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Research Article

Antifungal Efficacy of Cu-Based Nano-Chitosan on *Rhizopus stolonifer*, A Virulent Phytopathogen

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Abstract

Agro-scientists are giving endless efforts for synthesizing a bio-derived molecule that can act as a promising antifungal agent for combating a large number of phytopathogens. Harmful phytopathogens decrease crop yield and its quality. *Rhizopus stolonifer* is one such virulent phytopathogen that causes huge losses during the post-harvest period of crops. This pathogen mainly causes rot disease in fruits, crops, and vegetables. The second most abundantly available biological macromolecule, Chitosan and its metal-based nanoparticles stands as a potential antifungal agent for combating *Rhizopus stolonifer*. This study includes the synthesis of Cu chitosan nanoparticles (Cu-CNPs) and chitosan nanoparticles (CNPs) through the ionic gelation method and its characterization based on UV Vis spectrophotometer, FE-SEM, EDXS, and DLS. Cu-CNPs and CNPs were screened from 100-2000 µg/mL concentration against *R. stolonifer* for the assessment of its antifungal activity. Spore viability assay and lipid peroxidation of the pathogen using Cu-CNPs and CNPs were also determined. Generation of oxidative stress in the mycelium of the pathogen on the application of Cu-CNPs and CNPs was traced by fluorescence microscopy. Changes in the ultra-structure of the sporangium of *R. stolonifer* after treatment with Cu-CNPs and CNPs were visualized under SEM. Results showed that Cu-CNPs inhibit the growth of *R. stolonifer* at 2000 µg/mL and elevate malonaldehyde (MDA) content in the pathogen as a result of lipid peroxidation and produces defined damages on the sporangium membrane as observed under electron microscope. Fluorescence microscopy revealed the emission of high intensity of fluorescence due to the generation of oxidative stress in Cu-CNPs treated fungal mycelium.

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Introduction

Rhizopus stolonifer, a member of the phylum Zygomycota, commonly traced in tropical and subtropical zone, is one of the most virulent pathogens of crops and vegetables (Omoifo, 2011). This pathogen has a worldwide distribution and is mostly found in moldy materials. It subsists in both soil and air. It acts as a major parasite of plant tissue by causing rot disease (Baggio et al., 2015). This pathogen grows vigorously and spreads easily by means of a stolon. The stolon helps the fungus to grow vertically and horizontally (Estrada et al., 2019). They possess sporangiophores of about 2.5 mm long and 20 µm in diameter (Abe et al., 2010). *Rhizopus* causes huge loss of harvested crops during storage, transit, and while marketing. Usually, this pathogen occurs as a saprophyte or often as a facultative parasite in the stored plant products. This pathogen invades plant tissue through infection peg and emerges out from the wounds by producing aerial mycelium, sporangia, sporangiophore, stolon, and rhizoids (Diego, 1997). According to a field

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study by Holeta Agricultural Research Station, field crops suffered a loss of 32-52% due to the infection caused by *Rhizopus stolonifer*. Loss of industrial crops stands between 22 to 44% while horticultural crops showed a reduction in the yield of 35 to 62% (Shtienberg, 1997). *Rhizopus* infects vegetables, ornamental crops, fruits, seeds, grains and nuts. This pathogen majorly causes rot in sweet potatoes, sunflower, strawberry, cherries, peanuts, cucurbits and peaches. Under high level of moisture, they infect cereals and corns. Rhizomes of flower crops, bulbs and corms are also susceptible to this pathogen. Not only in plants but humans are also victimised through mucormycosis caused by *Rhizopus* spp. (Agrios, 2005). *Rhizopus* showed rapid growth and sporulate vigorously under humid conditions with mild temperature. It grows rapidly between 15 to 30ºC an optimal developmental temperature of 23 to 28ºC. The infected areas are characterized by soft watery mass covered with white hairy mycelia and black sporangiophores erected vertically (Agrios, 1997). Due to the comprehensive range of hosts of *Rhizopus stolonifer* and its fast colonization capability, it is important to target its control.

