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Inhibitory effect of Mycosynthesized gold nanoparticles on Hyphal branching of the phytopathogen *Rhizoctonia solani*

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Abstract

This study focuses on the antifungal activity of gold nanoparticles synthesized from selected edible mushrooms for the first time and its measured as effect on hyphal branching. The mycosynthesized gold nanoparticles was subjected to scanning electron microscopy which showed the particles are mostly spherical and the size ranges of the particles are 60=80nm. The different average ranges of gold nanoparticles (60-80nm) from those fungi were further more studied to examine their anti fungal activity on the well known plant pathogenic fungus *Rhizoctonia solani* Kuhn c. The various concentrations of the gold nanoparticles that were used were 0.04 µg/ml, 0.12 µg/m and 0.2 µg/ml. The hyphal branching of first, second and third order was observed and specific time points of 24 hours, 48 hours and 72 hours post inoculation of media. Here it has been found that both types of nanoparticles showed gradual increase in inhibitory effect with increasing concentration although EDMnd22 gold nanoparticle showed more inhibitory activity at the highest concentration than EDMnd21. The implication of use of nanoparticles as nanofungicide is also discussed.

Keywords: Gold nanoparticle, antifungal activity, hyphal branching

Introduction

Filamentous fungi including many edible mushrooms, are multicellular microorganisms which are mainly composed thread-like structures called hyphae (Balmant et al., 2015). The union of a set of a hyphae leads to the origin of a mycelium (Maier et al. 2009). Hyphae may be present in different structure like tubular, elongated and branched without septation, containing multiple nuclei. Specialized septate hyphae play a major role in growth of the fungus, absorption of nutrients and the production of spores from reproductive or aerial hyphae (Lin et al., 2015).

In recent years antimicrobial activity of various biogenic chemicals towards phytopathogen has been a major area of research. Among the disease causing microorganisms, fungi can cause major loss to the annual yield of various important crops (Basu et al, 2016, Chowdhury et al, 2017). Fungi are responsible for a wide range of diseases to crop plants with various symptoms including wilt, rot, blast, blight. On other hand, chemical pesticides are

highly toxic that can create major health problem through entering into the food chain and ground water (Boxi et al., 2016). Chemical fungicide pollutes the environment and they are not actually suitable for disease prevention in organic farm (He et al., 2010).

The control of pathogenic fungi has become more of a since over the years, fungi have developed resistance to the conventionally used safer traditional fungicide like Mancozeb, benzimidazole etc. (Pánek and Tomšovský, 2017). Nanomaterials or nanosystems for delivery of agrochemicals through encapsulation improved the solubility and stability from degradation in the environmental (Chowdhury et al. 2014). In contrast to the conventional fungicide the chances of developing resistant are much less in the case of nanoparticles-based fungicide. Nanoparticals like zinc, sulphur and silver may be also used in controlled and specific release of agrochemicals (Ray et al., 2011, Chowdhury et al., 2014). These types of nanosystems are also very effective to bind to the diseased parts of a plant and reducing runoff into environment.

Bacterial strain *Serratia sp* mediated AgNP was found to promising strong antifungal effect against *Bipolaris sorokinianan* which is the spot blotch pathogen of wheat (Mishra et al., 2014). This effect has been examined under both *in vitro* and *in vivo* conditions in wheat. Furthermore the chemically synthesized Ag doped hollow TiO2 nanoparticles has been reported as a potent green antifungal agent against many rotting disease of crops like potato, tomato etc and especially due to *Fusarium solani* and *Venturia inaequalis* (Boxi et al., 2016). This Ag doped hollow TiO2 nanoparticles have been also tested for photolytic activity in dark and light condition. Some metal nanoparticles like Copper nanoparticles can inhibit different plant pathogenic fungui like *Phoma destructiva*, *Curvularia lunata*, *Alternaria alternata* and *Fusarium oxysporum* under field conditions (Kanhed et al., 2013). Higher antifungal property has been reported in amphotericin coated silver nanoparticles (AmB-AgNPs) against *Candida albicans, Aspergillus niger* and *Fusarium culmorum* species in comparison to commercial fungicides (Tutaj et al.,2016).

