent inhibition was calculated. At 7 days after inoculation maximum inhibition of mycelial growth 71.67 per cent was observed in *ATPF-7* (GTLF field isolate), which was followed by *ATPF-4* (Kadiri field) 63.33 per cent and *T. harzianum* ATPFI 60.00 percent. Least inhibition was recorded in *T. harzianum* RDGF isolate 31.67 percent, respectively.

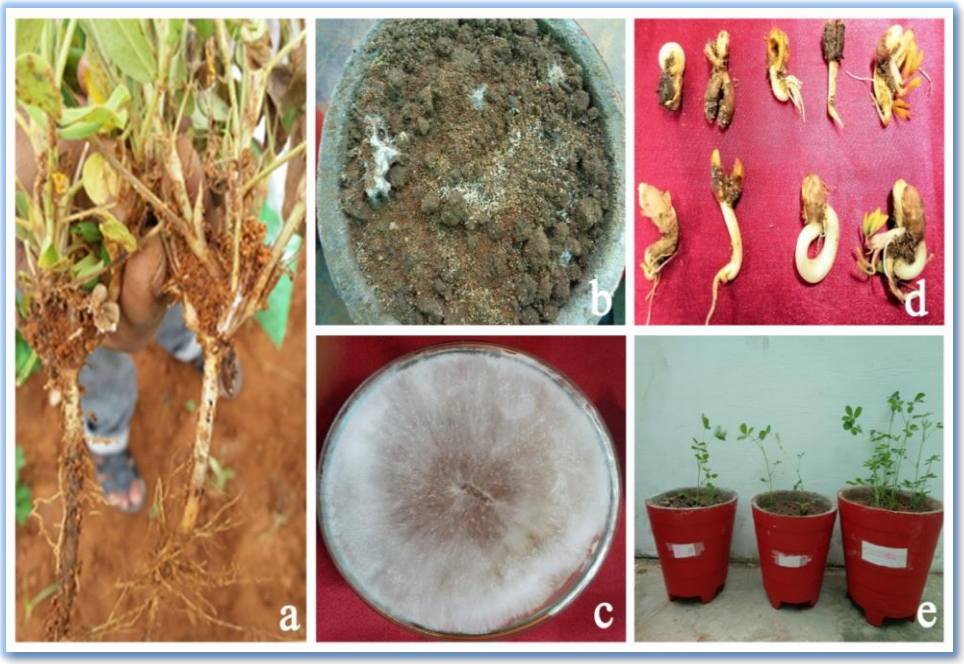

Fig. 1 showing Symptoms of stem rot of Groundnut.
a) Infected Groundnut plant with *Sclerotium rolfsi;* b) Sclerotium inoculated soil; c) Infected seedlings; d) Pure culture of *S. rolfsii* ; e) Pot culture studies

Some soil fungi isolated from the agricultural field soil were found to grow fast in dual culture with the pathogen i.e., *S. rolfsii*. In the present study the slow growth rate of the pathogen suggested a more rapid utilization of nutrients by the antagonists when grown together. Nutrient depletion, space and production of toxic substances (antibiotic and antibiotic like substances) by the fungi are known to play a dominant role in antagonism and these factors are usually governed by the physico- chemical nature of the environment (Burgess and Griffin, 1967). The present in vitro study results showing the positive antagonistic effect of the soil fungi which have restricted the growth of the pathogen under in vitro condition (i.e., *Trichoderma* sp.)

Number of Trichoderma species is known to be active hyperparasites of several soil fungi and hence they are used as a bio-control agent (Ekefan *et al*., 2009). Control of plant diseases using antagonistic microorganisms can be an effective means (Cook, 1993). Various plant diseases have been successfully controlled through bacterial and fungal antagonists (Cook and Baker, 1983]. Antagonistic microorganisms reduce growth, survival or infections caused by the pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. Antagonistic micro-organisms, such as Trichoderma, reduce growth, survival or infections caused by the pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion (Ponnusamykonar et al. 2011).

