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Natural Occurrence of Potential Fungal Biopesticide *Nomuraea Rileyi* (Farlow) Samson Associated with Agriculture Fields of Tamil Nadu, India and it's Compatibility with Metallic Nanoparticles

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Abstract

In the present study, natural occurrence of major fungal biopesticide *Nomuraea rileyi* (Farlow) Samson associated with agricultural field soil in an area around TamilNadu, India was studied adapting culture dependent method. Agricultural field soil samples were collected from ten different sites. Soil dilution method was used to isolate *N. rileyi*. A total of 123 isolates of *N.rileyi* were obtained. Among the 10 sampling sites, *Nomurea rileyi* was isolated from 4 sites belonging to Hasthampatty (Salem) Rajakoil (Vellore) Vadavali (Coimbatore), Pullarakottai (Viruthunagar), High frequency of fungal occurrence was recorded in vadavali (60%) followed by Pullarakottai (17%), Hasthampatty (13%,) Rajakoil (10%). Non *Nomurae rileyi* strains belong to *Beauveria* sp and *Metarhizium* sp were also isolated. Fungal occurrance was highly influenced by soil physico-chemical parameters. Effect of metallic nanoparticles such as silver, copper and the respective nanoparticles coated with chitosan on the post treatment persistance of *N. rileyi* was also studied.Distinct effcet on the growth of *N. rileyi* was recorded in copper nanoparticles with high concentration.

Keywords: *Nomuraea rileyi*; Natural occurrence; Agricultural field soil; Compatibility; Metallic nanoparticles

Introduction

Among the various microsymbions, fungi (mycorhizal and pathogens) is the major component of soil. Soil is also the major source of entomopathogenic fungi and the isolation of these fungi involves soil sampling since that is their natural habitat [1,2]. Entomopathogenic fungi are distributed in a wide range of habitats including aquatic forest, agricultural, pasture, desert, and urban habitats [3-5]. Their ability to regulate insect populations has been studied in tropical and temperate habitats [6-8]. Soil is considered an excellent environmental shelter for entomopathogenic fungi since it is protected from UV radiation and other adverse abiotic and biotic influences [9]. Fungal entomopathogens in the genera *Beauveria*, *Conidiobolus*, *Metarhizium* and *Isaria* (*Paecilomyces*) are commonly found in soil [9]. Occurrence of the entomopathogenic fungi was highly influenced by various factors geographical location, climatic conditions, habitat type, cropping system, and soil properties, as well as the effects of biotic factors [5,10,11]. It is increasingly recognized that the biodiversity of agro ecosystems delivers significant services, such as biological control of pests, to agricultural production [8]. The contribution of the entomopathogenic component of this biodiversity to the regulation of pest populations has often been ignored [12] and when it has been acknowledged, it has usually been discussed if the introduction of exotic strains of fungi, or the augmentation of endemic strains, is an appropriate biocontrol strategy [13]. Among the several existing entomogenous fungi, *Nomuraea rileyi* is a cosmopolitan species infecting many noctuids such as *Helicoverpa armigera*, *Spodoptera litura, Trichoplusia ni, Anticarsia gemmatalis, Pseudoplusia* includes and has a potential for development into mycoinsecticide and occurs in soils of various agro ecosystem.

Nanotechnology is significant for the comprehension, use, and control of matter at magnitudes of a minute scale, approaching atomic levels, with which to manufacture new substances, instruments, and frameworks. At present, silver and various metallic nanoparticles are in great use in the medicinal, pharmaceutical, agricultural industry and in water purification [14]. Increasing numbers of commercial products, from cosmetics to medicine, incorporate manufactured nano materials (MNMs) that can be accidentally or incidentally released to the environment and adversely affect the various biotic components in the ecosystem.

Gold, silver, and copper have been used mostly for the synthesis of stable dispersions of nanoparticles, which are useful in areas such as photography, catalysis, biological labelling, photonics, optoelectronics and surface-enhanced Raman scattering (SERS) detection. Concern over the potentially harmful effects of such nanoparticles has stimulated the advent of nanotoxicology as a unique and significant research discipline. However, the majority of the published nanotoxicology articles have focused on mammalian cytotoxicity or impacts to animals and bacteria, and only a few studies have considered the toxicity of nanoparticles to plants and other non target organism [15]. In the present study, the toxic effect of metallic nanoparticles on *Nomuraea rileyi* was discussed.

