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Antifungal effect of chitosan on certain soil borne fungal pathogens of Mulberry (*Morus* spp.)

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Abstract

Soil borne diseases occur in mulberry in alarming proportions rendering loss in mulberry leaf production and hence are very serious concern among sericulture farmers. Introduction of high yielding mulberry varieties, draught and low organic carbon in soil further intensified the disease. Due to the environmental concern and cost of chemical fungicides, farmers are reluctant for chemical control. Thus alternative methods are to be adopted to contain soil borne diseases. Chitosan and its derivatives are well known for their anti microbial properties and used in agricultural systems. A laboratory study has been conducted to evaluate the effect of chitosan on suppression of certain common soil borne pathogens of mulberry. The study showed significant ($P<0.01$) suppression of growth of all pathogens *viz. Fusarium solani, Fusarium oxysporum, Rhizoctonia bataticola* and *Botryodiplodia theobromae.* The suppression of *F. solani, R. bataticola* and *B. theobromae* was high (>65%) at 500 ppm. The fungal suppression was increased with increase in concentration of chitosan in all the cases. The mycelia biomass production of the fungus was also significantly less in presence of chitosan in the liquid medium with low mycelia production in high concentration of chitosan. Chitosan also influenced sporulation of the fungi. Sporulation was low in presence of chitosan compared with untreated control. The study shows chitosan is potential to suppress these fungal pathogens of mulberry and can be explored as an eco-compatible material for control of soil born diseases of mulberry.

Keywords: Antimicrobial property, chitosan, inhibition, mulberry, root rot pathogens

Introduction

Soil borne diseases such as dry root rot (*Fusarium* spp.), black root rot (*Botryodiplodia theobromae*) and char coal rot (*Rhizoctonia bataticola*) are major concern among sericulture farmers as the disease occurs in alarming proportions especially in southern India (Sharma *et al*., 2003). Draught situations and low organic carbon in the soil further added to the situation. The chemical fungicide application is not suitable for its control due to environmental considerations hence alternative methods are to be developed to contain the disease.

In recent decades, a greater knowledge of chitosan chemistry, and the increased availability of chitosan containing waste

materials from the seafood industry, has led to a variety of applications in the agriculture industry. Chitosan and its derivatives have been investigated as an antimicrobial material against a wide range of target organisms like algae, bacteria, yeasts and fungi in experiments involving *in vivo* and *in vitro* interactions with chitosan in different forms (solutions, films and composites). Chitosan, a natural substance, has proved to be effective in preventing fungal growth by directly interfering or by activating certain biological processes (El Ghaouth *et al*., 1992a). Chitosan is also known to elicit many plant defense responses by activating pathogenesis-related (PR) gene functions such as chitinases (Mauch *et al*., 1984; Benhamou and Theriault, 1992), chitosanase, b-glucanases, lignin (Notsu *et al*., 1994) and callose (Kauss *et al*., 1989). In addition to direct effects on plant nutrition and plant growth stimulation, chitin-derived products have also been shown toxic to plant pests and pathogens, induce plant defenses and stimulate the growth and activity of beneficial microbes. Chitin exhibited bacteriostatic effect on Gram-negative bacteria, *Escherichia coli*, *Vibrio cholerae*, *Shigella dysenteriae* and *Bacteroides fragilis* (Benhabiles *et al*., 2012). The effects of chitin and chitosan on disease incidence and severity of Fusarium yellows of celery showed significant reduction of incidence and severity by pre-plant chitin amendments to soil (Ashley *et al*., 1998). Chitin supplementation improved the biocontrol of the chitinolytic bacteria *Bacillus cereus* and reduced the severity of Botrytis grey mold disease in chickpea (Kishore and Pande 2007). The enzyme purified showed potent activity against the bacterial cultures with maximum inhibition against *Micrococcus luteus Staphylococcus aureus* and *Salmonella abony* (Alam and Mathur, 2014a) and fungi such as *Aspergillus niger* and *Candida albicans.* (Alam and Mathur, 2014b). As such, antimicrobial activity of chitinase, chitin, chitosan and chito-oligosaccharides was well established. However similar studies were not conducted in the mulberry cropping system. Since soil borne diseases of mulberry are a serious concern among sericulture farmers, soil amendment with eco-compatible material like chitosan may be an alternative to harmful chemicals. The present study has been conducted to evaluate chitosan for its mycotoxic effect to few soil borne pathogens of mulberry.

