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FZ performed the experiment, analyzed data, and prepared first draft of the manuscript; MZS wrote and revised the manuscript; MFRKP performed experiment; MAMA supervised the experiment; AK, MR wrote and edited the manuscript; MTI conceived and coordinated the project and edited the manuscript

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ORIGINAL RESEARCH PAPER

Chitosan biostimulant controls infection of cucumber by *Phytophthora capsici* through suppression of asexual reproduction of the pathogen

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Abstract
The biopolymer chitosan is a derivative of chitin, which can promote plant growth and protect plants from phytopathogens. This study aimed to evaluate the efficacy of chitosan as a biostimulant and a biorational agent to protect cucumber plants from damping-off disease caused by *Phytophthora capsici*. Cucumber seeds were treated with a range of chitosan concentrations, viz. 0, 125, 250, and 500 ppm, to evaluate effect on seed germination and fresh root and shoot weight of the seedlings. Chitosan significantly ($p \leq 0.05$) enhanced seed germination and root and shoot growth of cucumber in a dose-dependent manner up to 500 ppm. Application of in vitro chitosan suspension onto *P. capsici* mycelial plug suppressed growth of mycelia, formation of sporangia, and release of *P. capsici* zoospores at 125–500 ppm concentrations. Cucumber seedlings from chitosan-treated seeds showed enhanced resistance to damping-off disease caused by *P. capsici* compared to untreated control. Cucumber seedlings from 500 ppm chitosan seed treatment showed 100% disease resistance against damping off caused by *P. capsici*. These results suggest that chitosan could be used as a natural and environmentally safe alternative to a synthetic growth promoter and pesticide for sustainable production of cucumber.

Keywords
phytopathogen; damping-off; zoosporogenesis; biorational disease control

Introduction

Chitosan is the deacetylated derivative of chitin. The major sources of chitin are ciliates, amoebae, chrysophytes, some algae, yeasts, and the lower animals like crustaceans, worms, insects, and mollusks [1]. The biopolymer chitosan is the most abundant natural compound with a high capacity of controlling phytopathogens by plasma membrane permeabilization [2–4], preventing their growth and sporulation, decreasing spore viability and germination, and disrupting their cell wall. Chitosan's mode of action also includes induction of different defense mechanisms in host plants by up- or downregulating different biochemical pathways during plant–pathogen interactions. It is also a suitable product in sustainable agriculture due to its biodegradability, nontoxicity to environment, and biocompatibility [5]. Chitosan functions as an elicitor of plant defense response through activation of multiple beneficial metabolic pathways that may eventually contribute to enhanced yield [6]. Previous studies on chitosan reported

considerable positive effects, including plant growth promotion, production of different bioactive secondary metabolites, photosynthesis, activity of various enzymes via protein phosphorylation, generation of reactive oxygen species, biosynthesis of jasmonic acid, and expression of defense responsive genes in abiotic stress conditions [7,8].

Phytophthora capsici Leonian is a notorious phytopathogen, which commonly affects cucumber, pepper, tomato, and many other horticultural crops worldwide [9]. It infects the host plants primarily through asexually produced zoospores from the sporangia under favorable environmental condition. The motile biflagellate zoospores locate the host plants through chemotaxis to initiate infection. Therefore, production of sporangia, release of zoospores from sporangia (zoosporogenesis), and motility of the zoospores are three key asexual life stages, which are key determinants for pathogenesis and resultant disease severity on host by this notorious soilborne phytopathogen [10]. Disruption of any of these life stages eliminates the possibility of infection [11]. The reliability and efficacy of control of *Phytophthora* diseases even with modern fungicides are variable and often insufficient. Moreover, fungicide treatments are expensive and frequent fungicide use is deleterious to the environment, and may also lead to development of resistance in a pathogen population [12]. Cucumber is an economically important crop, which is susceptible to damping-off disease caused by *P. capsici*. Novel approaches are required to protect cucumber from the infection by the *P. capsici* pathogen. Several lines of evidence suggest that probiotic bacteria like *Pseudomonas*, *Bacillus*, *Burkholderia*, etc., are effective in controlling *P. capsici* [9,13]. But scant information is available on the effect of chitosan biopolymer application on *P. capsici* growth and disease control in cucumber. It is therefore important to determine the effect of chitosan on the growth and development of critical asexual differentiations such as sporangia formation, zoosporogenesis, and motility of *P. capsici*.