Researchers have proposed various strategies for controlling *Rhizopus stolonifer*. Strategies like crop rotation, the use of synthetic fungicides, and spraying of botanicals in the crops and foliage are age-old management practices of the farmers. Very recently, agro-researchers are trying to derive a potential biomolecule for controlling a broad spectrum of phytopathogens. In this context, nanotechnology has opened up new avenues for the management of crop diseases. Nanoparticles derived from a biological macromolecule- chitosan and its metal derivatives are known to inhibit a maximum number of fungal pathogens (Chouhan et al., 2022). CU nanoparticles (Cu-NPs) hold worldwide attention due to their broad array of antimicrobial properties. It was prescribed by Hippocrates in 400 BC for pulmonary disorders. For centuries CU is known to use for the purification of water. CU is bio-physically active owing to its mechanical, optical, electrical, thermal and catalytical property (Lee et al., 2009; Shende et al., 2015; Umer et al., 2014). CU and its nanoparticles hold strong reviews for having significant bioactivity against bacteria, fungi, viruses and nematodes (Ingle and Rai, 2016).

Chitosan is a biodegradable, biocompatible, non-toxic antifungal agent that provides greater permeability into the fungal membrane. Nanoparticles formed from chitosan are likely to be more permeable into the fungal membrane. It is already reported that chitosan is a promising antifungal agent due to its polycationic nature (Hans and Lowman, 2002). Owing to its polycationic nature, chitosan binds with the negatively charged components of the fungal membrane resulting in a disintegrated structure. Chitosan nanoparticles (CNPs) ensure greater encapsulation capacity with increased surface area (Sarkar and Acharya, 2020). Similarly, attempts have been made the synthesis of metal-conjugated nanochitosan for gain increment in the antifungal activity (Malerba and Cerana, 2016). Metal-conjugated nanoparticles such as (Cu-CNPs) are biologically more active due to their varied structural and functional properties. Metallic nanoparticles are known to generate ROS in pathogens. They cause protein and nucleic acid leakage of the pathogen, followed by hindering the membrane stability (Sathiyabama and Charles, 2015; Sathiyabama and Parthasarathy, 2016; Kong et al., 2010). Therefore, the present paper aims to delineate the hypothesis that Cu when incorporated in CNPs will enhance antifungal activity against *Rhizopus stolonifer*. Concomitantly, on application of Cu-CNPs there will be generation of oxidative stress and peroxidation of fungal lipid that will restrict its growth.

Materials and methods

Synthesis of CNPs and Cu-CNPs

Both the nanoparticles (CNPs and Cu-CNPs**)** were synthesized by following Ionic Gelation Method. Low molecular weight chitosan (80% N-deacetylation; 50,000-190,000 Da) was solubilized in 1% acetic acid solution (v/v) and continuously stirred for 30 mins. 40 mL of Sodium Tripolyphosphate (STPP) was added drop-wise into the chitosan solution with constant stirring. A milky white suspension is formed after 1 hr of further stirring. This solution is used as chitosan nanoparticles (CNPs) solution and stored at 4ºC for further use (Dananjaya et al., 2017a).

Cu-CNPs was prepared by the drop-wise addition of 10 mL of 4% $CuSO_4.5H_2O$ into low molecular weight chitosan solution made in 1% acetic acid solution. The solution was stirred for 20 mins and refluxed at 120ºC. 0.05 M ascorbic acid (0.5 mL) followed by 0.6 M NaOH was (2 mL) added into the solution and stirred for 15 mins. After further stirring, a light green colouration was observed. A quick brown colour appeared following the addition of 0.5 mL N_2H_4. Finally, STPP was added as mentioned above. This solution was centrifuged at 12,000 rpm for 10 mins and supernatant was discarded while the pellet obtained was resuspended in in same amount of distilled

water and used as nanoparticle solution (Usman et al., 2012).

