Efficacy of lectin-silver nanoparticle conjugate in the control of rice pathogen, *Curvularia lunata* (Wakker) Boedijn

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Abstract

The production of a new class of lectin-silver nanoparticle conjugates is experimentally demonstrated for the first time. The results revealed antifungal properties of lectins (glycoproteins of non immune origin) isolated from the edible and non-toxic mushroom, *Agaricus bisporus* (Lange) Pilat and lectin-silver nano conjugates which inhibited 89% of the conidial germination of the fungal pathogen of rice, *Curvularia lunata*. The antifungal activity of the conjugates was far superior to the activity exhibited by lectins or silver nanoparticles used individually. The utilization of lectins have several advantages, further these lectin-silver nanoparticle conjugates can be obtained as a stable powder that can be redispersed in water as desired.

Keywords: nanoparticle, rice pathogen, *Curvularia lunata*

Introduction

*Oryza sativa* plays a major role in the nutrition of the people around the world and after wheat it is the most important agricultural product (Yamaguchi et al., 2008). There are various factors that reduce rice production, the most important of which are pests, diseases and weeds (Yamaguchi et al., 2008). Diseases of rice are mainly caused by fungi, bacteria and viruses. There are several fungi which have been reported to affect rice cultivation in high humid and tropical temperatures. One of the most commonly encountered fungal genera is *Curvularia* sp. It may infect upto 80% of seeds and cause grain discoloration. In severe infections, *Curvularia* may weaken seedlings and cause leaf spot (Ou, 1985). The most common species infecting rice is *C.lunata*, however, *C. affinis, C. geniculata, C. oryzae* and *C.pallescens* may also be involved in black kernel disease. Due to the adverse effects of chemical pesticides, other areas of plant disease control were explored. The most important one being the biological control of plant pathogens. Research on the mechanisms of biocontrol employed by effective bacterial and fungal strains has revealed a variety of natural products that can be exploited for the development of disease control measures.

Lectins, a well-known class of multivalent carbohydrate binding proteins of non-immune origin which recognize diverse sugar structures with a high degree of specificity in a non-catalytic manner are wide spread in distribution (Sharon and Lis, 1989). Plant and animal lectins are subjected to extensive studies (Rini and Lobsanov, 1999; Kaur et al., 2006; Tanaka et al., 2009; Fuji et al., 2009) and very little information is available on lectins from fungi (Guillot and Konska, 1997; Wang et al., 1998; Konska, 2006). In the last few years, mushroom and other fungal lectins have attracted wide attention due to their antitumor, antiproliferative and immunomodulatory activities (She et al., 1998; Wang et al., 2000). Lectins have been isolated from several common edible mushrooms, including *Agaricus bisporus, Flammulina velutipes, Ganoderma lucidum, Grifola frondosa, Hericium erinaceum, Pleurotus ostreatus*, and *Volvariella volvacea* (Pemberton, 1984; Ng, 2004). In recent years, Silver nanoparticles have been extensively studied and various approaches have been employed for the preparation of metal...
nanoparticles (Ghosh et al., 1996; Yin et al., 2003; Jiang et al., 2005). Silver in ionic or nanoparticle forms has a high antimicrobial activity and is therefore widely used. There have been relatively few studies on the applicability of silver to control plant diseases. In the present study, lectins or glycoproteins of nonimmune origin were isolated from Agaricus bisporus and their antagonistic effects against the rice pathogen, Curvularia lunata, were experimentally analysed and documented. A novel method of conjugating lectins with silver nanoparticles was also attempted since the antibacterial and antifungal nature of silver nanoparticles has already been well established through various research papers.

Materials and methods

Conjugation of silver nanoparticles and lectins

Isolation of lectin from Agaricus bisporus mycelium was done according to (Li et al., 2010). Silver Nitrate (AgNO₃) 6 mM solution was mixed with equal volume of lectin solution. To the above solution 1ml of KOH (10M) was added and boiled for 5-10 minutes in a boiling water bath. A corresponding control was maintained. Colour change was observed in both the tubes. The conjugate was dialyzed for 24 hours. The dialysate was centrifuged thrice to remove any unbound particles. The pellet was dried and taken for characterization and analysis. UV-VIS and FT-IR spectral analysis was carried out.