Materials and Methods

Sampling Sources

Agricultural field soil was collected from the different parts of Tamil Nadu belonging to Nanmangalam (Kanchipuram), Hasthampatty (Salem), Rajakoil (Vellore), Veeranam Thiruvanamalai,Mattuthanvani (Madurai), Vadavalli (Coimbatore), Pullarakottai (Viruthunagar), Pullarakottai Road (Viruthunagar) Pondicherry, Cunnur (Nilgiris).

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For each soil sampling site, 1.5 to 2 kg soil was collected from 5 points randomly and mixed to obtain homogenous sample, the homogenized soil samples were kept in sterile polythene bags and brought to the laboratory.

Physicochemical of soil analysis

Before microbial analysis, soil aggregates were broken by hands, trays with soil were kept open until moisture was at equilibrium [2]. Soil texture pH electrical conductivity organic matter nitrate, phosphorous, potassium, calcium, magnesium sulphur, sodium, zinc, iron, copper were determined for all soil collected. These measurements were determined in national agro foundation at Taramani Tamil Nadu, India.

Isolation of *N. rileyi*

Soil dilution method was adopted for natural occurrence of *N. Rileyi*. 1 gm of homogenized sample was suspended in 99 ml of sterilized distilled water mixed well and serially diluted. 1 ml of aliquots was transferred to sterile petriplates, 20 ml of sterile molten CTC (chloramphenicol, Thiabendazole, cycloheximide media)+PDA Agar media consisting of potato dextrose agar supplemented with 0.5 gm/l Chloramphenicol 0.01 gm, Thiabendazole 0.25 gm, Cyclohexamide was added, allowed to solidify. The seeded plates were incubated at 25°C in an incubator for 3-7 days. Fungal colonies were isolated after the incubation period, respective fungal colonies were purified and the pure culture was stored on CTC media agar slant. Identification of fungal culture was determined by morphological characteristics and microscopic examination of the spores by lactophenol cotton blue staining.

Evaluation of toxic effect of metallic nanoparticles on the post treatment resistance of *N.rileyi*

In the present study, chitosan coated metallic nanoparticles such as silver; copper was selected to evaluate toxic effect.

Synthesis of silver and copper nanoparticles: Silver nanoparticles were synthesized by chemical reduction of 1 mM silver nitrate with 1 mM sodium borohydride as reducing agent. Synthesis of silver nanoparticles was confirmed by the conversion of the reaction mixture into brown colour and further characterization of the synthesized silver nanoparticles was carried out with determination of Plasmon absorption maxima with UV-VIS spectroscopy and particle morphology with electron microscopy (SEM). Similarly copper nanoparticles were synthesized by chemical reduction of 1 mM copper sulphate (Sigma, analytical grade) with 1 mM sodium borohydride (Sigma, analytical grade) as reducing agent. Synthesis of copper nanoparticles was confirmed by the conversion of the reaction mixture into green colour and further characterization of the synthesized nanoparticles was carried out with determination of Plasmon absorption maxima with UV-Vis spectroscopy and particle morphology with Scanning Electron Microscopy (TEM) and energy dispersive X-ray spectroscopy. Synthesized nanoparticles were purified by successive centrifugation by 10,000 rpm and the collected pellets were washed thrice with deionised water, the washed suspension thus obtained was freeze dried.

Characterization

Determination of Plasmon absorption maxima of the reaction mixture with UV-VIS spectra is the primary confirmation of the synthesis of nanoparticles. UV VIS absorption spectrum was carried out with Thermo scientific spectrascan UV 2700 spectrophotometer operating in the transmission mode. Scanning Electron Microscopy (SEM) images were recorded by using Carl zeiss subra (Germany scanning electron microscope equipped with an Energy-dispersive Spectrum (EDS) capability.