Characterisation of CNPs and Cu-CNPs

UV-Vis (Aligent Technologies, Carry 100 UV-Vis) spectral absorbance of CNPs and Cu-CNPs were obtained by scanning the nanoparticle solutions in the range of 200-800 nm wavelength. The surface morphology of both the nanoparticles were scanned in JSM-7900F Schottky Field Emission Scanning Electron Microscope (FE-SEM); JEOL, with an accelerating voltage of 0.1-30 kV. The existence of elements in Cu-CNPs down to boron were scanned through EDXS analysis. EXDS was particularly performed to confirm the presence of CU in the synthesized nanoparticle. The measurement of hydrodynamic particle size and its distribution were determined through Dynamic Light Scattering (DLS).

Poisoned Food Assay

The effect of CNPs and Cu-CNPs on mycelial growth of *Rhizopus stolonifer* was determined through a poisoned food assay. A series of concentrations (100, 500, 1000, 1500 and 2000 µg/mL) of both nanoparticles were prepared in the form of solution. Nanoparticle solutions were sterilized and mixed with 20 mL of autoclaved Potato Dextrose Agar (PDA) media by maintaining the final required concentration of the nanoparticles in the Petri plates. The experiment was carried out in sterilized petri plates of 90 mm diameter. A 5 mm diameter of mycelial disc was excised from 7 day old culture and placed in the center of the nanoparticles treated plates. A control plate was prepared without the treatment of nanoparticles. Plates were incubated at 28-30ºC and monitored for days until the control plate showed full radial mycelial growth. Three replicates were for each plate. Fungal growth inhibition was measured by using the following formula and it is expressed as percent inhibition of radial growth (PIRG) (Dananjaya et al., 2017a):

$$\text{PIRG (\%)} = \frac{RGC_{Control\ plate} - RGC_{treated\ plate}}{RGC_{Control\ plate}} \times 100$$

Where, RGC – denotes radial growth of fungal colony

Spore viability assay

The viability of the *R. stolonifer* spore was estimated quantitatively by XTT 2,3- Bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino) carbonyl]-2H-tetrazolium hydroxide. An electron coupling agent Menadione was used in this assay. Spore suspension containing 1×10^6 conidia/mL was prepared in a Czapeck liquid medium. Spore suspension of 100 µL was cultured in 96 well flat bottom microplates for 6 hrs at 28-30ºC. Following incubation, equal amounts of both nanoparticles under different concentrations ranging from 100-2000 µg/mL were added to the suspension. The suspension was further incubated for 4 hrs. 50 µL XTT solution mixed with 7 µL of 25 µM menadione was added to the resulting suspension. Optical density was measured at 450 nm after 3 hrs of incubation at 28-30ºC (Alcaraz et al., 2016).

Determination of Lipid Peroxidation of R. stolonifer

Lipid peroxidation of *Rhizopus stolonifer* after the application of CNPs and Cu-CNPs, was quantitatively measured by malonaldehyde (MDA) estimation. MDA is the indicator of lipid peroxidation. The pathogen was treated with aforesaid concentrations of both the nanoparticles and cultured in potato dextrose broth (PDB) for 7 days at 28ºC. After incubation the harvested mycelial mat when thoroughly washed with sterile distilled water and dried with sterile blotting paper. One gram of mycelial mat was homogenized in 10 mL of chilled 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 12,000 rpm for 10 mins. The pellet was discarded and the supernatant was used for MDA estimation. 100 µL of the mycelial homogenate was mixed with 0.335% (w/v) thiobarbituric acid and 10% of trichloroacetic acid in equal volume. The reaction mixture was boiled in a water bath for 10-15 mins. The absorbance of the color formed in the reaction mixture was measured spectrophotometrically at 530 nm using molar absorption co-efficient 1.56×10^5 (Subban et al., 2019).