Nowadays nanotechnological research is focused on the green synthesis of nanoparticles which is less expensive, environment friendly, less toxic, easy to synthesize and have promising biomedical applications (Malarkodi et al., 2013). Using edible mushrooms for the synthesis of nanoparticles has many advantages and can be utilized for large scale production (Ray et al., 2011, Chowdhury et al. 2014). Among the metal nanoparticles, gold nanoparticle have greater potential compred to other types of nanoparticles in various field of application specially in the medicinal field. In a recent study gold nanoparticles synthesised from *Vetiveria zizanioides* and *Cannabis sativa* have been shown to have fungicidal activity against different fungus like *Penicillium sp*, *Mucor sp*, *Fusarium sp*., *Aspergillus flavus* , *A. fumigates*. Gold nanoparticles have high antifungal efficacy and so they are suitable for preparation of antifungal medicines (Swain et al., 2016). It has been also found that the antifungal activity of green synthesized gold nanoparticles is more effective than the chemically synthesized gold nanoparticles (Paria, D. and Pal, R., 2014).

The present paper deals with gold nanoparticles mycosynthesized from two different edible mushrooms and their effects in branching patterns of *R. solani* which is a plant pathogenic fungus causing root and stem rot of various monocot and dicot crops.

Materials and methods

Source of gold nanoparticles

The edible mushrooms EDMnd21 (unpublished, patent pending) and EDMnd22 (unpublished, patent pending)

were used to synthesis protein capped gold nanoparticles. Chloroauric acid purchased from sigma Aldrich, India, was added in the cell free filtrate of the mushrooms, to the final concentration of 1mM.

Scanning electron microscopy

To determine the size and shape of the gold nanoparticles, on copper grid and examined by using a scanning electron microscopy (Spectrophotometer FEI, Model: Quanta 200).

Fungal material

Rhizoctonia solani Kuhn c, pathogenic fungus causing rice sheath blight disease, used in this study to determine the inhibitory effect of the nanoparticles, was obtained from Chinsura Rice Station, West Bengal, India. The fungus was first cultured on PDA media in petriplates. The inoculated culture was grown in 28ºC temperature and kept in dark. The white actively growing hyphae from the periphery of the mycelial colony were mainly used for inoculation of the nanoparticle containing media.

Maintenance of *R. Solani*

R. Solani was sub cultured on the potato dextrose agar for 15 days. The sub cultured plates were incubated under 20 ºC in dark for 10 days. After 10 days fully grown culture plates were stored in 4ºC for storage.

Preparation of different dilutions of the nanoparticles

Gold nanoparticle with the average diameter range 60nm -80nm from EDMnd21, was diluted to a final concentration of 0.04 µg/ml, 0.12 µg/m and 0.2 µg/ml. Form EDMnd22 mediated gold nanoparticles were diluted to a final concentration of 0.02µg/ml, 0.07µg/m and 0.12µg/ml concentration. The nanoparticles were added to potato dextrose agar media such that the above concentrations were obtained.

Preparation of media with nanoparticles

Potato dextrose agar was used for the preparation of experimental plates. 2% agar was added to solidify the media. These PDA media with different concentration of Gold nanoparticle were poured in thin layers. For control and different concentration of gold nanoparticle sets were done in triplicate. These experimental plates were kept in 28°C temperature after inoculation.

Analysis of order of hyphal branching

Firstly the plates were prepared with various different concentrations of gold nanoparticles by EDMnd21 that 0.04µg/ml,0.12 µg/ml and 0.2 µg/ml. Secondly, different concentrations of gold nanoparticles has been prepared by EDMnd21 that were 0.04µg/ml,0.12 µg/ml and 0.2 µg/ml. Then the mycelia discs of 3mm diameter were taken from 10 days old fungal culture with sterile cork borer. These mycelia disc were used as fungal inoculum that was placed at the centre of the media. Inoculated PDA were kept in petriplate under aseptic condition. The following observation of order of hyphal branching were recorded at various time period that were 24, 48, 72 hrs. Then the order branching were quantified under compound microscope (Leica DMLS, 20x-100x) number 20x magnification.

