



Biological preparation of chitosan nanoparticles and its in vitro antifungal efficacy against some phytopathogenic fungi

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ARTICLE INFO

Article history:

Received 29 February 2016

Received in revised form 20 April 2016

Accepted 11 May 2016

Available online 13 May 2016

Keywords:

Chitosan nanoparticle
Biological preparation
Inhibition
Phytopathogen
Growth promoter
Chickpea seedlings

ABSTRACT

The aim of the present study was to prepare Chitosan nanoparticles through biological method with high antifungal activities. Chitosan nanoparticles were prepared by the addition of anionic proteins isolated from *Penicillium oxalicum* culture to chitosan solutions. The formation of chitosan nanoparticles was preliminary confirmed by UV–vis spectrophotometric analysis. The physico-chemical properties of the chitosan nanoparticles were determined by size and zeta potential analysis, FTIR analysis, HRTEM and XRD pattern. The chitosan nanoparticles were evaluated for its potential to inhibit the growth of phytopathogens viz., *Pyricularia grisea*, *Alternaria solani*, *Fusarium oxysporum*. It is evident from our results that chitosan nanoparticles inhibit the growth of phytopathogens tested. Chitosan nanoparticle treated chickpea seeds showed positive morphological effects such as enhanced germination%, seed vigor index and vegetative biomass of seedlings. All these results indicate that chitosan nanoparticle can be used further under field condition to protect various crops from the devastating fungal pathogens as well as growth promoters.

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1. Introduction

Phytopathogenic fungi cause severe damages to many crops world wide (Nair et al., 2010). They affect economically important crops at all age levels and results in qualitative and quantitative losses in agriculture (Agrios, 2000). Farmers use chemical fungicides to control the phytopathogenic fungi. Excessive and indiscriminate use of Agrochemicals leads to deterioration of soil health, degradation of agro-ecosystem, environmental pollution and resistance in pathogens (Kashyap, Xiang, & Heiden, 2015). Hence, effective control of the fungal pathogens is of serious concern. Therefore, development of technologies that improve food productivity without causing any adverse impact on the ecosystem is the pressing need. In this context, nanotechnology has emerged as a technological advancement that can transform the agricultural sector by providing novel tools for the management of biotic and abiotic stress (Chena & Yada, 2011; Khot, Sankaran, Maja, Ehsani, & Schuster, 2012) to increase the food production. Nanomaterials have been utilized in agriculture, mostly in crop protection due to their size-dependent qualities, high surface to volume ratio and unique optical properties.

Chitosan, a bio-polymer known for its broad spectrum antimicrobial activities (Dash, Chiellini, Ottenbrite, & Chiellini, 2011;

Pichyangkura & Chadchawan, 2015). However, the low solubility of bulk chitosan in aqueous media limits its wide spectrum activity as an antimicrobial agent (Saharan et al., 2015). Therefore, various strategies have been employed to enhance its antifungal potential (Li et al., 2012; Qin et al., 2012). Chitosan has immense structural possibilities for chemical and mechanical modification to generate novel properties and functions, to use in the field of agriculture.

Chitosan based nanoparticles has gained considerable interest due to its biocompatibility, biodegradability, high permeability, cost-effectiveness and non-toxic property (Prabaharan & Mano, 2006; Prabaharan, Reis, & Mano, 2007; Shukla, Mishra, Arotiba, & Mamba, 2013). There are few reports that chitosan based nanoparticles show antifungal activity towards some phytopathogenic fungi (Kashyap et al., 2015; Saharan et al., 2013). Recently we have reported that chitosan nanoparticle prepared through chemical method protected rice plants from blast fungus (Manikandan & Sathiyabama, 2016). Generally, chitosan nanoparticles were prepared through physical as well as chemical methods, which shows variation in stability, size, shape and biological activity (Calvo, Remunan-Lopez, Vila-Jato, & Alonol, 1997; Liu & Gao, 2009; Saharan et al., 2015). Hence, it is important to improve their physico-chemical parameters for better stability and increased biological activities. Though, chitosan nanoparticle prepared through ionic gelation by using TPP is considered safe, it yields nanoparticles with variable sizes (250–400 nm) and charge. This heterogeneity in size further affects their physico-chemical characters (Kamat, Maratha, Ghormade, Bodas, & Paknikar, 2015). The biological preparation

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of chitosan nanoparticles yields nanoparticles with size less than 100 nm. In this study, we report for the first time, the biological preparation of stable chitosan nanoparticle and its antifungal effect on some phytopathogenic fungi. The prepared chitosan nanoparticles were also evaluated for seedling growth of chickpea.

2. Materials and methods

2.1. Biological preparation of chitosan nanoparticles

Penicillium oxalicum, an endophytic fungus (Parthasarathy & Sathiyabama, 2014) was grown in Czepak Dox broth (CDB) medium for 3 days. The extracellular proteins produced by the fungus were precipitated with 80% (w/v) ammonium sulphate saturation. The precipitated proteins (5 mg) were passed through pre-saturated CM-cellulose column (30 × 2.5 cm) as described earlier (Sathiyabama & Balasubramanian, 2000). The unbound proteins eluted from the column were collected and checked for the protein content. This was used for the preparation of chitosan nanoparticle.

Chitosan was dissolved at 0.5% (w/v) with 1% (v/v) acetic acid and pH was adjusted to 4.8. The anionic proteins (180 µg/ml) eluted from the void volume of the column (6 ml) were added to the chitosan solution (15 ml) under magnetic stirring for 30 min and kept overnight at room temperature. After incubation, the colloidal suspension was centrifuged at 10,000 G for 10 min. The precipitate was washed twice to remove the unreacted substance and then freeze-dried.