Analysis of CNPs and Cu-CNPs on oxidative stress production upon treatment on R. stolonifer

In order to check the generation of oxidative stress in the fungal hyphae a non-fluorescent probe molecule, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFH-DA) was used. This molecule is non polar hydrophobic in nature and it is easily permeable to the fungal cell. The intracellular esterase present inside the cell results in the deacetylation of the probe into 2',7'-dichlorodihydrofluorescein (DCFH). This deacetylated product gets oxidized by reactive oxygen species produced in the cell as a result of nanoparticle treatment. The oxidized product emits green fluorescence from the fungal hyphae when observed under a fluorescence microscope. The fungal hyphae were treated with the previously

mentioned range of concentrations of both the nanoparticles for 3 days, incubated at 28ºC in PDB. After incubation, the cultural were centrifuged at 10,000 rpm for 12 minutes to obtain the hyphal cells. The hyphal cells were washed with phosphate buffer saline (PBS) 3 times. The hyphae remained suspended in PBS. In a separate microcentrifuge tube, 40 µL of hyphal suspension was mixed with an equal volume of non-fluorescent probe dissolved in methanol under dark conditions. The tubes were incubated for 2 hours in the dark at 28ºC with frequent shaking. The generation of oxidative stress in the cells was observed under a fluorescence microscope with excitation filter of 485 nm and an emission filter of 525 nm (Chen et al., 2020).

Analysis of ultrastructural changes on sporangium of R. stolonifer on CNPs and Cu-CNPs treatment

The changes in the ultra-structural level on the sporangium of *R. stolonifer* were observed under Scanning Electron Microscopy (SEM) after the application of both nanoparticles at 2000 µg/mL concentration. Sporulated fungal cultures produced in PDB were treated with the nanoparticles and incubated for 48 hrs at 28-30ºC. The fungal tissue was pre-treated with glutaraldehyde and washed with graded alcohol before examining under microscope (Dananjaya et al., 2017b).

Results and Discussion

Characterisation of CNPs and Cu-CNPs

The synthesized nanoparticle solutions were scanned in the range of 200-800 nm in a UV-Visible spectrophotometer for the assessment of their optical properties using water as a reference. The results for UV-visible absorption spectroscopy showed characteristics of absorption peaks for non-metallic CNPs at 300 nm and Cu-CNPs at 550 nm. The CNPs nanoparticles showed agglomeration with rough porous surface morphology having sharp and pointed edges as revealed in FE-SEM analysis. Cu-CNPs showed spherical nano-disc morphology with irregular shape and surface topography. EDXS is an X-ray non-destructive analysis particularly used for the identification of the elemental profile of the tested material. The presence of metal ions (Cu) in the Cu-CNPs confirms it as a metal-conjugated nanoparticle. The hydrodynamic sizes of all the synthesized nanoparticles were measured through DLS analysis and it was observed that all the nanoparticles lie between 50 to 500 nm in range. The average size of non-metallic nano chitosan ranges between 40-70 nm. The hydrodynamic size of Cu-CNPs showed a size between 75-520 nm.