Synthesis and characterization of chitosan coated nanoparticles

In a typical procedure of chitosan stabilized silver nanoparticles, 5 ml of 0.1 M silver nitrate, 1 ml of tri sodium citrate of 0.1 M and 1 ml of 0.1 M sodium borohydride, 10 ml of a solution containing chitosan (6.92 mg mL^{-1}) was mixed and kept under magnetic stirrer for 3 hours to obtain homogeneous solution. The homogenous thus obtained was transferred to the screw cap vial and incubated for 12 h at 95°C. The colour of the solution changed from colourless to light yellow, finally to yellowish brown which primarily confirmed the coating of chitosan with silver. Similarly, chitosan coated copper nanoparticles were synthesized by reduction of 10 ml of 0.1 M copper sulphate, 2.5 ml of 0.1 M tri sodium citrate, 1 ml of 0.1 M sodium borohydride 10 ml of a solution containing chitosan (6.92 mg mL^{-1}) separately were mixed and kept under magnetic stirrer for 3 hours to obtain homogeneous solution The homogenous thus obtained was transferred to the screw cap vial and incubated for 12 hours at 95°C. The colour of the solution changed from colourless to light yellow, and finally to yellowish brown which primarily confirmed the stabilization of chitosan with copper. After the preliminary confirmation, the reaction mixture was freeze dried and characterized with SEM, FTIR and EDAX.

Pot assay

Fertile loam soil was collected from the garden of Sathyabama University in sterile polythene bag, kept in ice box brought to the laboratory. Homogenized soil was sterilized by autoclaving at 121°C for 15 minutes by autoclaving. Plastic pot of diameter of 16 cm was filled with 1.5 kg of sterilized soil and inoculated with 1 ml of *N. rileyi* spore suspension, (1.0×10^8 spores/ml) mixed well. The different concentration of nanoparticles was added into each pots already inoculated with spore suspension of *N. rileyi*. The pots were closed with cheese cloth inoculation at 25°C. Every 10 days interval, 1 gm of soil sample from each Nanoparticles treated pot was taken in a sterile Petri plate, serially diluted and plated on sterile CTC media. Plates were incubated at 25°C for 3-7 days.

Effects of nanoparticles on spore germination of *N. rileyi*

Spore germination inhibition was carried out by microscopic method to determine the effect of Nanoparticles on spore germination of *N. rileyi*. In this method one drop of sterile CTC media was added to clean microscopic slide, a drop of fungal spore suspension previously incubated with different concentration of Nanoparticles was added, mixed well by sterile tooth pick. The slides were then transferred to the sterile petriplates lined with sterile filter paper and a thin layer of cotton. The plates were incubated at 25°C. The slides were examined for spore germination under microscope. Spore germination inhibition (%) was calculated by following formula:

Spore Germination Inhibition (%) = {(No of spore germination in control)-No. of Spores germination in treatment)} * 100 No of spore germination in treatment

Result and Discussion

Among the 10 sampling sites, *Nomurea rileyi* was isolated from 4

sites belonging to Hasthampatty (Salem), Rajakoil (Vellore), Vadavali (Coimbatore), Pullarakottai (Virruthagar), High frequency of distribution was recorded in vadavali (60%) followed by Pullarakottai (17%), Hasthampatty (13%) and Rajakoil (10%) (Figure 1). Non *N. rileyi* such as *Beauveria* sp. and *Metarhizium* sp. were also isolated associated with *N. rileyi* in the sampling sites. Soil physico-chemical parameters highly influenced the natural occurrence of *Nomurea rileyi*. *Nomurea rileyi* isolated from respective soil samples reveals high organic matter, available nitrogen and phosphorous (Tables 1 and 2).This may favour the viability of the fungal spore and thus improved the natural occurrence of *Nomurea rileyi*. Moreover the presence of other trace element may affect the fungal distribution. High content of available zinc may inhibited the occurrence of *Nomurea rileyi*. *N. rileyi* was not obtained from any soil sample consists of more availbale zinc, available iron,available copper and available mangnese. Electrical conductivity and pH did not effect the natural occurrence of *Nomurea rileyi*. Natural occurrance of entomopathogenic fungi associated with soil was carrried out in various parts of the world [2,16,17].

Natural occurrence of the entomopathogenic fungi is highly influenced by various physico chemical parameters of the soil. The effect of soil factors (organic matter, clay, sand, silt content, and pH) and geographical location (latitude, longitude and altitude) on the occurrence of entomopathogenic fungi has been reported. They reported that frequency of occurrence was found to be more in soils contain high organic matter and total nitrogen content. Moreover, soil pH and geographical distribution did not affect the occurrence. In the present study, soils revealed the occurrence *N. rileyi* showed more organic matter and the soil pH did not cause any distinct effect.