Results and discussion

Scanning electron microscopy of Gold nanoparticles

For examined the surface morphology of nanoparticles scanning electron microscopy has been utilized. Fig 1,a show the size gold nanoparticles produced by the cell free filtrate of EDMnd21to be mostly 60nm. In this set gold nanoparticle were mostly spherical. The second set of EDMnd22 also representing almost spherical and mono dispersal nanoparticles most being of size size 80 nm.

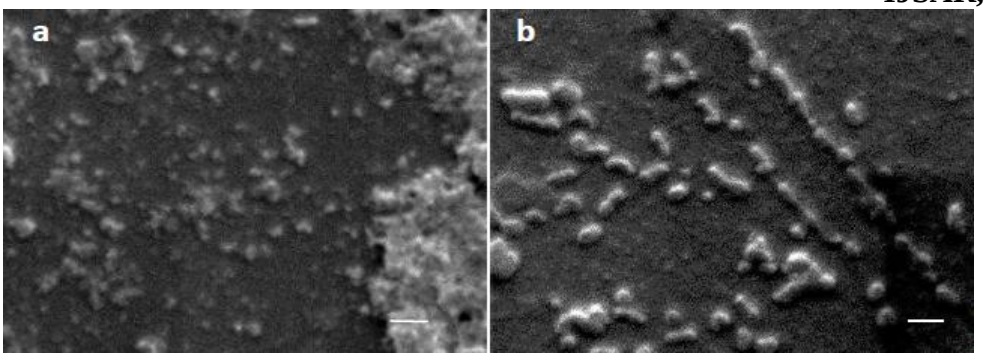

Figure 1: Scanning electron micrograph of (a) EDMnd21 Gold nanoparticles and (b) EDMnd22 Gold nanoparticles at 50,000 magnification (bar=100nm).

Hyphal branching analysis of *Rhizoctonia solani* in control sets

The control sets of the experiments (i.e. without nanoparticles) were analysed under compound microscope at 24hr, 48hr and 72 hr time points. In Figure (2 a-c) the pictures showing normally growing first order, second order and third order of branching. In the control sets representing the number of third order branches was present nearly same in contrast to the first order and the second order of branches. At the 72 hr observation all three order branches including the third order of braches were clearly present (Figure 2d).

Effect of EDMnd21 Gold nanoparticles on hyphal branching of *Rhizoctonia solani*

At first the hyphal branching pattern of the experimental sets using media supplemented with 0.04µg/ml of EDMnd21 Gold nanoparticles was examined at 24hr, 48hr and 72hr time points. Here the branches of different order were less in number with 0.04µg/ml of Gold nanoparticles concentration (Figure 2 e-h). The hyphae of *R. solani* treated with 0.12 µg/ml concentration did not showed significant difference from the previous concentration (Figure 2i-l). There was significantly less number of secondary and tertiary branches at this higher concentration of gold nanoparticles. Finally, the highest inhibitory effect on hyphal branching of different orders was found at 0.2 µg/ml concentration of gold nanoparticles (Figure 2 m-p). All the orders of branching specially the second order branching were significantly reduced compared to other sets. In contrast to the control sets treated sets with the concentration 0.2 µg/ml of gold nanoparticle were seen to have significant antifungal effect on hyphal branching of *R. solani* therefore signifying that these gold nanoparticles have increasing antifungal effect on *R. solani* with increasing concentration.