The objectives of this study were to evaluate (***i***) inhibitory effects of chitosan on production of sporangia, zoosporogenesis, and motility of *P. capsici* zoospores; (***ii***) effect of chitosan on seed germination and growth of cucumber seedlings; and (***iii***) effective dose of chitosan for control of *P. capsici* infection in cucumber.

Material and methods

Sources of chitosan and *P. capsici* isolate

Practical grade chitosan ($C_{12}H_{24}N_2O_9$) biopolymer (poly β-1,4-D-glucosamine) available in powder form was purchased from Sigma (Sigma-Aldrich, CAS No. 9012-76-4). It was commercially prepared by alkaline deacetylation of chitin obtained from shrimp shells (*Pandalus borealis*). The degree of deacetylation was ≥75% with low viscosity. The strain of *P. capsici* was kindly provided by Professor Dr. W. Yuanchao of Nanjing Agricultural University, China, which was isolated from the *Capsicum annuum* cultivated agricultural soil of Nanjing, China. This micro-organism was preserved in sterilized water at room temperature and cultured on V8 juice agar media on a regular basis.

Effect of chitosan on sporangia development, zoosporogenesis, and motility of zoospores

For sporangia development, the *P. capsici* isolate was maintained on 30 mL sterile Carrot Piece Agar (CPA) media in 90-mm Petri dishes at 25°C in the dark. Colonies were regularly subcultured by transferring a plug of hyphal tips into a fresh agar plate and incubating it in the same environmental condition mentioned above. To induce sporangia formation, agar cultures from 2-week-old Petri dishes were cut into pieces, covered with sterile distilled water, and kept in Petri dishes at 25°C in the dark [9,13]. To induce zoospore release, cultures with sporangia were placed at 4°C for 30 min, and then incubated at room temperature for another 30 min. The sporangia and release of zoospores were checked under a compound light microscope and photos were taken using a digital camera attached to the microscope. Zoospores remained motile for 10–12 h in sterile water, during which time they were used for bioassays.

Production of sporangia and zoosporogenesis of *P. capsici* under varying concentrations of chitosan (0, 125, 250, and 500 ppm) were assessed every 15 minutes after the treatment [13,14].The motility behavior and viability of zoospores were tested by the "homogenous solution method" [13–15]. Briefly, 40 µL of chitosan suspension was directly added to 360 µL of zoospore suspension (3×10^5 mL^{-1}) placed in a multidish. The motility of zoospores for each chitosan concentration was observed under a compound light microscope at 40× magnification and data were recorded compared to the control suspensions (zoospore suspensions in sterile distilled water or with 2% DMSO). Quantification of changes in motility and lysis of zoospores in time-course was carried out as described earlier [16]. Data are expressed as percentage of zoospores released (calculated as a proportion of the control value, 100%), percentage inhibition of motility (of the tested zoospores) and percent lysis, and are shown as mean values ± standard error of the mean. The experimental design was completely randomized with five replicates per treatment and each bioassay was repeated three times. Data from five replications were subjected to analysis of variance and separation of means as shown in the data analysis section.