Biological control is an effective, eco-friendly, and alternative approach for management of any disease. Similar trend was observed when the test pathogen was placed at periphery where, maximum per cent inhibition of mycelial growth in ATPF-7 Isolate (92.15%), followed by ATPF-4 (57.97%) and least inhibition of mycelial growth was observed in Bacillus subtilis (10.74%). Similarly (Manu, 2012). reported least inhibition by *B. Subtilis* and *P. fluorescens* as against higher inhibition by Trichoderma spp. (Parmar, 2015) screened the six Trichoderma strains among them *T. viride* (NBAII Tv 23) inhibited 61 per cent growth of *S. rolfsii* followed by *T. harzianum* (NBAII Th 1) 55 per cent, respectively (Nagamma and Nagaraja, 2015).

3.1 Isolation and evaluation of bio-control agents

The 30 mycoflora isolated from rhizosphere were identified as *Trichoderma* spp. based on their colony and morphological characteristics. The results were in coordination with earlier reports of (Sreenivasa Murtrhy et al. 2018). The Cultural (colony characteristics), Morphological and Biochemical characterization of Fungal bio control agents shows moderate plant growth promoting activities in terms of phosphate solubilisation, Indole production, Exopolysaccharides and ammonia production etc.

3.2 Evaluation of the antagonistic activity of micro flora against S. rolfsii in dual culture under in vitro

The antagonistic potential of isolated bio-control agents was assessed based on their ability to arrest the pathogen growth in dual culture technique. The effect of these native antagonists on the mycelial growth of the pathogen was calculated and expressed as percent inhibition (Nene and Thapliyal, 1993) (Fig.2 & Table.1).

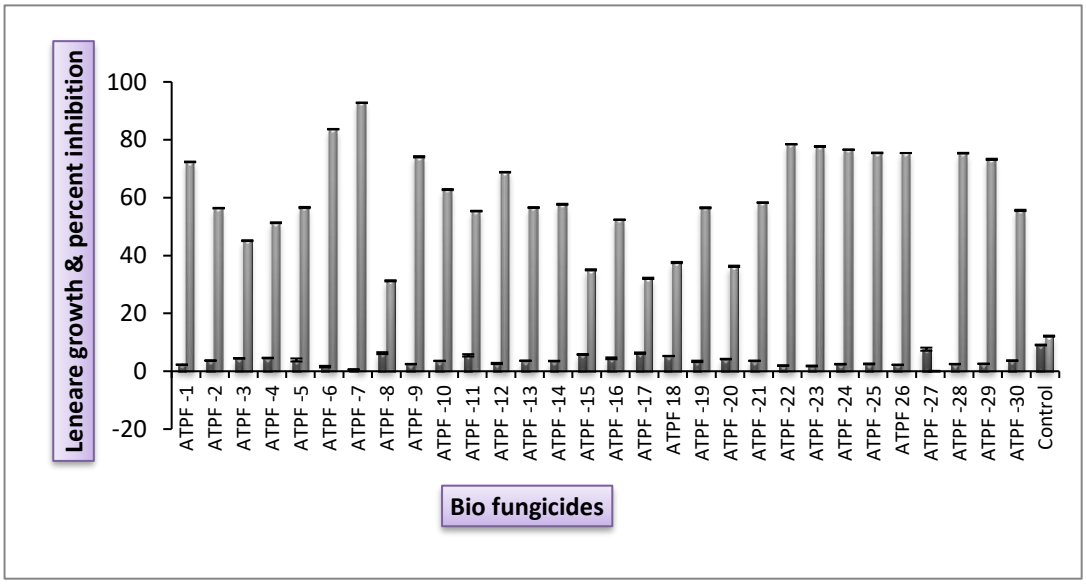

Fig. 2 Antagonistic activity of fungal bio-fungicides

Table 1 List of bio-agents used for *in vitro* evaluation (ATPF-1 TO ATPF-30), Against *Sclerotium rolfsii* causing stem rot of Groundnut.