Evaluation of toxic effect of nanoparticles on post treatment presence of *N. rileyi* Synthesis and characterization of metallic nanoparticles

Silver nanoparticles synthesis adopting chemical reduction was primarily confirmed by colour change of the reaction mixture from pale yellow to brown which clearly indicates formation (Figure 2a). Synthesized silver nanoparticles characterized by UV-Vis spectroscopy which reveals a strong broad surface Plasmon peak located at 420 nm (Figure 2b). Particle morphology size and shape with Transmission electron microscopy reveals spherical particles with the size of 19-21 nm (Figure 3a). In EDAX, strong signals from the silver particles were observed (42.44% in mass), while weaker signals from C, O, Al and S atoms are also recorded which confirmed silver nanoparticles (Figure 3b). CuNPs synthesized by chemical reduction method with copper sulphate reduced with sodium borohydride as a reducing agent adapting one phase synthesis. CuNPs were formed immediately after the addition of sodium borohydride to the copper sulphate solution and the synthesis was primarily confirmed by the colour change of the reaction mixture into green colour (Figure 4a).

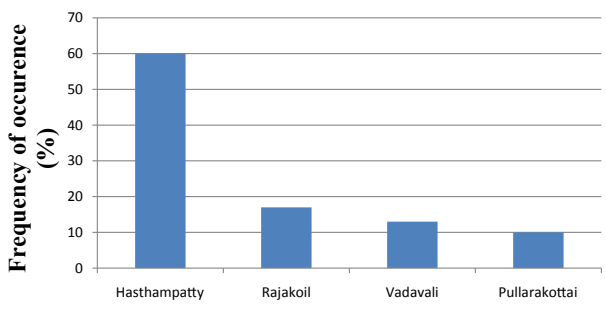

Figure 1: Frequency of occurrence of N.rileyi associated with agricultural field soil

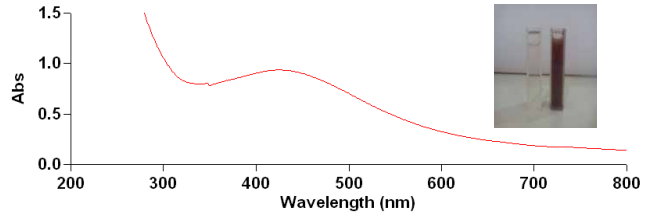

Figure 2: Characterization of synthesized silver nanoparticles (a) reaction mixture, (b) UV-VIS absorption spectra of the synthesized nanoparticles.

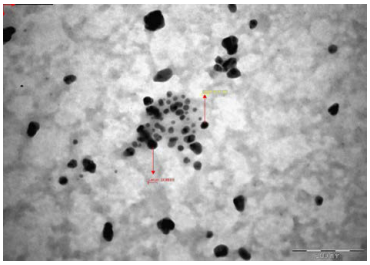

Figure 3a: TEM micrograph of synthesized nanoparticles.

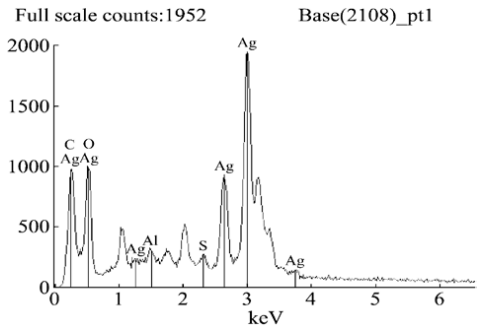

Figure 3b: EDAX spectra of synthesized nanoparticles.

Characterization of this synthesized particle was further studied by the Plasmon absorption maxima at 610 nm with UV-VIS Spectrophotometer which can be attributed to the plasma resonance absorption of non-oxidized the copper particles (Figure 4b). Size and shape with SEM which reveals 132 nm (Figure 5a) presence of elemental copper as strong peak was confirmed by EDAX (Figure 5b). Chitosan stabilized respective nanoparticles were synthesized by chemical reduction of respective metal salt precursor with nontoxic and biocompatible polymer chitosan which primarily confirmed by FTIR, SEM and EDAX. When the FTIR spectrum of free and stabilized nanoparticles was compared, it was found that almost the all the absorbed peaks were modified upon coating with chitosan. FTIR spectra of chitosan coated silver and copper nanoparticles are presented in Figure 6a and 6b.

