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Effect of nitrogenous polysaccharides in sporulation of entomopathogenic fungus, *Lecanicillium lecanii* (Zimmermann) Zare and Gams

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Abstract

Chitin is a biodegradable, nitrogenous polysaccharide widely spread in nature, mainly in crustacean shells. Chitin and some of its derivatives have insecticidal as well as fungicidal properties, but little is known about their effect on fungal biocontrol agents. Lecanicillium lecanii (Zimmermann) Zare and Gams is a potential entomopathogen effective against sucking pests. In this work, we studied the effect of nitrogenous polysaccharides like chitin and chitosan both in pure and crude form at concentrations ranging from 0.5 – 5 %. This was carried out under liquid state fermentation. Chitin and chitosan at all the tested concentrations increased the conidiation as well as pathogenicity of L. lecanii with the retention of conidial viability.

Keywords-chitin; chitosan; Lecanicillium lecanii; conidiation; pathogenicity

I. INTRODUCTION

Biological control agents like entomopathogenic fungi, offer an environmentally safe alternative to chemical pesticides [1]. *Lecanicillium* spp. has been used to control aphids and insects in field as well as in greenhouses [2, 3]. *Lecanicillium lecanii* is primarily entomopathogenic to aphids and scales. Many isolates of this fungus demonstrate pathogenicity to several species of aphids such as *Aphis gossypii* (Glover), *Macrosiphum euphorbiae* (Thomas), *Brevicoryne brassicae* (L.) and *Myzus persicae* (Sulzer) [4, 5, and 6]. Conidia from these biocontrol agents are the propagules that initiate pathogenesis and are involved in disease transmission [7]. As conidiation is a highly successful reproductive process, increasing sporulation of these entomopathogenic fungi will lead to an improvement in their biocontrol potential.

Chitin is a natural nitrogenous polysaccharide composed of β- 1, 4 N-acetyl-D-glucosamine units. It is highly distributed in nature, as a constituent of insect exoskeletons, shells of crustaceans and fungal cell walls [8]. It is the second most abuntant polysaccharide in nature next to cellulose [9]. Chitosan is a partially deacetylated form of chitin consisting of β - 1, 4 – glucosamine subunits. Chitin has been used to enhance the efficiency of biological control agents like entomopathogenic fungi. It was found to increase chitinase production of entomomopathogens when used as a sole carbon source in the culture media [10]. Antifungal activity of chitosan on plant pathogenic fungi has been widely studied. But, the effect of chitin and chitosan on the growth, viability and pathogenicity of entomopathogens is little known.

In this paper, we demonstrate that nitrogenous polysaccharides like chitin and chitosan can be utilized to significantly enhance conidiation of *L. lecanii*. We also show that conidiation promotion by chitin and chitosan does not affect pathogenicity and viability of conidia.

II. MATERIALS AND METHODS

A. Fungal isolate and culture conditions

L. lecanii isolate (V.l.8) was obtained from National Bureau of Agricultural Insect Resources (NBAIR). The fungus was subcultured and maintained in Sabouraud Dextrose Agar (SDA) under refrigeration and mass production for laboratory experiments were done in Sabouraud Dextrose Broth (SDB).

B. Media amendments

The basic media SDB was supplemented with crude as well as extra pure forms of chitin and chitosan at concentrations viz., 0.5, 1, 2 and 5 %. Crude chitin and chitosan of low molecular mass was obtained from MATYAFED Chitosan Plant, Kerala and extrapure grades were purchased from Himedia. The media supplements were added to 100 ml SDB and sterilized under 15 lbs pressure (121° C) for 15 minutes. The flasks with media were then seeded with 1 ml of 14 day old culture of *L. lecanii* and incubated at room temperature for 14 days. SDB without any supplements served as control. Each treatment was replicated thrice. The biomass yield, spore count, colony forming units (cfu) and mortality to test insect were observed on 14 days after inoculation.

C. Determination of fungal biomass

The 14 day old culture was filtered through a pre-dried and weighed Whatmann no 1 filter paper and the mat collected was dried at 100° C for 24 hr and weighed. The difference in weight was recorded [11].