Effect of EDMnd22 Gold nanoparticles on hyphal branching of *Rhizoctonia solani*

With the concentration of 0.02µg/ml gold nanoparticle showed significant inhibition in hyphal branching (Figure 3 e-g). The graph of Fig 3 h. Shows that the third order branching was delayed to start and that that less new ones arose at 72 hrs than an 48 hrs point. At 0.074µg/ml concentration, the third order of branches were completely suppressed (figure 3.i-l). Fresh development of second order branching was reduced. At the highest concentration of gold nanoparticle 0.124µg/ml, there was clear suppression of all orders of braching in *R. solani* especially third order of branching (Fig 3. m-p). From the above data, the antifungal activity has been found from the lowest concentration of 0.02µg/ml and the effect increased with increasing concentration of gold nanoparticle.

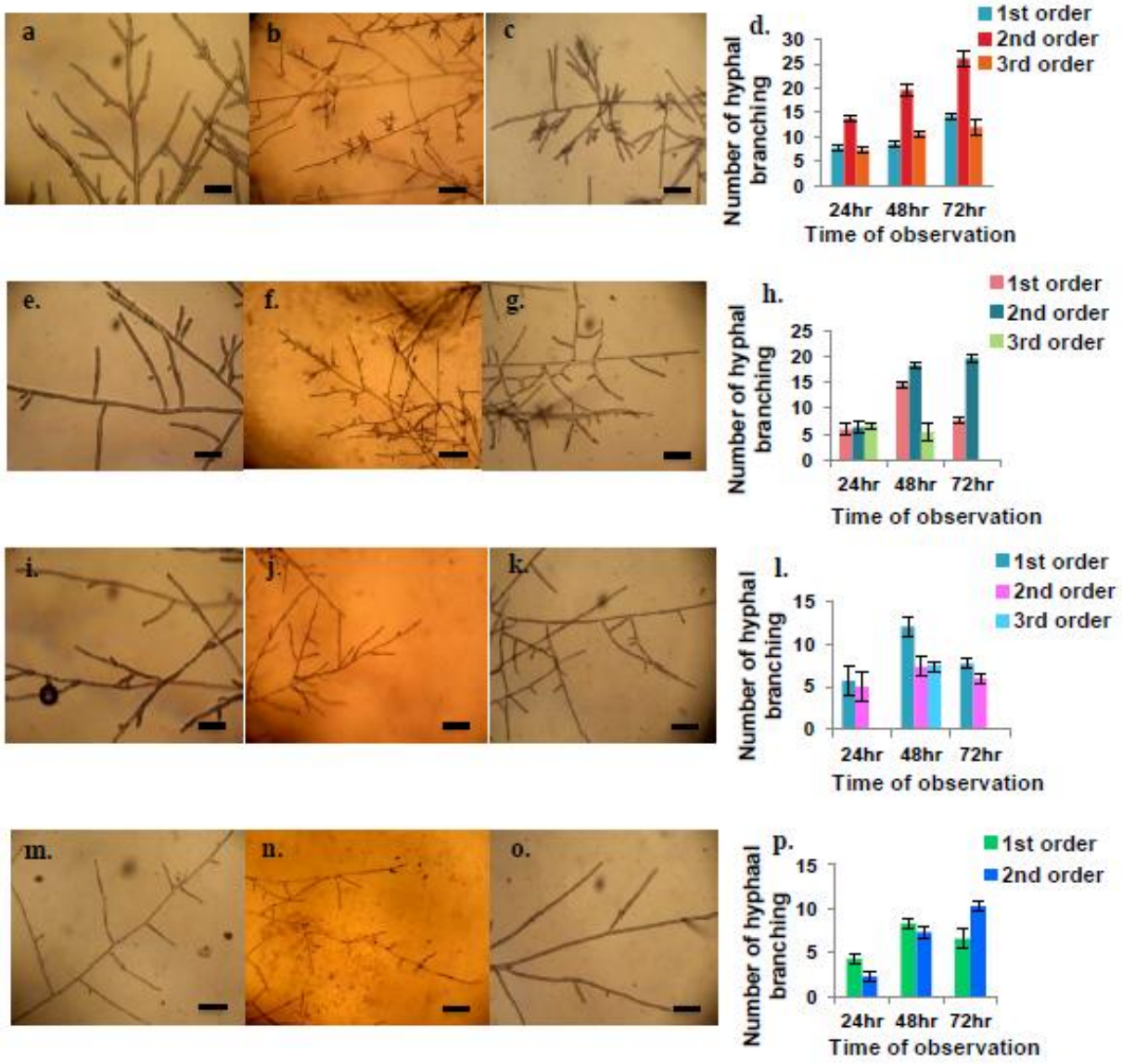