Bio-Fungicides	*Linear growth *S. rolfsii* (cm)	Percent inhibition of mycelial growth of *S. rolfsii*
ATPF -1	2.26±0.04	72.34±0.12
ATPF -2	3.68±0.05	56.36±0.10
ATPF -3	4.45±0.08	45.12±0.16
ATPF -4	4.58±0.10	51.32±0.15
ATPF -5	3.89±0.53	56.58±0.21
ATPF -6	1.59±0.28	83.65±0.09
ATPF -7	0.58±0.05	92.78±0.08
ATPF -8	6.25±0.34	31.24±0.21
ATPF -9	2.46±0.08	74.12±0.23
ATPF -10	3.59±0.06	62.78±0.18
ATPF -11	4.20±0.41	55.34±0.12
ATPF -12	2.68+0.21	68.78±0.11
ATPF -13	3.63±0.08	56.57±0.17
ATPF -14	3.52±0.10	57.68±0.19
ATPF -15	5.79±0.08	35.04±0.20
ATPF -16	4.46±0.32	52.36±0.09
ATPF -17	6.25±0.25	32.10±0.23
ATPF 18	5.26±0.06	37.57±0.21
ATPF -19	3.41±0.25	56.48±0.19
ATPF -20	5.49±0.10	36.25±0.24
ATPF -21	3.61±0.08	58.28±0.12
ATPF -22	1.95±0.12	78.45±0.08
ATPF -23	1.81±0.15	77.68±0.10
ATPF -24	2.43±0.12	76.57±0.09
ATPF -25	2.54±0.14	75.46±0.11
ATPF 26	2.23±0.06	75.42±0.19
ATPF -27	7.62±0.54	12.12±0.14

ATPF -28	2.47±0.08	75.35±0.13
ATPF -29	2.58±0.06	73.23±0.21
ATPF -30	3.67±0.08	55.58±0.23
Control	9.06±0.14	0.00±0.02
CD (0.05)	-	1.5801
S.Em±	-	1.0738

3.3 Mass multiplication of bio agent (ATPF-07)

The potential antagonistic bio pesticide *Hypocrea koningii* (ATPF-07) was mass multiplied on potato dextrose Broth at 28±2°C for 96 hours and applied to the soil @ 20 ml Kg^{-1} soil.

3.4 Development of talc-based formulations

One kg of talc powder was taken in a metal tray Add 15 gr. of $CaCO_3$ for adjusting pH-7 Add 10 gr. Of carboxy methyl cellulose Mix well and Autoclave it for 30minutes at 120ºC Mix 400 gr. Of fungal suspension (Bio control) dry up to 35% moisture content overnight under aseptic condition. Pack the mixture in poly propylene bags and sealed the talc-based formulations of potential bio pesticide ATPF-07 were developed; air dried and packed in polythene covers. The formulations were stored at room temperature until they were used for field evaluation purpose (Fig. 3)

3.5 UV-visible spectrum

The absorption spectrum was recorded for the sample in the range of 700-800nm the spectrum showed the absorbance range at 297nm corresponding to characterize the LSPR band of copper Nano particles. The protein and bio metrics present in the fungi may lead to the change in the absorbance UV-vis micro graph, whereas there is no absorbance peak seen in the Uv-visible spectra clearly agree with the result enlightened that the synthesized Nano particles were found to be symmetrical with spherical in nature. Surface Plasmon resonance is clearly found in the optical spectra and was in visible region. Since the intensity of the Plasmon resonance band depends on particle size, shape, metallic material and its surrounding environment, the number particles cannot be related linearly to the absorbance intensities (Fig.4a & b) (Supraja and Prasad, 2017).