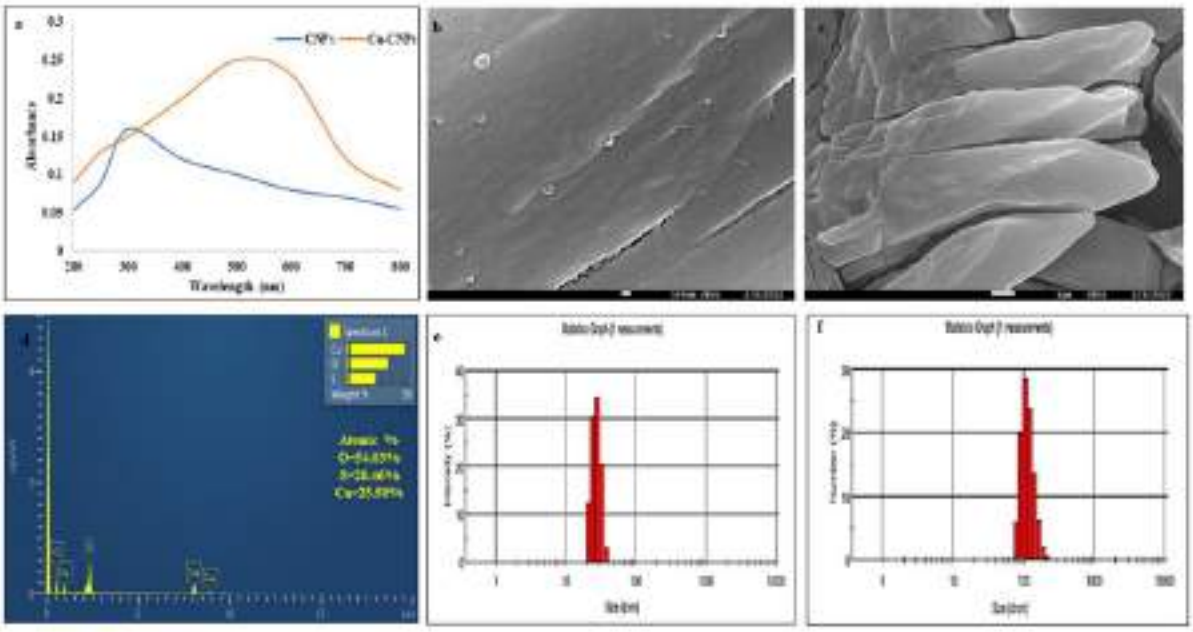

Fig. 1 Characterization of CNPs and Cu-CNPs through (a) UV-Vis spectra; FE-SEM analysis of (b) CNPs and (c) Cu-CNPs; (d) EDXS analysis of Cu-CNPs; DLS analysis of (e) CNPs and (f) Cu-CNPs

Poisoned Food Assay

The antifungal efficiency of both the nanoparticles were evaluated by poisoning the growing media of the fungal pathogen with chitosan-based nanoparticles. This assay reveals that in comparison to CNPs, Cu-CNPs showed complete inhibition (100% PIRG) of *R. stolonifer* at 2000 µg/mL. While, CNPs treated plate at 2000 µg/mL showed

only 10% of fungal growth inhibition. The density of the fungal mat was found to be progressively reduced in Cu-CNPs treated plates with an increasing range of concentration. It was also observed that the formation of sporangium or the sporulation process of the pathogen was triggered by the increasing concentration of both the chitosan-based nanoparticles. The fact is already established that metal nanoparticles when combined with chitosan, the bioactivity is increased manifolds (Reddy et al., 2008). There are reports that chitosan and its metal-based nanoparticles are highly efficient in controlling the ramification of phytopathogens (Yanat and Schroen, 2021). Researchers have successfully explained that CU-based nanoparticles significantly inhibited the growth of a range of fungal pathogens (Ingle and Rai, 2016).