Antifungal activity of silver-lectin conjugate

1ml of the culture containing 1x 10⁵ spores were centrifuged to obtain the pellet. From the stock concentration of conjugate 50, 100, 250, 500 and 750 µg/ml concentration was made. 100 µl of each of these concentrations were added to Curvularia lunata spores. These were incubated for 12 hrs and spread onto PDA plates. The colony forming units were observed after 24 hrs of incubation. A minimum inhibitory concentration test (MIC₅₀) was also done.

UV Spectral analysis of lectin-silver conjugate

The synthesized air-dried lectin-silver conjugate was analysed in UV-VIS spectrophotometer UV-2450 (Shimadzu) between 200-400 nm for maximum absorption peaks.

FT-IR Spectral analysis of lectin-silver conjugate

In Fourier transform infrared (FTIR) spectroscopy measurements, the conjugate particles synthesized were air dried and mixed with potassium bromide in the ratio of 1: 100. FTIR spectrum of samples was recorded on Shimadzu IR Prestige-21 FTIR instrument with a diffuse reflectance mode (DRS-8000) attachment. All measurements were carried out in the range of 400-4000 cm⁻¹ at a resolution of 4 cm⁻¹. This range was used to study the fundamental vibrations and associated rotational vibrational structure.

Scanning Electron Microscopy

An aliquote of 10 µl of silver-lectin conjugate treated (24 hrs of incubation) conidia of Curvularia lunata as mentioned above was applied onto fresh leaf bits of Papatlal and Ponni variety of Oryza Sativa. After an incubation of 48 hrs, the leaf bits were dried in a dessicator for 72 hrs and taken for SEM analysis. The leaf bits were dried using a critical point drier and placed onto a stub and observed in Hitachi S-4500 SEM machine and photographed.

Scanning Atomics Force Microscopy

Curvularia lunata conidia were treated with silver-lectin conjugate (300 µg/ml-MIC₅₀) and incubated for 24 hrs. The interaction of conjugate with the conidial surface was studied using SAFM and photographed.

Results

Conjugation of silver nanoparticles and lectins

A direct method of preparing silver capped glycoprotein conjugate was attempted using the lectins and silver nanoparticles. In the blank sample pale creamish solution was observed during the reaction whereas in the conjugation reaction, a quick gradation of colour change was observed. The colour varied from pale brown to dark brown (Fig. 1). According to the literature cited, this colour change indicated the reduction of silver ions by glycoproteins. The size of
the conjugate particle was measured to be 24 µm (0.024 nm).

**Fig 1.** Conjugation of silver nanoparticles and lectins

![Conjugation of silver nanoparticles and lectins](image)

Note the colour change in the reaction mixture T - treated; C - control

**Antifungal activity of conjugate**

An *in vitro* petridish assay procedure was followed to test the antifungal activity of lectin-silver conjugate. The conidia of *Curvularia lunata* were treated with various concentrations of Lectin-silver conjugate and incubated at room temperature. After 24 hours although the first three concentrations of 50, 100 and 250 µg did not show significant inhibition percentage, the last two concentrations of 500 and 750 µg showing considerable inhibition ranging from 73-88% was noted (Fig. 2). Compared to the individual lectin and silver results, the 750 µg/ml of the conjugate showed much higher inhibition range. The Minimum inhibitory concentration was carried out between a series of concentrations ranging from 250 to 500 µg/ml. Thus an MIC<sub>50</sub> of 300 µg/ml was effective in inhibition of conidial germination (Fig. 3).

**UV Spectrum of conjugate**

In the UV spectral analysis of the lectin-silver conjugate, the band pattern between 300 nm to 400 nm shows a dip which is present in the corresponding UV spectrum of lectin. The absorption peak of silver nanoparticle was shifted from 417 to 454 nm (Fig. 4).
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**Fig. 5.** FT-IR Spectrum of lectin-silver conjugate

**FTIR Spectrum of conjugate**

The bands seen at 3417 cm\(^{-1}\) and 2923 cm\(^{-1}\) were assigned to be stretching vibrations of primary and secondary amines respectively, while the corresponding bending vibrations were seen at 1639 cm\(^{-1}\) and 1509 cm\(^{-1}\) respectively. The two bands observed at 1277 cm\(^{-1}\) and 1027 cm\(^{-1}\) can be assigned to the C-N stretching vibration of aromatics and aliphatics amines respectively (Fig. 5).