Fig. 3 Talc based formulations from *Hypocrea koningii*

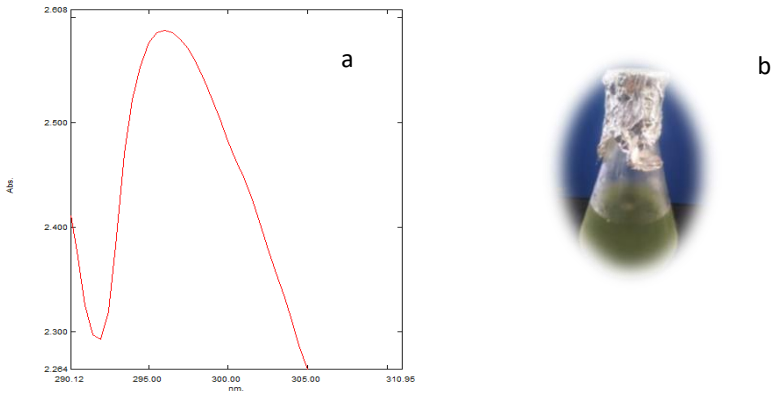

Fig. 4 UV –Visible spectrum of a) Control, b) Copper nanoparticles synthesized from *Hypocrea koningii*

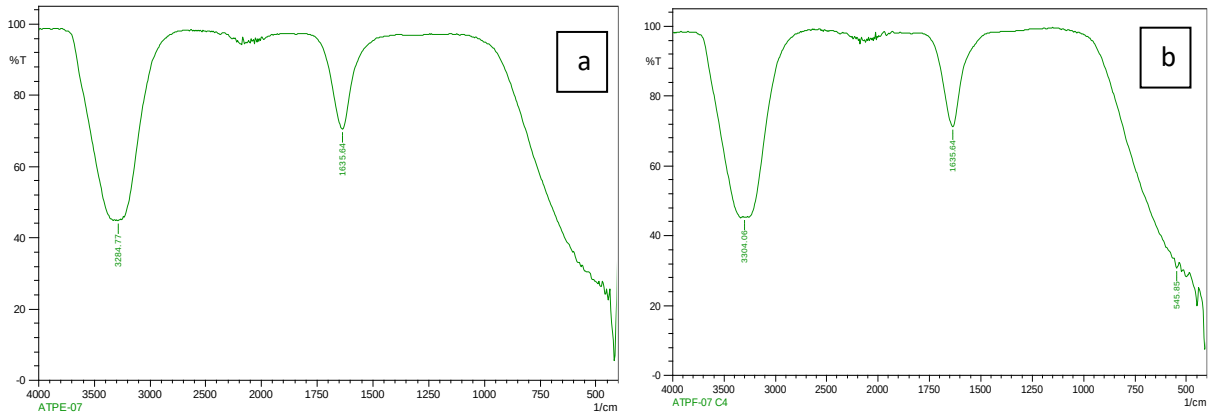

Fig. 5 FT-IR spectrum of a) Control, b) Copper nanoparticles synthesized from *Hypocrea koningii*

3.6 FT-IR spectroscopy

Whereas in control, the peak present at 3284.77cm-1 indicating C-H stretching Vibration of Lipids Absorbance present in fungi (*Hypocrea koningii*), 1635.64 cm-1 peak indicating C-N stretching vibration of amide (protein) present in fungal sample. In FTIR changes in weak number of the functional groups was observed due to reduction and stabilization of copper on comparing IR stretching frequency of functional present in Hypocrea koningii by CuNPs (Fig. 5a)

The functional groups – amines, carbonyls, alkyl halides, lipids, amide, the spectrum shows the bands for the functional groups located as 3304.06, 1635.64, 545.85 cm^{-1} in the presence of CuNPs. Whereas in control (*Hypocrea koningii*) 3284.77 and 1635.64^{-1}. The peak present at 3304.06 indicating the presence of N-H bend stretching vibration of alkyl halides. It was recorded that copper binds to Oxygen moiety indicated by C-Br at 545.85 cm^{-1} (Fig. 5b) (Kapil et al. 2018)