Materials and methods

Isolation and purification of the pathogens associated with soil borne diseases of mulberry

Soil borne pathogens were isolated from root samples of mulberry infected with diseases. The infected plants were up rooted and the roots were carefully cut from the stump and were washed in running water in the laboratory to remove the adhered soil particles and again washed with sterile distilled water thoroughly. The rotted roots were cut into bits of 0.5 mm in sterile condition. The bits were placed in sterile petri-plates and then subjected to surface sterilization with 0.1% $HgCl_2$ solution for one minute and thoroughly washed with sterile distilled water and blotted with sterile tissue paper. The Potato Dextrose Agar (PDA) medium was prepared adding 39 ml PDA powder (Himedia) in 1 liter water followed by sterilizing at 120°C and 15psi. The molten PDA was poured in 9 mm diameter petri-plates and allowed to cool under UV in a laminar flow chamber. Bits of roots were then transferred to the petri-plates containing solid medium. The petri-plates were then sealed and incubated in a BOD incubator for seven days at 28±2°C. The fungal colonies formed in the medium were sub-cultured. The pure cultures were then studied under microscope and based on the cultural and morphological characters of

the fungi were identified as *Fusarium solani, Fusarium oxysporum, Rhizoctonia bataticola* and *Botryodiplodia theobromae.* The pure cultures of these fungi were sub cultured and maintained for further studies.

Preparation of chitosan stock solution

Chitosan solution was prepared according to the method described by Borges Jr. *et al*. (2000) and Palma-Guerrero *et al*. (2008). Purified Chitosan powder (Sigam Aldrich) was weighed accurately 100-500 ppm and dissolved in 0.25 N HCl under continuous stirring. The pH of the medium was adjusted to 5.6 and the solution was autoclaved at 120°C and 15 lb/inch² for 20 minutes.

Effect of chitosan against pathogens associated with root rot disease of mulberry

In vitro evaluation of chitosan was done in solid and liquid media

The growth of fungi in chitosan amended medium was assessed separately for each fungus. Prepared 60 ml PDA in 200 ml conical flasks and sterilized in an autoclave at 121°C for 15 psi for 15 minutes. Different concentrations of chitosan stock solution was pipetted out and added to conical flasks containing sterilized PDA media and mixed well. These were then poured in three (20 ml each) petri-plates. After solidification under UV, seven days old culture of fungi were taken and made into 0.5 cm diameter discs using a cork borer and these bits were placed in the petri-plates containing PDA amended with chitosan in various concentrations (100, 200, 300, 400 and 500 ppm). A control also maintained without amending chitin for comparison. Three replications were kept against each concentration and control for each fungus. The petri-plates were then incubated at 28±2°C in a BOD incubator. The plates were observed in an interval of 5th and 10th day after inoculation and the radial growths were measured. The inhibition (%) was calculated following the formula:

$$\text{Inhibition (\%)} = \frac{\text{Growth in control- Growth in treatment}}{\text{Growth in control}} \times 100$$

In case of liquid media, fungi were grown in liquid PDB (Hi media) in 200 ml conical flasks. Each conical flask was added with 100 ml PDB and sterilized. Chitosan stock solution was pipetteed out and made different concentrations in conical flasks containing PDB media. Seven days old culture of root rot associated fungi (*F. solani, F. oxysporum, R. bataticola* and *B. theobromae)* were taken and made into 0.5 cm diameter discs using a cork borer and these bits were transferred into the conical flasks containing PDB media amended with different concentrations of chitosan in sterile condition. The PDB media inoculated with test fungi without adding chitosan served as control. Three replications were maintained against each concentration and control for each fungus. The whole set was incubated at 28±2℃ for 15 days in BOD incubator. After incubation the conical flasks was observed for sporulation and mycelia growth of the organisms. The flask containing media along with the culture was filtered using Whatman filter paper. The filter paper containing mycelia was allowed to dry in an oven for 5 hrs at 60℃. The filter paper containing mycelia was weighed and deducted the weight of Whatman filter paper to get the weight of mycelia. The sporulation was graded visually as high (+++), medium (++) and low (+) based on spore concentration.