D. Determination of spore count

The spore count was enumerated from 14 day old culture using a Naeubaer haemocytometer and calculated using the formula,

Spores / ml = (n) x 10^4 spores per ml, where 'n' is the average number of spores in the four 1 mm corner squares of haemocytometer.

E. Estimation of viability and virulence

Viability of the cultures were determined by assessing cfu using dilution plate method on SDA. One ml of culture was diluted in 99 ml sterile water and plated on each Petri dish and 15 ml molten media was added and gently rotated for uniform spreading of spore suspension and incubated at room temperature. Three replications were maintained for each treatment. Cfu was estimated after 7 days of incubation, as follows

$$\text{cfu} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{Volume of sample plated (ml)}}$$

To test virulence, 14 day old culture was applied on test insect, black pea aphid *Aphis craccivora* under laboratory conditions. Tender cowpea leaf was kept upside down in a 9 cm plastic petriplate with a filter paper moistened to saturation level. Leaf petiole was covered with moistened cotton to maintain turgidity. Second instar nymphs of *A. craccivora* collected from laboratory culture were released into the petriplate using camel hairbrush @ 50 aphids per plate. Three replications were maintained for each treatment. The spore suspension was applied using an atomizer. Test

insects sprayed with water alone served as control. Care was taken not to drown the insects in spore suspension. Observations were recorded upto seven days after treatment. Pathogenicity of *L. lecanii* was confirmed by reisolating the fungi from cadavers and examining under microscope.

F. Statistical Analysis

All data were analyzed using analysis of variance in completely randomized design by using computer programme Excel.

III. RESULTS AND DISCUSSION

3.1. Effect of media supplements in growth and sporulation

In general, sporulation is favoured by nutritional conditions that restrict growth [12] but in our study, we observed that all concentrations of chitosan and higher levels of chitin, slightly promoted the fungal growth along with the increase in conidiation (Figure 1). The low concentrations of chitin had no or minor inhibition over the growth of fungus.

Enumeration of spore count revealed that both crude and extrapure forms of chitin and chitosan could enhance sporulation of the fungus at all the tested concentrations. The conidiation was improved ten fold ranging from 1.7 – 9.3 x 10^8 spores ml^{-1} compared to control where the spore load was 3.6 x 10^7 spores ml^{-1}. *L. lecanii* showed a chitosan concentration-dependent conidiation increase (Figure 2). Maximum sporulation was obtained with chitosan extrapure 5 % with spore yield of 9.3 x 10^8 spores ml^{-1} followed by chitosan extrapure 2 % with 7.8 x 10^8 spores ml^{-1}. Extrapure chitosan was found to be the best media supplement to enhance sporulation twenty five times than the control. In the case of chitin, highest conidiation was observed for crude chitin 5 % with a spore yield of 3.0 x 10^8 spores ml^{-1} which is significantly greater than the control. Chitosan, both crude and extrapure forms at all tested concentrations were found to be superior to chitin. Chitin crude 5 % with 3.0 x 10^8 spores ml^{-1} was on par with chitosan crude 0.5 % with 3.7 x 10^8 spores ml^{-1}. Palma-Guerrero and coworkers [13] observed a similar increase in sporulation of entomopathogenic fungi, *Beauveria bassiana* and *L. psalliotae* with highest tested concentrations of chitosan.