3.7 X-Ray Diffraction

The XRD pattern of CuNPs powder. The peaks observed at 2θ values of 33.39°, 38.67º, 50.49°, 58,67º and 74.18° correspond to (110), (111), (202), (113) and (200) planes of metallic Cu Apart from the metallic Cu peaks, several other diffraction peaks appeared at 36.54° and 61.6° representing the formation of cubic copper (I) oxide nanocrystals. The result was explained that CuNPs might be formed by oxidation when the solution was evaporated at 150°C for 2 hours at atmosphere. Because of the biomass residue, other crystallographic impurities were also observed in the XRD profile. The size of CuNPs according to the XRD was about 50.2 nm. This result was consistent with the TEM study, whereas in control there is no any peak indication is seen (Fig. 6a & 6b) (Kayal et al., 2016).

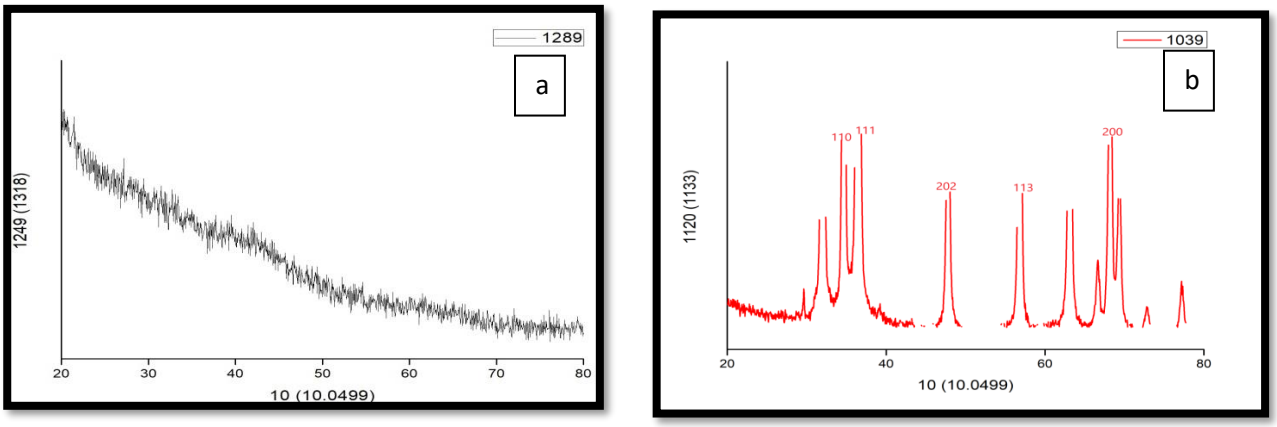

Fig. 6 XRD of a) Control, b) Copper nanoparticles synthesized from *Hypocrea koningii*

The particle size distribution in the *Hypocrea koningii* - 282.6 nm
Zeta potential - -3.1 mV
Electrophoretic mobility mean - -0.00024 (m2/ vs