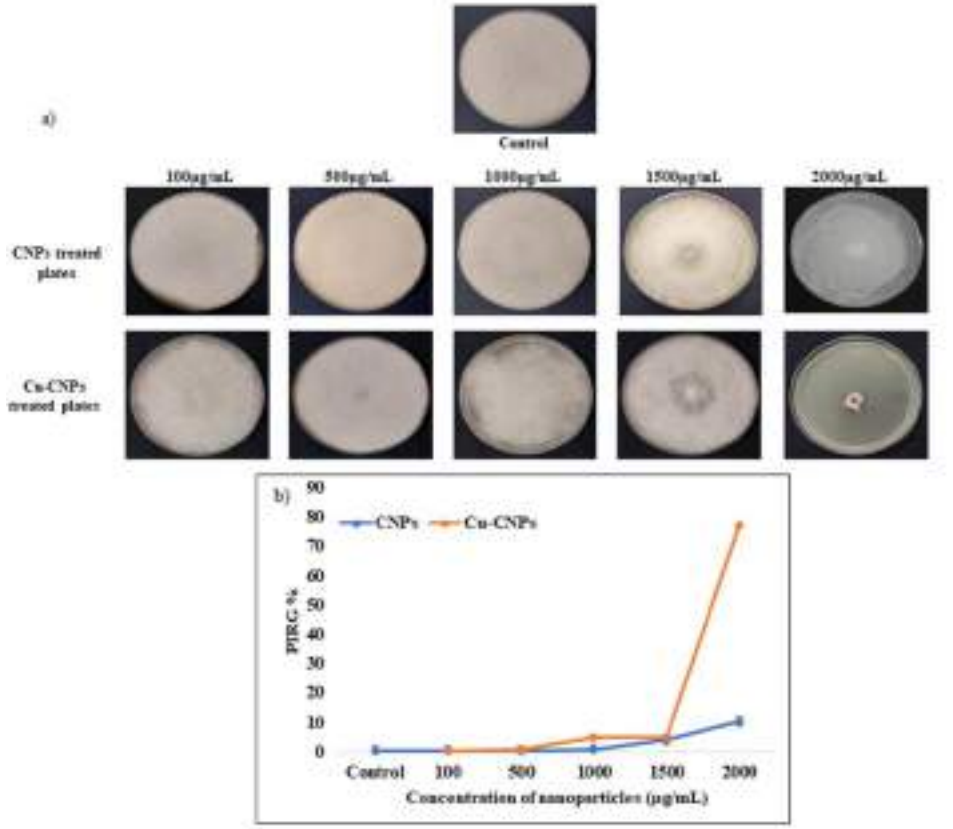

Fig. 2 Characterization of CNPs and Cu-CNPs through (a) UV-Vis spectra; FE-SEM analysis of (b) CNPs and (c) Cu-CNPs; (d) EDXS analysis of Cu-CNPs; DLS analysis of (e) CNPs and (f) Cu-CNPs

Spore viability assay

Spores of *R. stolonifer* treated with Cu-CNPs were found non-viable at 2000 µg/mL. The viability percentage of spores treated with 2000 µg/mL of Cu-CNPs was 1.88%. On the other hand, CNPs treated spores showed 12.56% of viable spores at the highest treated concentration. 50% of the spores were found viable at 1500 µg/mL of Cu-CNPs. Whereas, the untreated spores showed 100% viability in the growth medium. A number of scientists have proved that metal and metal-derived nanoparticles stand as strong antifungal agent (Chen *et al*., 2016). Research by Triawan et al., 2015 revealed that when spores of *R. stolonifer* are treated with magnesium oxide and zinc oxide nanoparticles, the viability of the spores and their propagation is completely inhibited. In order to completely triggered the propagation of a phytopathogen, it is important to trigger its propagative unit i.e., spore (Judelson and Blanco, 2005). The viability of the spore is sensitive to various abiotic stresses (Li et al., 2010). The application of the synthesized Cu-CNPs can significantly generate cellular stress and check the viability of the spore.

Lipid Peroxidation of R. stolonifer

The fungal tissue treated with 2000 µg/mL of Cu-CNPs showed 80% of MDA production as a result of lipid peroxidation due to nanoparticle treatment. Alike of Cu-CNPs, CNPs also showed 75% of MDA production in the fungal tissue. It was also observed that at each treated concentration of both the nanoparticles, the production of MDA was found higher in Cu-CNPs treated fungal tissue in comparison to CNPs treated fungal tissue. In

contrary, the untreated fungal tissue showed no MDA production. The fungal tissues are composed of polyunsaturated fatty acid which contains methylene groups in them. These methylene groups are highly affected by free radicals or ROS which are produced in the fungal tissue as a result of the application of nanoparticles. ROS generated in the fungal tissue results in the oxidation of fungal membrane lipids thereby causing membrane destabilization. The oxidation of the membrane lipid produces an aldehyde product called MDA. The more is this generation of stress in the fungal tissue upon exposure of the nanoparticles the more will be the MDA content in the fungal tissue (Kalagatur et al., 2018). Our result depicts that Cu-CNPs can significantly elevate the MDA content in the fungal tissue with increasing concentration.