The IR spectra of the chitosan capped Nano silver shows prominent peaks at 3788 cm^{-1}, 3427.4005 cm^{-1} corresponding to O – H stretching,

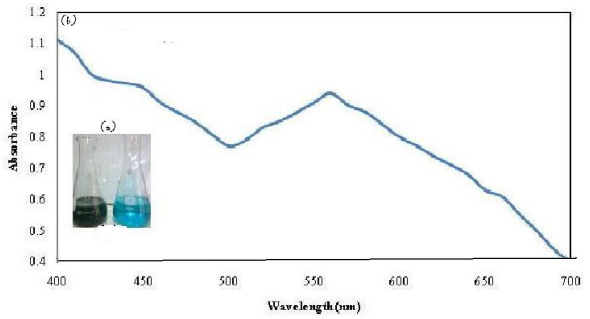

Figure 4: (a) Synthesized Copper nanoparticles Reaction mixture (b) UV-VIS absorption spectra of the synthesized nanoparticles.

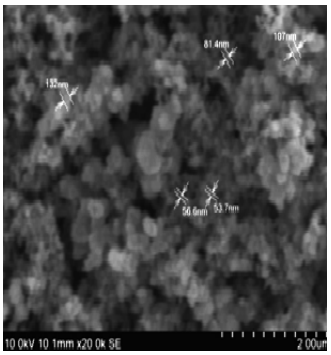

Figure 5a: SEM image

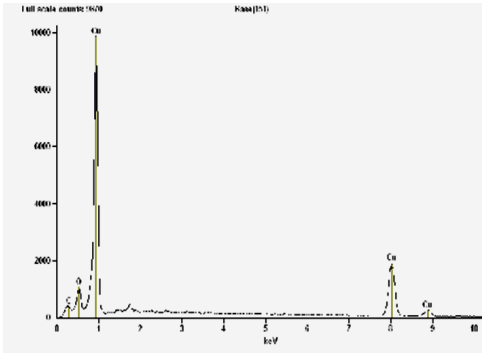

Figure 5b: EDAX spectra of copper nanoparticles.

strong polymerization, at 2928.1733 cm^{-1} for aliphatic C – H stretching. Peaks at approximately 2369.0387 cm^{-1}, 2345.3187 cm^{-1} represent N – H stretching vibration. Peaks at 1637.5845 cm^{-1} and 1389.4649 cm^{-1} represent N – H bending and a peak at 1026.6957 represent C – N vibration in aliphatic compounds. A is also observed at 617.7611 cm^{-1} showing the presence of inorganic metal ions (silver ions). The IR spectra of the Chitosan capped Nano copper shows prominent peaks at 3432.35 cm^{-1} corresponding to strong polymerization. Peaks at approximately 2044.52 315 cm^{-1} represent N – H stretching vibration. Peaks at 1633.03 cm^{-1} represent N – H bending and a peak at 1384.37 cm^{-1} represents O – H bending and C – H stretching.

A peak is also observed at 1091.66 cm^{-1} showing C – O – C stretching in polysaccharide of chitosan. Finally the presence of inorganic metal ions (copper ions) is shown by a peak at 463.13 cm^{-1}. The SEM analyzer built in with and EDAX analyzer allows a quantitative deduction on localization of elements in the nano specimens Scanning electron microscopy (SEM) study of chitosan stabilized copper nanoparticles reveals. The uniform spherical smooth morphology. within the size range of 101.78 nanometer and electron dense thin chitosan coating shell of diameter 3-5 nanometer (Figure 7a) Such size distribution analysis primarily confirms that the particles are well dispersed and less aggregated The EDAX images illustrated the presence of large amounts of C, O, N (Figure 7b). SEM and EDAX analysis of chitosan stabilized copper nanoparticles shows the spherical particles with electron dense thin chitosan coating shell of 50-65 nm diameter (Figure 8a) EDAX analysis further confirms the high amount of C, O, N and Cu (Figure 8b).