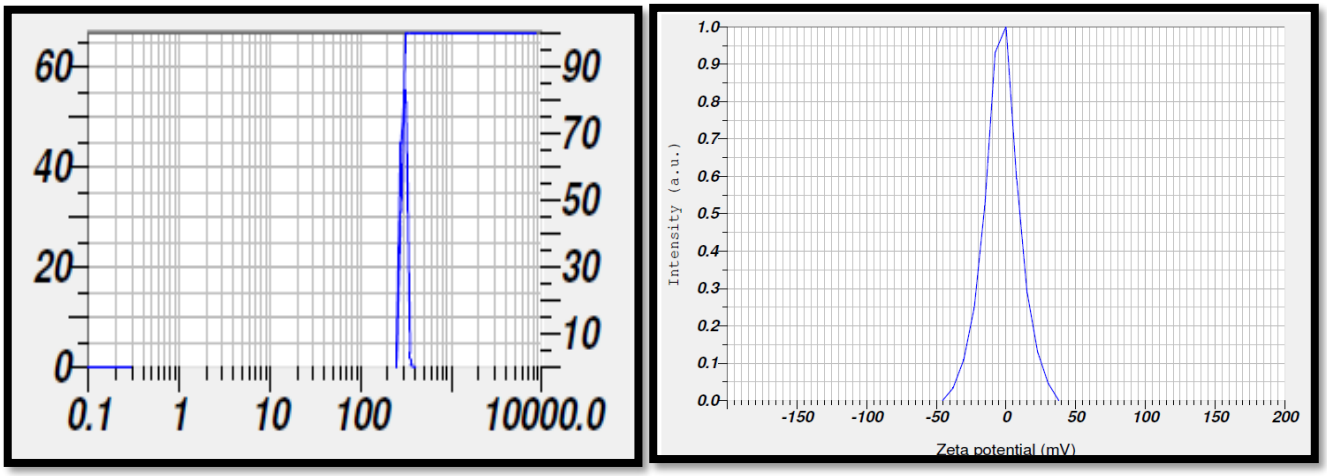

Fig. 7a Control showing DLS of *Hypocrea koningii*

3.8 DLS (Dynamic Light Scattering) Analysis

Particle size and zeta potential values were measured using Nano particle S-Z-100(HORIBA). The particle size distribution in the *Hypocrea koningii* recorded as 282.6 nm and the Zeta potential is -3.1mV and electrophoretic mobility mean is -0.00024(m^2/vs. however if the particle has low zeta potential values, then there will be no force to frequent the particles coming together by flocculating (Fig. 7a).

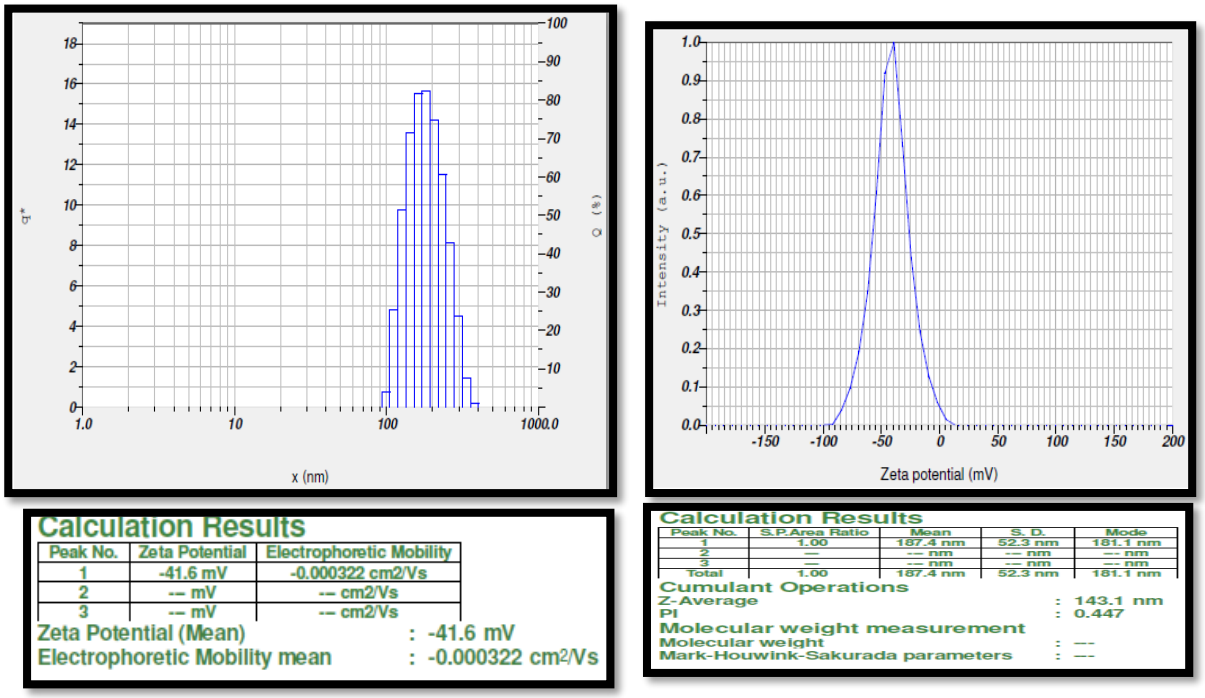

Fig. 7b DLS analysis of Copper nanoparticles synthesized from *Hypocrea koningii*