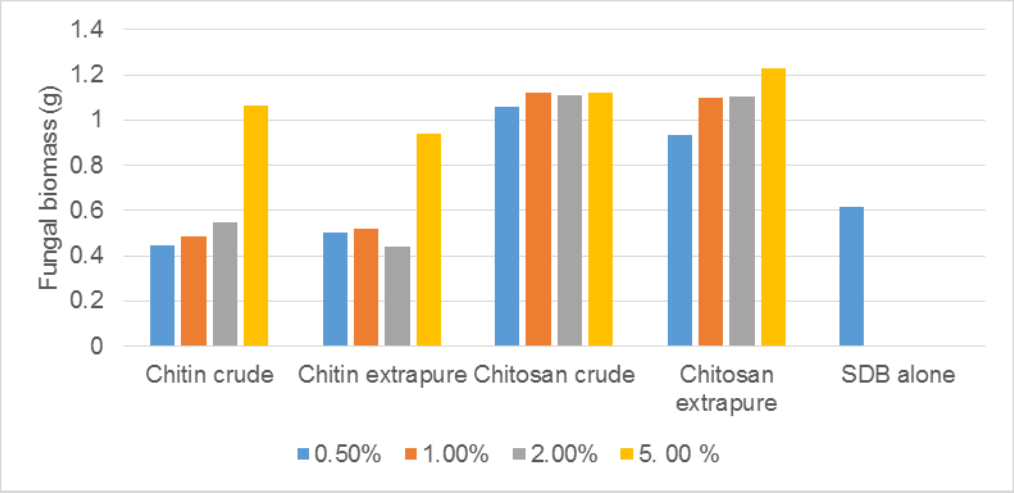

Figure 1. Effect of chitin and chitosan on biomass yield of L. lecanii

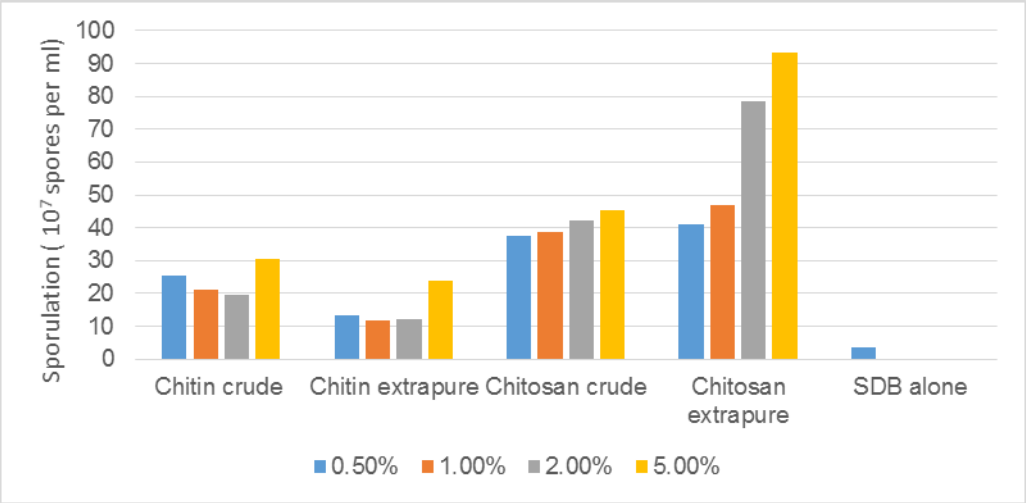

Figure 2. Effect of chitin and chitosan on sporulation of L. lecanii

3.2. Effect of chitin and chitosan on conidial viability and pathogenicity

Conidia harvested from the chitin and chitosan amended media were tested for viability by assessing the number of cfus. The number of cfu did not vary significantly in the amended media and control (Figure 3). Pathogenicity was enhanced with the addition of chitin and chitosan to the media. While assessing cumulative percent mortality of *A. craccivora*, cent percent mortality was observed within 72 hours of treatment with chitin crude 5 %, chitin extrapure 5% and chitosan extrapure 5 %. Lowest mortality obtained was for chitosan crude 0.5 % with 76.66 % mortality. The corresponding percentage mortality of *A. craccivora* in unamended SDB was 36.66 %.

Higher virulence of entomopathogenic fungi can be due to higher chitinolytic activity [14]. Samuels and coworkers [15] also found detectable amounts of extracellular chitinase, lipase and protease in highly pathogenic strains of entomopathogens. Bidochka and Khachatourians [16] reported high chitinolytic activity when *B. bassiana* was cultivated with media containing insect cuticle. It was also reported that shrimp waste silage, a source of chitin was an efficient inducer of N-acetyl-hexosaminidse for *Verticillium* (*Lecanicillium*) *lecanii* [17]. Though we did not test the chitinolytic activity, the enhanced pathogenicity may be attributed to chitinase production as evident from the earlier reports.