Toxic effect

Distinct effect on post treatment or viability on *N.rileyi* was not observed in silver nanoparticles treatment. In free silver nanoparticles treatment, 24×10^3, 21×10^3, 19×10^3, 19×10^3 and 18×10^3 CFU/g of *N. rileyi* was recorded in the respective concentration. Similar finding was reported in chitosan coated silver nanoparticles treatment. Colony count was not recorded in free copper nanoparticles at the concentration of 1000, 750µg /ml and similar effect was recorded in chitosan coated copper nanoparticles (Table 3). Spore germination was not inhibited in all tested concentration of both free silver and chitosan coated silver nanoparticles treatment (Table 4).

Significant inhibition in spore germination ($P>0.05$) was recorded in all the concentration of free and copper nanoparticles. Nanotechnology is currently employed as a tool to explore the darkest avenues of medical sciences in several ways like imaging, sensing, targeted drug delivery, gene delivery systems and artificial implants [18]. In present situation, silver and various metallic nanoparticles nanoparticles are in great use in the medicinal, pharmaceutical, agricultural industry and in water purification [14].

Increasing numbers of commercial products, from cosmetics to medicine, incorporate manufactured nanomaterials (MNMs) that can be accidentally or incidentally released to the environment. Concern over the potentially harmful effects of such nanoparticles has stimulated

S.no	Parameters	Hasthampatty	Rajakoil	Vadavali	Pullarakottai
1	pH	7.95	7.50	7.80	7.95
2	Electrical conductivity(ms/cm)	0.600	0.865	0.425	0.418
3	Organic matter (%)	2.33	2.57	2.74	0.78
4	Nitrate nitrogen (ppm)	24.9	20.1	1606	27.1
5	Available phosphorous(ppm)	237.7	388.0	31.6	314.6
6	Potassium exchangeable k(ppm)	93	314	246	234
7	Calcium exchangeable (ppm)	1932	2277	2401	2579
8	Magnesium exchangeable (ppm)	511	477	356	644
9	Sulphur available s as so4 ((ppm)	49.3	58.5	25.4	83.1
10	Sodium exchangeable Na((ppm)	302	329	142	585
11	Zinc available Zn (ppm)	2.15	2.22	0.68	1.55
12	Manganese available Mn (ppm)	4.72	5.25	21.57	10.88
13	Iron available Fe (ppm)	1.36	0.79	1.24	1.18
14	Copper available	1.84	0.45	1.91	1.83

Table1: Physico chemical parameters of soil samples collected from different agricultural fields of Tamil Nadu

S.no	Parameters	Nanmangalam	Veeranam	Pullarakottai Road	Mattuthavani	Pondicherry	Cunnor
1	Ph	7.2	7.30	8.10	5.40	6.64	5.75
2	Electrical conductivity(ms/cm)	0.995	0.885	0.715	0.076	0.168	0.040
3	Organic matter (%)	0.44	0.47	0.60	0.13	1.07	0.80
4	Nitrate nitrogen (ppm)	17.4	29.1	5.9	7.7	9.1	5.7
5	Available phosphorous(ppm)	189.6	293.5	7.9	56.1	347.0	8.6
6	Potassium exchangeable k(ppm)	58	219	104	43	59	37
7	Calcium exchangeable (ppm)	1728	2249	3101	1162	1914	418
8	Magnesium exchangeable (ppm)	412	463	674	327	262	134
9	Sulphur available s as so4 ((ppm)	67.0	60.9	3.5	3.9	11.3	4.4
10	Sodium exchangeable Na((ppm)	226	315	122	104	149	135
11	Zinc available Zn (ppm)	4.00	2.47	0.26	1.29	5.00	0.26
12	Manganese available Mn (ppm)	5.74	5.95	7.53	6.67	38.23	6.37
13	Iron available Fe (ppm)	11.24	0.94	0.87	224.00	26.51	22.39
14	Copper available	2.55	0.78	1.37	4.88	2.79	2.12

Table 2 : Physico chemical parameters of soil samples collected from different agricultural fields of Tamil Nadu.