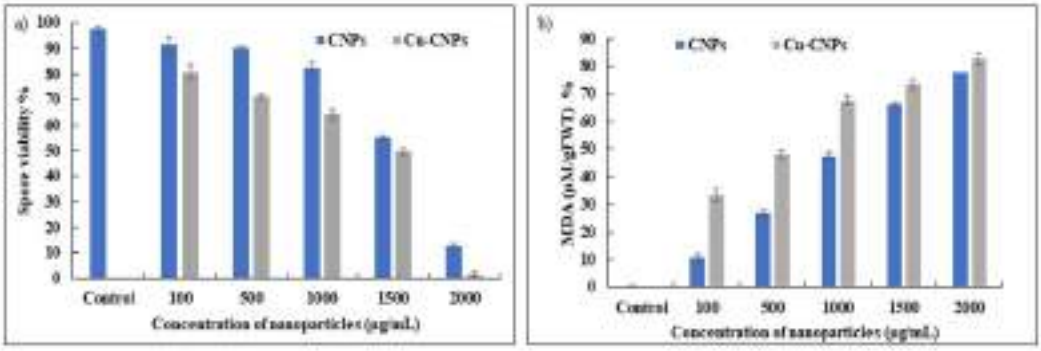

Fig. 3 Effect of different concentrations of CNPs and Cu-CNPs on (a) spore viability percentage and (b) MDA percentage of *R. stolonifer*

Analysis of oxidative stress production on R. stolonifer

The generation of oxidative stress in the fungal mycelium was determined through DCFH staining method. The fungal mycelium incubated with Cu-CNPs showed maximum intensity of fluorescence due to the generation of cellular oxidative stress. The fungal mycelium treated with CNPs showed moderate intensity of fluorescence as a result of the generation of minimal oxidative stress in the mycelium. The fungal mycelium without any treatment showed no fluorescence when observed under the microscope. This assay significantly proved that Cu-CNPs at 2000 µg/mL generate maximum cellular oxidative stress in the fungal pathogen in comparison to CNPs. The emission of high intensity of fluorescence is directly proportional to the generation of oxidative stress in the fungal pathogen (Kumar et al., 2016; LeBel et al., 1992). The level of fluorescence emitted in the fungal mycelium treated with Cu-CNPs hindered the integrity of the fungal membrane and thereby affect the functionality of the pathogen (Kalagatur et al., 2018).

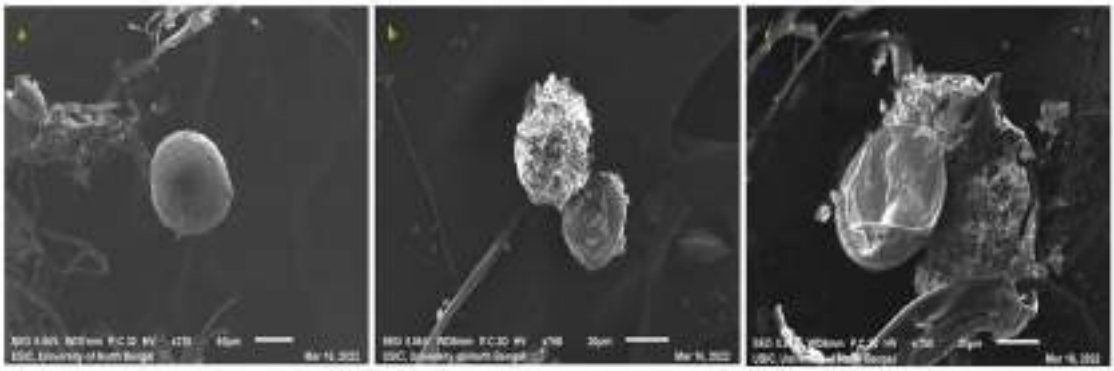

Figure 4. Morphological changes of *R. stolonifer* sporangium directly exposed to (a) distilled water; (b) CNPs (2000 µg/mL); (c) Cu-CNPs (2000 µg/mL) observed under scanning electron microscope (SEM)

Analysis of ultrastructural changes on sporangium of R. stolonifer

Under Scanning Electron Microscopy (SEM), it was observed that the application of Cu-CNPs at 2000 µg/mL produces define damages in the sporangium and spores of *R. stolonifer*. The sporangium membrane was completely damaged due to protoplasmic leakage and generation of membrane destabilization as a result of Cu-CNPs treatment. CNPs at 2000 µg/mL was observed to create moderate damage in the sporangium and its membrane. No membrane damages were observed in the sporangium of the untreated set. It contains completely intact undamaged sporangium.