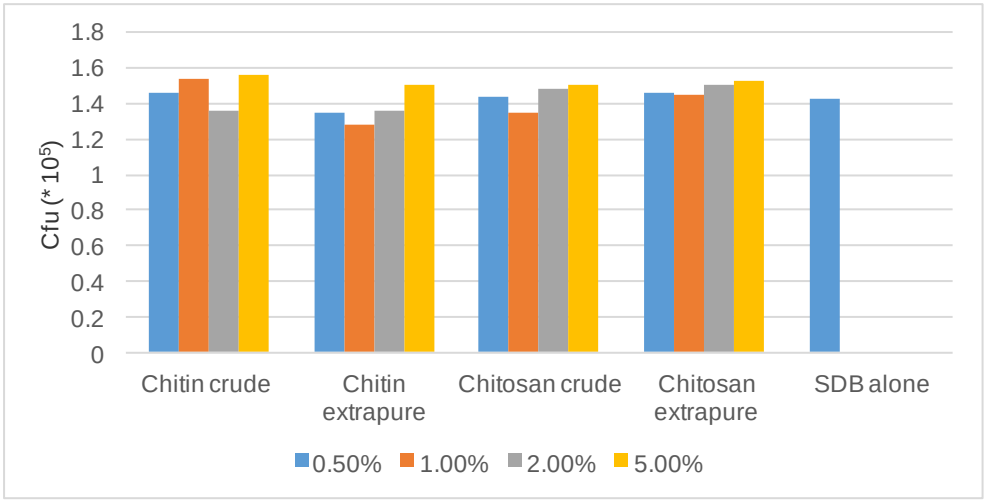

Figure 3. Effect of chitin and chitosan at different concentrations on viability of L. lecanii

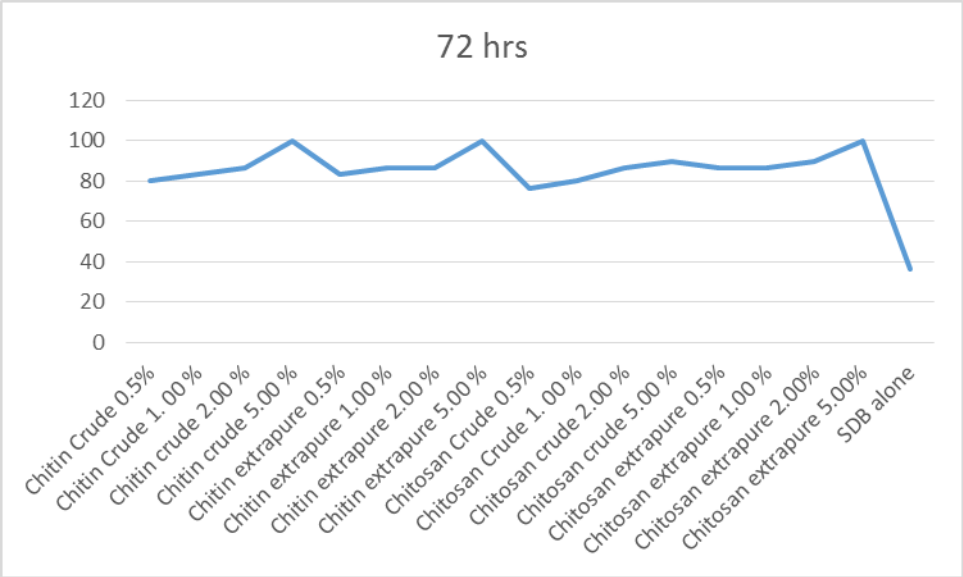

Figure 4. Cumulative percent mortality of Aphis craccivora after 72 hrs of treatment with amended media

IV. CONCLUSION

This paper shows that nitrogenous polysaccharides viz., chitin and chitosan, can be used as a conidiation inducer for entomopathogenic fungi. The increase in sporulation without reducing spore viability and pathogenicity make these compounds suitable media supplements which can be widely exploited in the increase conidia production with wide applications in biological control.

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