The data were subjected for analysis of variance (ANOVA) and the means were compared for significant difference.

Results and discussion

Chitosan significantly ($P<0.01$) reduced the growth of *F.solani* in solid medium. Average reduction in growth was found higher with increase in concentration with

maximum reduction in 500 ppm (44.47 mm) and least (25.47 mm) in 100 ppm with average inhibition of 65.57% and 40.46% respectively against untreated control. Similarly, there was a significant variation in growth of the fungi between days after inoculation with average growth of 19.26 mm and 29.41 mm respectively at 5 and 10 days after inoculation. There was significant interaction between chitosan concentration and days after inoculation (Table 1). The growth of fungi was slow in the medium amended with chitosan. The mycelia biomass reduced with the increase in concentration.

Concentrations of chitosan significantly inhibited the growth of *F. oxysporum*. The concentration of chitosan directly influenced growth of the fungi with least growth in 500 ppm (17.08 mm) and higher n 100 ppm (29.50 mm). However, the growth was maximum (44.47 mm) in untreated control. The inhibition of the fungi was higher (57.60%) in 500 ppm and low (27.82%) at 100 ppm compared with the control. There was significant variation in growth of the fungus with 18.61 mm and 34.03 mm respectively 5 and 10 days after inoculation.

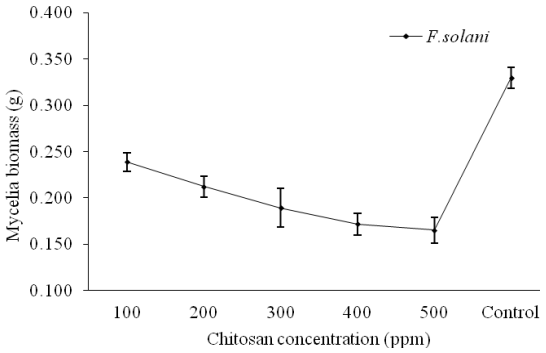

Fig. 1: Effect of various chitosan on mycelia production of *F. solani*.

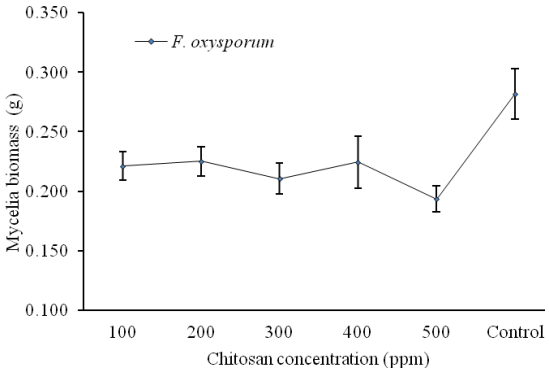

Fig. 2: Effect of various chitosan on mycelia production of *F. oxysporum*.

Table 1: Effect of chitosan on growth of *F. solani* in solid medium.

Chitosan Conc. (ppm)	5- days after inoculation		10- days after inoculation		Average	
	Mycelia growth (mm)	Inhibition (%)	Mycelia growth (mm)	Inhibition (%)	Growth (mm)	Inhibition (%)
100	21.4±0.2	32.57	29.6±0.1	48.35	25.47	40.46
200	19.3±0.1	39.05	26.4±0.2	53.78	22.89	46.42
300	17.1±0.2	46.23	23.6±0.5	58.83	20.31	52.53
400	14.5±0.2	54.29	21.1±0.3	63.10	17.81	58.70
500	11.6±0.3	63.57	18.6±0.2	67.57	15.06	65.57
Control	31.7±0.1	0.00	57.2±0.3	0.00	44.47	0.00
Mean	19.26		29.41			

CD P<0.01: Trt - 0.55; Days - 5.04; Trt x Days - 0.05

Table 2: Effect of chitosan on growth of *F. oxysporum* in solid medium.