2.2. Characterization of chitosan nanoparticles

UV-visible spectra were recorded using a Shimadzu UV-vis 1800 Spectrophotometer for the preliminary confirmation of nanoparticle formation. The structural features of chitosan nanoparticles were performed in a Nicolet 560 FTIR Spectrophotometer in a range between 400 and 4000 cm⁻¹ using a KBR pellet technique. Particle size, polydispersity index (PDI) and zeta potential (surface charge) were analysed by dynamic light scattering (DLS) using the Zetasizer (Malvern, UK) at 25 °C in triplicate. The size and morphology of the chitosan nanoparticles were examined by HRTEM (JEOL model 1200 EX). X-ray diffraction studies were performed using an X-ray diffractometer (Rku Ultima III XRD) with CuKα1 radiation to determine the structure of the sample. The X-ray source was operated at 40KV and 40 mA. Diffraction intensity was measured in the reflection mode at a scanning rate of 2/min for 2θ = 10–70°.

2.3. Determination of antifungal activity

Antifungal activity of chitosan nanoparticles (100 µg) against some phytopathogenic fungi viz., *Fusarium oxysporum* f.sp. *ciceri*, *Pyricularia grisea*, *Alternaria solani* was evaluated by disc diffusion method, under *in vitro* condition. Experiments were done in triplicate and repeated twice. % of inhibition was calculated using the formula:

$$\% \text{inhibition} = \frac{\text{mycelial growth in control} - \text{mycelial growth in treatment}}{\text{mycelial growth in control}} \times 100.$$

2.4. Effect of chitosan nanoparticles on seed germination

The seeds of chickpea were surface sterilized with 1% sodium hypochlorite for 10 min and washed thoroughly with sterile distilled water. They were placed in sterile petri plates (90 × 15 mm Borosil, India) lined with filter paper. The filter paper was made

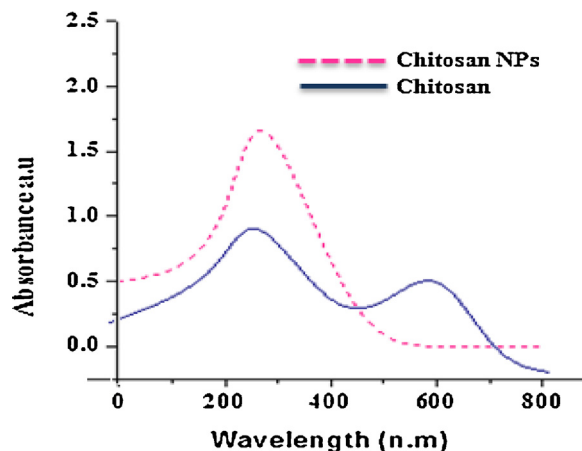


Fig. 1. UV-vis spectrum of chitosan nanoparticle and chitosan.

wet with 5 ml chitosan nanoparticle (0.1%) solution. Seeds incubated with sterile distilled water served as control. Experiments were performed in triplicates with 50 seeds each. The petri plates were sealed and incubated at 28 ± 2 °C under dark for initial five days and at 8 h light and 16 h dark condition for a further 5 days. Seedling vigour index (SVI) was calculated by the formula:

$$\text{SeedVigourIndex} = \% \text{germination} \times \text{seedlinglength}.$$

3. Results and discussion

The current challenges of sustainability, food security and climate change warrants continuous innovation in the agricultural sector. This extends nanotechnology as a new source to improve existing crop management techniques (Parisi, Vigani, & Rodriguez-Cerezo, 2015). The use of nanomaterials in particular, biological nanoparticles are receiving more attention due to their high specificity and improved function. They are often biocompatible and have reproducible structure. Chitosan nanoparticles are used worldwide for various applications including drug delivery, agriculture, etc. Chitosan nanoparticles are prepared by physical as well as chemical methods (Calvo et al., 1997; Kashyap et al., 2015; Manikandan & Sathiyabama, 2016; Shukla et al., 2013). In this study, we report the biological preparation of chitosan nanoparticles. During the purification of extracellular proteins produced by *P. oxalicum* (an endophytic fungus), we collected the unbound protein fraction eluted from CM-Cellulose column chromatography. This fraction was added to the chitosan solution to aid the formation of chitosan nanoparticle. The formation of nanoparticles was analysed through UV-vis spectrophotometer which showed a single peak at 285 nm (Fig. 1).

FTIR analysis (Fig. 2) shows the presence of bands at 3434.9 cm⁻¹ (due to overlap of O–H and N–H stretching), 1602.8 cm⁻¹ (carbonyl stretch N–H amide linkage protein), 1564.18 cm⁻¹ (N–H amide group), 1403.55 cm⁻¹ (N–H secondary amines) 1385.05 cm⁻¹ (N=O nitrogroup), 1125.87 cm⁻¹ (C–O stretching), 895.15 cm⁻¹, 832.48 cm⁻¹ (N–H primary and secondary amines). The bands at 1602.8, 1564.18, 1403.5 indicate the binding of protein to chitosan. The chitosan spectrum showed characteristic bands of amide (1623.30 cm⁻¹) and amino (1157.45 cm⁻¹) groups (Han, Zhou, Yin, Yang, & Nie, 2010).

The average size of the nanoparticle measured by zetasizer was 89.8 nm with a narrow size distribution (PDI = 0.225) (Fig. 3a), which implies the monodisperse nature in aqueous solution and is vital for uniform biological activities (Van, Minh, & Anh, 2013). The addition of anionic proteins to chitosan solution at pH 4.8 results

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