**Scanning Atomic Force Microscopy**

Scanning Atomic Force Microscopy imaging of the fungal conidia was performed in the contact mode using a Pico Scan 5100 LE machine purchased from Agilent Technologies. The images showed the presence of conjugated nanoparticles on the surface of the treated conidia whereas the control conidia showed a smooth surface. The image of glowing particles on the surface of conidia indicated the presence of lectin-silver conjugate (Fig 6).

The scanning electron microscopic studies were carried out in Hitachi S-4500 SEM machine. From the electron micrographs it was clearly visible that there was a difference in the colony forming capabilities of the conidia. The control conidia colonized at a much higher percentage than the treated conidia. Several hyphae without the formation of the conidia were visible in the treated sample. The surface of the untreated conidia was smooth, whereas the conjugate treated conidia appeared to be porous and the shape of the conidia also looked distorted. In some the conjugate treated conidia the adherence of the larger conjugate particle was evident.

**Fig. 7.** Scanning Atomic Force Microscopy of *Curvularia lunata* conidial surface treated with lectin-silver conjugate
Discussion

In the present study, a novel method of conjugating lectins with silver nanoparticles was done. This was done to help better the application methods of silver as well as lectin in controlling plant diseases. So far the protocols adapted for realizing such conjugates have relied on the traditional organic chemistry synthesis for the preparation of glycolipids and the standard reported methods for obtaining nanoparticles. For example, reduction of metal ions by an appropriate reducing agent in the presence of the glycolipid or a post synthetic conjugation of these metal nanoparticles with the glycolipids concludes the preparation of a glycolipid metal nanoparticle conjugate (Singh et al., 2009). While the nanosystems possess size dependent optoelectronic, magnetic and fluorescent properties, the carbohydrates bring on board biocompatibility, selective interactions with various proteins and molecules and render the conjugates water dispersible. The shift in the absorption bands of the silver and lectins in the UV spectrum of conjugate indicate the binding of lectins to silver nanoparticles. The presence of amine linkages in the conjugate spectrum confirms the presence of lectins in the conjugate and they play a key role in the reduction of silver to silver ions. The vibrations within 2859 cm\(^{-1}\) and 2923 cm\(^{-1}\) show the presence of ‘CH\(_3\)’ groups which indicates complex formation between silver nanoparticles and lectins and thus adsorption onto the conidial surface.

An in vitro study of the in planta evaluations of lectin-silver conjugate was done by scanning electron microscopic studies. Since lectins can only control the mycelial colonization ability, these structural changes can be attributed to the silver nanoparticles present in the conjugate. Silver nanoparticles may directly attach to and penetrate the cell membrane to kill spores, although penetration of silver nanoparticles into microbial cell membranes is not completely understood (Morones et al., 2005). In planta evaluations with the help of SEM also showed marked differences between the treated and untreated leaf bits. A high range of fungal colonization was visible in the control whereas in the conjugate treated leaf sample, very less colonization was seen as well some of the hyphae did not produce germinating spores. This non spore formation may be due to the activity of nanoparticles. The silver nanos are known to produce pores on the surface and also inhibit the cell wall formation and deposition of wall materials. It is postulated that a similar mode of action may be responsible for the inhibitory action in the present study. The conidia also looked deformed in the treated leaf. The AFM images indicated the presence of conjugate on the surface of the conidia. This was done to confirm the attachment of the conjugate to the conidial surface. The present study, evaluation of the lectin silver conjugate in controlling Curvularia lunata has resulted in a positive control on the pathogen.

Further investigation on the mode of action of these lectin-silver conjugate may throw more light into the use and implementation of lectin-silver nanos in control of plant pathogens. To summarise the infection of paddy by Curvularia lunata causes discolouration of the grains, which in turn highly reduces the marketing value of rice cereal. In the past few years nanotechnology has emerged as a highly evolving field which can be utilized in the control of pathogens causing harm to their respective hosts. Conjugation of the Agaricus lectin with the silver nanoparticles done presently is a novel method of attempting to control the fungal pathogen, Curvularia in a very efficient and ethical way. Further studies on the mode of action and in vivo evaluations need to be conducted to analyse the extent of inhibition and the degree of toxicity in order to dispense this formulation for field trials.

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