Chitosan Conc. (ppm)	5- days after inoculation		10- days after inoculation		Average	
	Mycelia growth (mm)	Inhibition (%)	Mycelia growth (mm)	Inhibition (%)	Growth (mm)	Inhibition (%)
100	3.03±0.03	12.80	5.36±0.04	7.21	4.19	10.01
200	2.82±0.02	18.88	5.07±0.05	12.21	3.94	15.55
300	2.36±.03	32.00	4.82±0.03	16.63	3.59	24.32
400	2.13±0.01	38.56	4.04±0.01	30.10	3.09	34.33
500	1.67±0.03	52.00	3.56±0.01	38.46	2.61	45.23
Control	3.47.03	0.00	5.78±0.01	0.00	4.63	0.00
Mean	18.61		34.03			

CD P<0.01: Trt- 0.54; Days – 5.06; Trt x Days - 0.79

Table 3: Effect of chitosan on growth of *R. bataticola* in solid medium.

Chitin Conc. (ppm)	5- days after inoculation		10-days after inoculation		Average	
	Mycelia growth (mm)	Inhibition (%)	Mycelia growth (mm)	Inhibition (%)	Growth (mm)	Inhibition (%)
100	78.50±0.16	1.87	81.66±0.09	6.49	80.08	4.18
200	75.50±0.09	5.62	77.66±0.17	11.06	76.58	8.35
300	53.50±0.44	33.12	58.50±0.19	33.01	56.00	33.07
400	43.00±1.68	46.25	50.83±0.76	41.79	46.92	44.02
500	19.33±0.63	75.83	37.50±0.25	57.061	28.42	66.45
Control	80.00±0.01	0.00	87.33±0.34	0.000	83.67	0.00
Mean	58.31		65.58			

CD P<0.01: Trt - 7.34; Days - NS; Trt x Days - 9.54 (NS= Not significant)

In the liquid medium, the fungi showed slow growth in presence of chitosan. The biomass of mycelia was least at 500 ppm (0.193 g) which was 31.36% less than the fungal mass observed in untreated control. Sporulation was high (+++) in control and fungi failed to sporulate in higher concentrations of chitosan.

Various concentrations of chitosan influenced on the growth of *R.bataticola* significantly. Minimum growth was observed at higher concentration of chitosan with 66.45% inhibition followed by 44.02% and 33.07% at 400 ppm and 300 ppm respectively compared with control. The inhibition at 100 ppm and 200 ppm was <10 and were not varied significantly. Though there was reduction in fungal growth in days after inoculation, significant reduction between the days after inoculation was not observed while interaction between days after inoculation and concentration of chitosan was significant. Different concentrations of chitosan suppressed the production of mycelia of *R.bataticola* (Fig.3) in liquid medium with least growth at 500 ppm (0.302 g) and maximum (0.363 g) in control. The fungi sporulated in all cases with low (+) in 400 ppm and 500 ppm, medium (++) in 200 ppm and 300 ppm while high in control (+++).

Regarding *B.theobromae*, chitin significantly suppressed growth of the fungus with significantly less mean growth at 500 ppm (14.02 mm) and higher growth was found in control (73.00 mm).

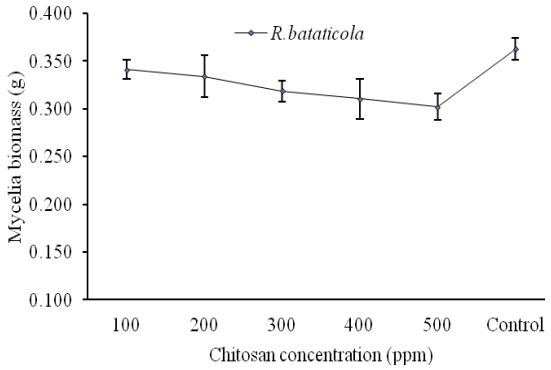

Fig. 3: Effect of various chitosan on mycelia production of *R. bataticola*.

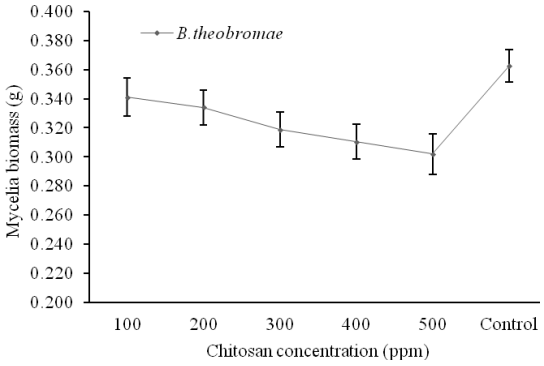

Fig. 4: Effect of various chitosan on mycelia production of *B. theobromae*.