Figure 2: Inhibitory effects on hyphal branching of EDMnd21 Gold nanoparticles against *Rhizoctonia solani*. (a,b,c) The hyphae shows number of 1st, 2nd , 3rd order branches of the experimental sets without Gold nanoparticle at 24hrs, 48hr and 72hr. (d) The graph shows 1st, 2nd , 3rd order branches of the experimental sets where *R. solani* were grown on PDA without Gold nanoparticle. (e,f,g) Hyphal branching occurred on PDA treated with 0.04µg/ml Gold nanoparticle at 24hr,48hr and 78hr. (h) The graph representing 1st, 2nd , 3rd order branches of the experimental sets treated with 0.04µg/ml Gold nanoparticle at 24hr,48hr and 78hr. (i,j,k) Hyphae showing 1st, 2nd , 3rd order branches treated with 0.12 µg/ml Gold nanoparticles at 24hr,48hr,72 hr. (l) The graph represents the quantification of the number of 1st, 2nd , 3rd order branches with 0.12 µg/ml Gold nanoparticles at 24hr,48hr and 72hr. (m,n,o) 1st, 2nd , 3rd order hyphal branches with 0.2 µg/ml Gold nanoparticle treatment at 24hrs, 48hr and 72hr. (p) The graph showing quantification of the number of 1st, 2nd , 3rd order branches with 0.2 µg/ml Gold nanoparticles treatment.(bars=50µm).