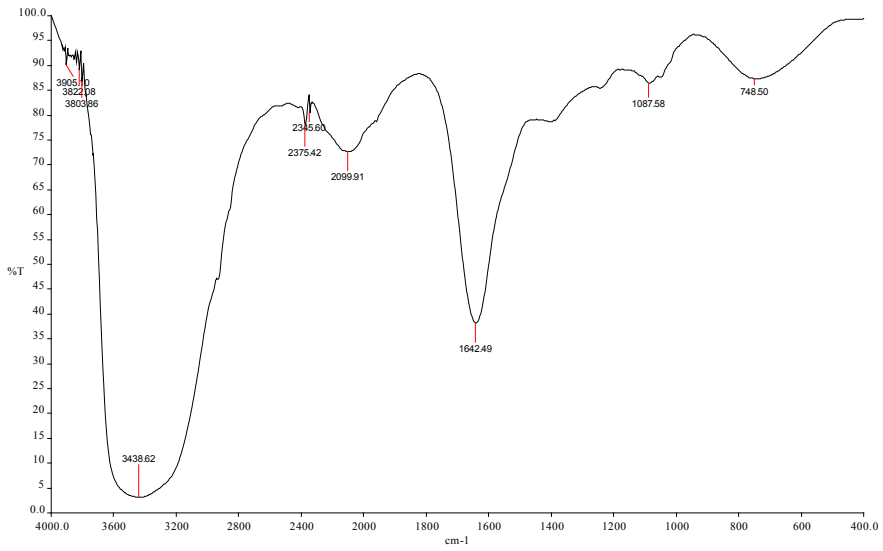

Figure 6a: FTIR spectra of chitosan coated silver nanoparticles.

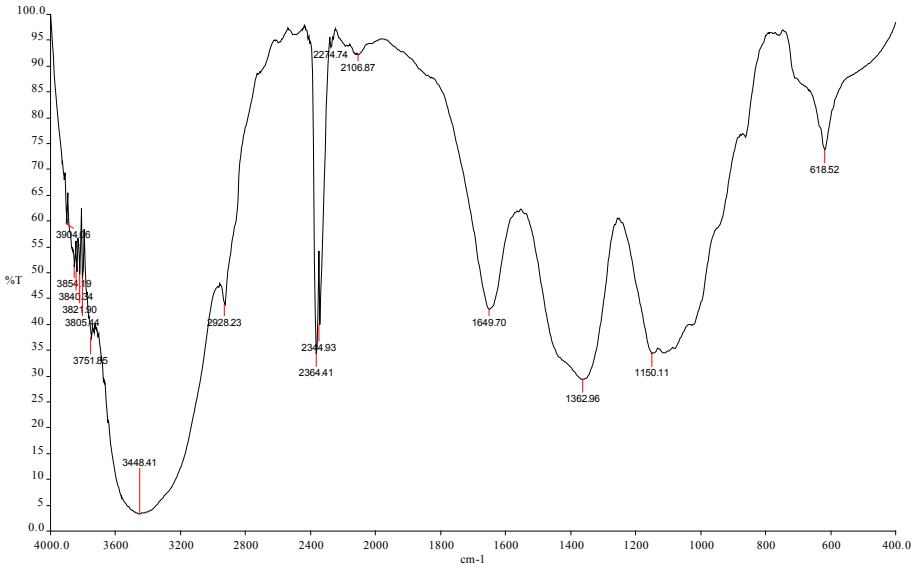

Figure 6b: FTIR spectra of chitosan coated copper nanoparticles.

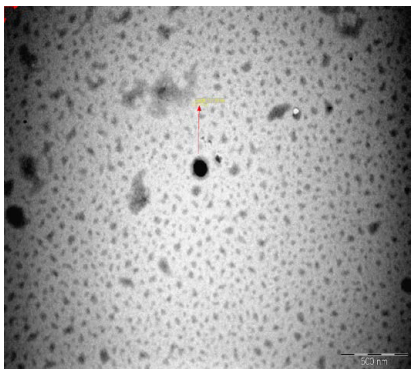

Figure 7a: SEM image of chitosan coated silver nanoparticle.