Irreversible damages are produced in the sporangium of the pathogen as a result of the treatment of nanoparticles. Due to membrane destabilization, cellular protoplasmic leakage occurs that produces various surface abnormalities as a result of which the Cu-CNPs treated sporangium looks like a punctured ball (Dananjaya et al., 2017a). The polycationic molecule chitosan contains multiple positive charges in it. Due to the amalgamation of Cu ion into the chitosan network the resulting molecule is highly positively charged in nature. On the other hand, the fungal membrane contains negatively charged membrane components. The interaction between super positively charged nanoparticles and negatively charged fungal cellular components leads to ionic imbalance (Goy et al., 2009). It is suggested that chitosan nanoparticles enter into the fungal cell and binds with the fungal DNA, thus inhibiting protein synthesis (Kulikov et al., 2014).

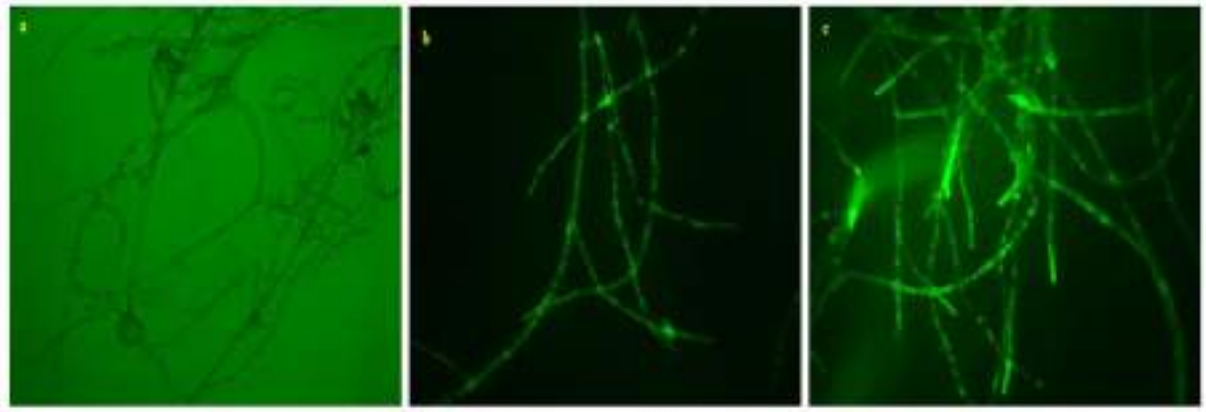

Figure 5. Fluorescence microscopic observations showing generation of oxidative stress in *R. stolonifer* mycelium due to the effect of (a) distilled water; (b) CNPs (2000 µg/mL) and; (c) Cu-CNPs (2000 µg/mL)

Conclusion

CNPs and Cu-CNPs were successfully synthesized by ionic gelation method and characterized as active nanoparticles. The application of Cu-CNPs against *R. stolonifer* showed progressive reduction of the growth of the pathogen. At 2000 µg/mL, Cu-CNPs completely terminates the growth of the pathogen in PDA media. At the same concentration the spores of the pathogen were found completely non-viable. Cu-CNPs generates maximum MDA level in the fungal tissue as a result of the peroxidation of the membrane lipids. The fluorescence assay further confirms the generation of oxidative stress through the emission of high intensity of fluorescence. Observations under SEM concludes that Cu-CNPs produces defined membrane damages of *R. stolonifer*. Thus, it can be concluded that Cu-CNPs can be used as a potential antifungal agent against the virulent phytopathogen, *R. stolonifer*.

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