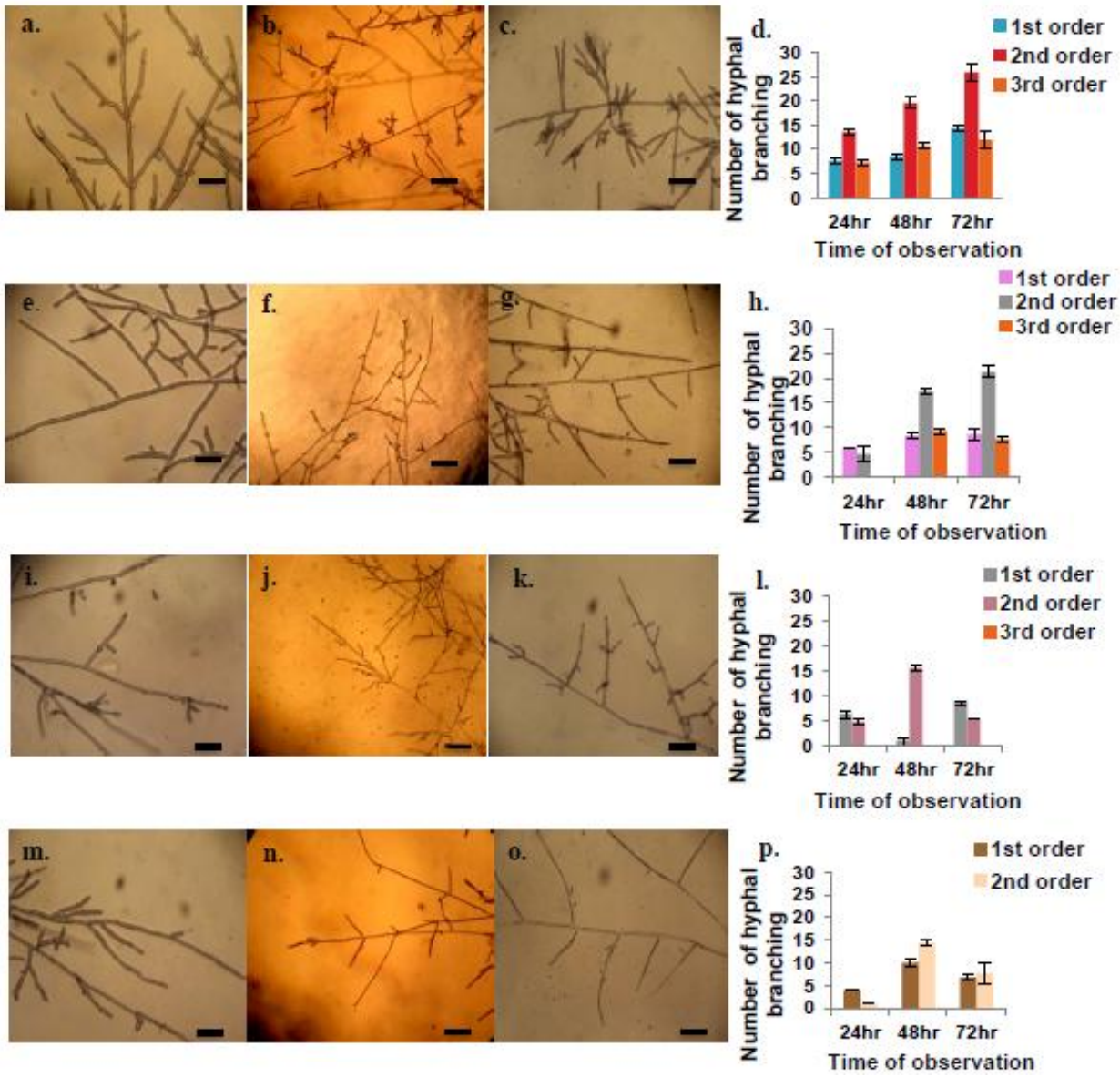

Figure 3: Inhibitory effect on hyphal branching of EDMnd22 Gold nanoparticles against *Rhizoctonia solani*. (a,b,c) Hyphae showing 1st, 2nd, 3rd order branches of *R. solani* in the experimental sets, without Gold nanoparticle at 24hrs,48hr and 72hr interval observations. (d) The graph showing the experimental sets where *R solani* were grown on PDA without Gold nanoparticle. (e,f,g) Number of 1st, 2nd, 3rd order branches occurred on PDA treated with 0.02µg/ml Gold nanoparticle at 24hr,48hr and 78hr. (h) The graph corresponding to the number of 1st, 2nd, 3rd order branches treated by 0.02µg/ml concentration of Gold nanoparticle . (i,j,k) Hyphal branching shows the number of 1st, 2nd , 3rd order branches, treated with 0.07 µg/ml Gold nanoparticles at 24hr,48hr,72 hr . (l) The graph shows the quantification of the number of 1st, 2nd , 3rd order branches with 0.07 µg/ml Gold nanoparticles at 24hr, 48hr and 72hr. (m,n,o) Hyphae showing1st, 2nd , 3rd order branches with the treatment of 0.12µg/ml Gold nanoparticles. (p) The graph showing quantification of number of hyphal branches with 0.12 µg/ml Gold nanoparticles concentration. (bars=50µm).

Conclusion

This work was undertaken to examine the antifungal effect of gold nanoparticles from fungus EDMnd21 and EDMnd22. The morphology of these two different gold nanoparticles from two different fungi is mostly spherical as seen under scanning electron microscope. Nanoparticles were used to investigate their effect on hyphal branching of pathogenic fungus *R. Solani.* Interestingly the nanoparticles showed inhibitory effect on different orders of hyphal branching. Therefore all these concentrations can be utilised as an antifungal agent like fungicide or antifungal medicines for further studies either alone or in combination with conventional fungicides. Nanoparticle has the capacity to easily penetrate the fungal cell wall due to their shape and nanometer size. For this feature these goldnanoparticles have the potential to be used for fungicide delivery into pathogens under field conditions and also in therapy on target tissues.

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