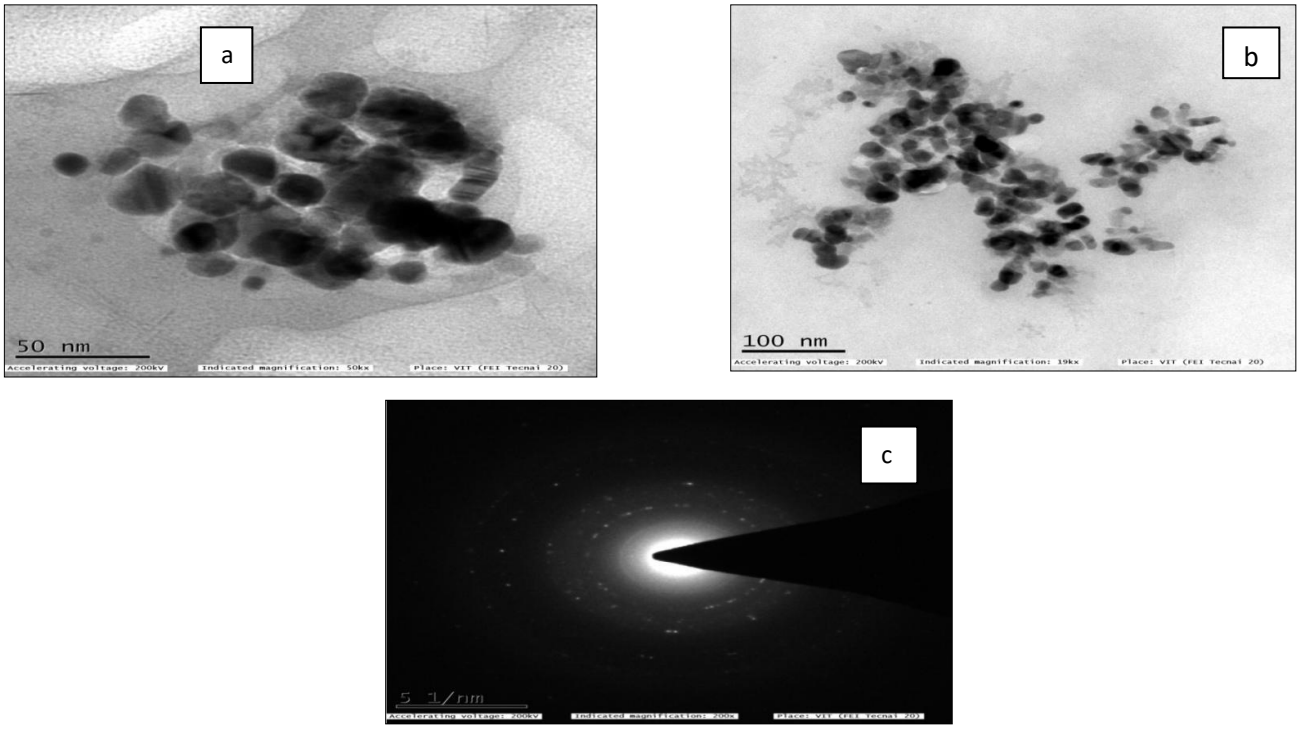

Fig. 8 TEM analysis of Copper nanoparticles synthesized from *Hypocrea koningii*

Particle size and zeta potential values were measured using Nano particle S-Z-100(HORIBA). The zeta potential spectra for the *Hypocrea koningii* Bio-control agent extract mediated copper Nano particles were recovered as -41.6 mV by electrophoretic mobility is 0.000322 (m^2 /vs by the particle size of NPS were 143.1 mm (Fig. 7b) (Supraja et al. 2018)