The suppression of growth was 81.10% at 500 ppm chitosan compared with untreated control. The growth of fungus also significantly varied with days after inoculation with higher growth in 10 day after inoculation as well as interaction between treatments and days. The mycelia production was least (0.302 g) at 500 ppm and the fungus could not sporulate. However, in 100 and 200 ppm the fungus showed medium (++), while in 300 and 400 ppm low (+) sporulation (Fig. 4).

In the present study, we investigated the effect of chitosan in controlling soil borne pathogens of mulberry in *in vitro*. The growth rate of *F. solani, F. oxysporum, R. bataticola* and *B. theobromae* was reduced in PDA amended with chitosan at 100-500 ppm concentrations in various degrees. There was a significant influence of chitosan on growth of pathogens. A significant suppression of growth was observed in high concentration of chitosan. Also the chitosan reduced mycelia production in the liquid medium due to chitosan interruption of chitosan, fungi failed to sporulate normally. This shows the interference of chitosan on the normal metabolic and reproductive activities of these fungi.

Table 4: Effect of chitosan on growth of *B.theobromae* in solid medium.

Chitin Conc. (ppm)	5- days after inoculation		10-days after inoculation		Average	
	Mycelia growth (mm)	Inhibition (%)	Mycelia growth (mm)	Inhibition (%)	Growth (mm)	Inhibition (%)
100	31.33±0.09	45.66	56.33±0.54	36.22	43.83	40.95
200	29.33±0.63	49.13	50.50±0.29	42.83	39.92	45.98
300	22.16±0.17	61.56	45.66±0.28	48.30	33.92	54.93
400	18.50±0.16	67.91	30.33±0.16	65.66	24.42	66.79
500	9.83±0.16	82.95	18.33±0.17	79.24	14.08	81.10
Control	57.66±0.25	0.00	88.3±30.12	0.00	73.00	0.00
Mean	28.14		48.25			

CD P<0.01: Trt - 0.70; Days - 6.40; Trt x Days - 0.99

Effects of chitosan derivatives against bacterial and fungal strains have been reported (Liman *et al*., 2011), and the inhibition was correlated with its concentration (El-Ghaouth *et al*., 1992). Chitosan derivative has been widely used to reduce the growth of *Rhizoctonia* sp. (Elmer and LaMondia, 1994). In the present study the effectiveness of chitosan was found depending on the concentration as the effectiveness was increased with the dose of chitosan. In earlier reports, many workers found chitosan have inhibitory effects on several plant pathogenic fungi including different species of *Fusarium* (Kim *et al*., 2016; Stamford *et al*., 2009). Bell *et al*. (1998) found inhibition of radial growth of *F. oxysporum* f.sp. *apii* on PDA plates amended with different concentrations of chitosan where the inhibition was progressively higher with concentrations. The present study showed antifungal properties of chitosan against all the fungal pathogens studied.

The chitosan activity against fungus is assumed to be fungistatic rather than fungicidal with a potential to communicate regulatory changes in both the host and fungus (Raafat *et al*., 2008) The antifungic mechanism of chitosan involves cell wall morphogenesis with chitosan molecules interfering directly with fungal growth (El-Ghaouth *et al.,* 1992b). Antifungal activity of chitosan in the present study may be attributed to the interaction with outer cellular components, and cytoplasmic membrane or with cytoplasmic constituents. The mechanisms underlying the antimicrobial activity of chitosan have only been studied comparatively recently and the amount of information available is limited, although increasing. Several studies purport to have identified such mechanisms; but only few were supported by experimental evidence.

In mulberry, soil borne diseases are very serious since it causes large scale mortality of plants. In diseases like root rot, due to the microbial complex, control of the disease is very difficult unless the controlling chemicals targets all the organisms associated with the disease. Though several chemical control methods are available these methods could not successfully contain soil borne diseases. The increasing environmental awareness and cost of chemical fungicides makes the farmers reluctant to resort hazardous chemicals for plant protection. The chitosan which showed effectiveness against all the pathogens in this study is organic in nature, nontoxic and economical and hence could be explored its antifungal potential as an eco-compatible material for management of soil borne diseases in mulberry.

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