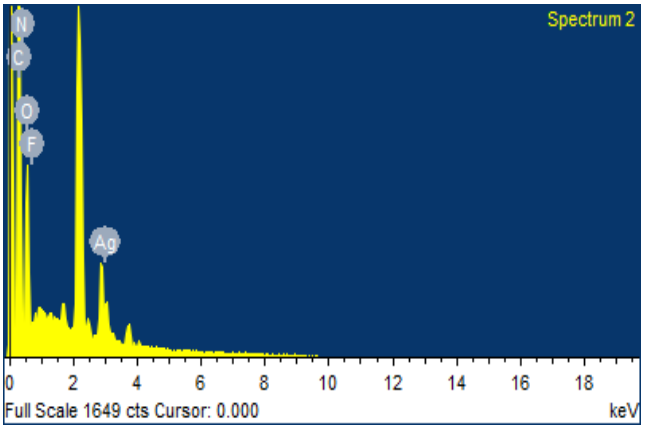

Figure 7b: EDAX image of chitosan coated silver nanoparticles.

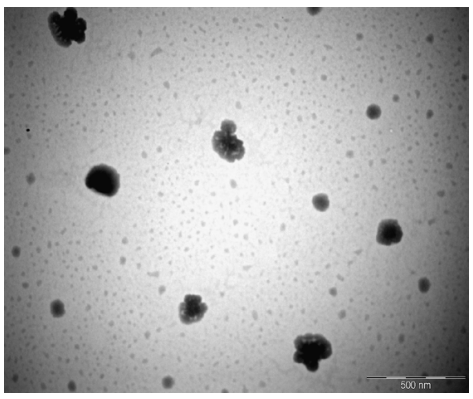

Figure 8a: Chitosan coated copper nanoparticles.

S.No	Treatment	Colony count (CFU/g)				
		100	250	500	750	1000
1	Control	$24X10^3$				
2	Free silver nanoparticles(AgNps)	$24X10^3$	$21X10^3$	$19 X10^3$	$19X10^3$	$18X10^3$
3	Chitosan coated silver nanoparticles (CS-AgNps)	$23X10^3$	$21X10^3$	$19X10^3$	$19X10^3$	$18X10^3$
4	Free copper silver nanoparticles (CuNps)	$11X10^2$	$11X10^2$	$7X10^1$	0.0	0 .0
5	Chitosan coated copper nanoparticles (CS-CuNps)	$10X10^2$	$8X10^2$	$7X10^1$	0.0	0 .0

Table 3: Effect of nanoparticles on the post treatment persistence of *N.rileyi*.

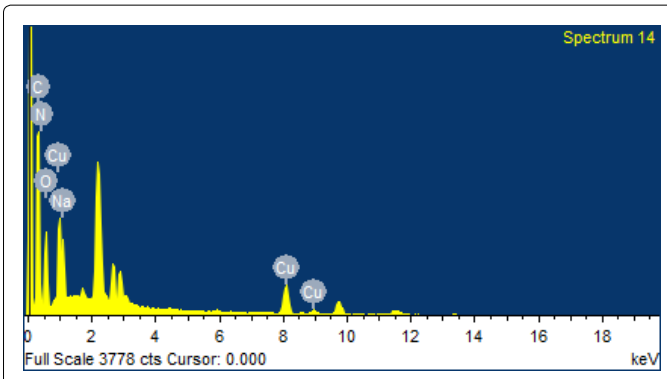

Figure 8b: EDAX image of chitosan coated copper nanoparticles

S.No	Treatment	Germination inhibition(%)				
		100	250	500	750	1000
1	Control	0.0				
2	Free silver nanoparticles(AgNps)	0.0	0.0	0.0	0.0	0.0
3	Chitosan coated silver nanoparticles (CS-AgNps)	0.0	0.0	0.0	0.0	0.0
4	Free copper silver nanoparticles (CuNps)	10.0	25.0	65.0	75.0	90.0
5	Chitosan coated copper nanoparticles (CS-CuNps)	20.0	45.0	70.0	80.0	98.0

Table 4: Effect of nanoparticles on the spore germination inhibition (%) of *N.rileyi*

the advent of nanotoxicology as a unique and significant research discipline. However, the majority of the published nanotoxicology articles have focused on mammalian cytotoxicity or impacts to animals and bacteria, and only a few studies have considered the toxicity of MNMs to plants and other non target organism [15]. This is the first report of studying compatibility of N.rileyi with nanoparticles. Further study under field trail will be used to understand the non target effect.

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