3.9 Transmission Electron Microscopy

Surface morphology of *Hypocrea koningii* extract mediated synthesized copper nanoparticles was studied from the TEM micrograph. It is evident that CuNPs were spherical in shape and were poly-dispersed very slight agglomeration is seen due to the presence of mycelium in fungi. The measured average size of CuNPs was 50-100 nm and occasional agglomeration of the CuNPs has been observed (Fig.8) (Sreekanth et al. 2014).

3.10 Energy Dispersive X-ray (*EDX)*

The quantitative and qualitative analysis of elements may be concerned in the formation of copper nanoparticles. They were identified by EDX analysis. Due to the Surface Plasmon Resonance, the copper nanoparticle shows the absorption peaks of higher counts some other peaks also found it is due to the presence of proteins present in *Hypocrea koningii* (Fig.9).

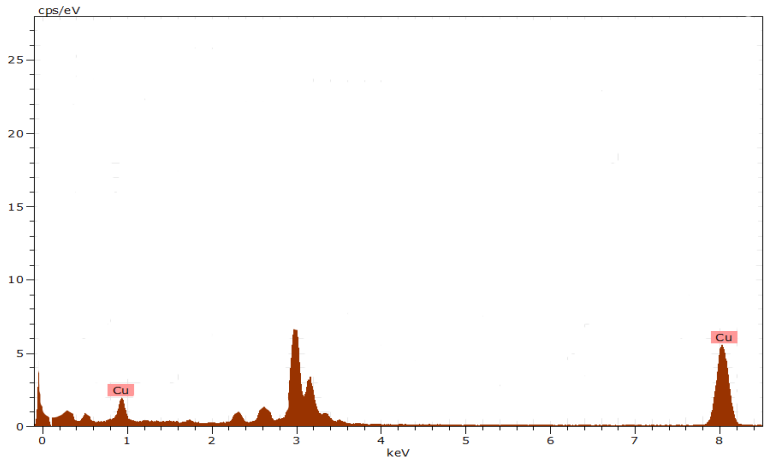

Fig. 9 EDX analysis of Copper nanoparticles synthesized from *Hypocrea koningii*

4. CONCLUSIONS

The idea of a sustainable agricultural practice and environmental protection is enhancing the need of biocontrol as an alternative technique to avoid chemical hazards on both human beings and beneficial soil microorganisms.

- From the *in-vitro* findings, it can be suggested that the antagonist *Hypocrea koningii* (*Trichoderma koningii)* can be used as a bio-control agent against *S. rolfsii* under pot culture studies.
- It is also revealed that the microorganisms that naturally remain in the soil are having more or less similar potential antagonistic effect on the various crop diseases caused by various pathogens. And some of them can be used as potential bio-control agents under field condition to decrease the disease incidence and to increase crop productivity.
- In addition, *Trichoderma* Enhances yield along with quality of produce. Boost germination rate. Increase in shoot & Root length solubilizing various insoluble forms of Phosphates Augment Nitrogen fixing. Promote healthy growth in early stages of crop. Increase Dry matter Production substantially. Provide natural long-term immunity to crops and soil.
- As the *Hypocrea koningii* has several benefits to crop plants. The efficient usage of antagonist was needful. To utilize the antagonist effectively copper Nanoparticles are biosynthesized by reducing copper nitrate. The size and surface Plasmon of CuNPs are compatible to enhance the efficacy of antagonist. However, CuNPs talc-based powder formulation was developed and commercialized.
- Therefore, further work should be taken up to explore the possibility of the usage of the CuNPs talc-based powder formulation under field condition.

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