Effect of chitosan on seed germination and root and shoot length of cucumber

To test growth promoting effects of chitosan on cucumber, four concentrations of chitosan were prepared, viz. 0, 125, 250, and 500 ppm [7]. One gram (ca. 30 seeds) of cucumber seeds were weighed and surface sterilized following a standard protocol for each replicate of each chitosan concentration [17]. All seeds were soaked in four varying concentrations (0, 125, 250, and 500 ppm) of chitosan suspension for an hour and then dried overnight at room temperature. The chitosan treated seeds (20 seeds per dish) were placed in 9-cm Petri dishes containing sterilized water soaked filter paper and incubated at 25°C at 95% relative humidity for germination to occur. The germination percentage of the seeds was calculated at day 7 by using the following formula [17]:

$$Germination\ (\%) = \frac{Number\ of\ seeds\ germinated}{Total\ number\ of\ seeds\ sown} \times 100\%$$

After germination, the root length and shoot length of the seedlings were measured compared with the untreated control. Five replications were maintained for each treatment for evaluating growth promoting effects of chitosan on cucumber.

Suppression of *P. capsici* disease in cucumber by chitosan

A pot experiment was conducted in the net house of the Department of Plant Pathology of Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh, to assess the efficacy of chitosan on suppression of disease caused by *P. capsici* in cucumber. Soil was collected from the experimental plot of the Department of Plant Pathology and sterilized twice in an autoclave at 121°C under 1.1 kg/cm^2 pressure for 40 min. Soil was mixed thoroughly between autoclaving and laid in a thin layer on the tray for efficient sterilization. Surface sterilized seeds of cucumber 'Baromashi' (Lal Teer Seed Co. Ltd.) were dipped into 0, 125, 250, and 500 ppm chitosan suspension followed by sowing in plastic pots (10 × 5 cm size) containing 200 g of sterilized soil (one seed/pot). After germination, seedlings were inoculated with 500 mL *P. capsici* zoospore suspension (ca. 1×10^6/mL) by injecting into the rhizosphere [13]. Ten replications were maintained for each treatment. After inoculation, the seedlings were incubated overnight in a humid chamber to facilitate infection. Seven days after inoculation, number of infected seedlings and percent of disease suppression were calculated by using the following formula [17]:

$$Protection\ (\%) = 100 - Plant\ disease\ incidence\ (\mathrm{PDI})$$

where:

$$PDI = \frac{Number\ of\ infected\ plants}{Total\ number\ of\ seeds\ sown} \times 100\%$$

Statistical analysis

Data obtained from different replicates and experiments as percent (%) were transformed using angular transformation (arcsine of square-rooted value) prior to the analysis using SPSS version 15 software and back transformed data are presented. Means were separated by using Fisher's protected least significant difference test at $p \leq 0.05$.

Results

Chitosan suppresses development of *P. capsici* sporangia

To assess the effect of chitosan on production of sporangia, we tested four doses of chitosan. Production of sporangia from *P. capsici* mycelia significantly reduced within 15 minutes of chitosan treatment (Fig. 1). The relative percentage of sporangia formation was severely affected by higher doses of chitosan. No significant differences in the relative sporangia production were observed at 125 ppm chitosan compared to 0 ppm or untreated control. Sporangia development process in *P. capsici* was completely inhibited by chitosan at 500 ppm (Fig. 1).

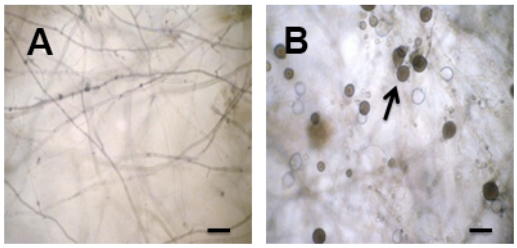

Fig. 1 Reduction of sporangial development by chitosan application: (**A**) 500 ppm chitosan and (**B**) 0 ppm chitosan (control). Photographs were taken 3 days after chitosan treatment.

Zoospore production

Chitosan application significantly reduced the release of zoopores from the sporangia of *P. capsici* in a dose dependent manner (Fig. 2). The lowest relative percentage of released zoospores was found in the Petri dishes treated with 500 ppm of chitosan (Fig. 2). Apparently, 50% of the zoospore production was suppressed by the application of 250 ppm chitosan. Chitosan treatment at 125 ppm reduced about 20% of zoospore release compared to untreated control (0 ppm chitosan).

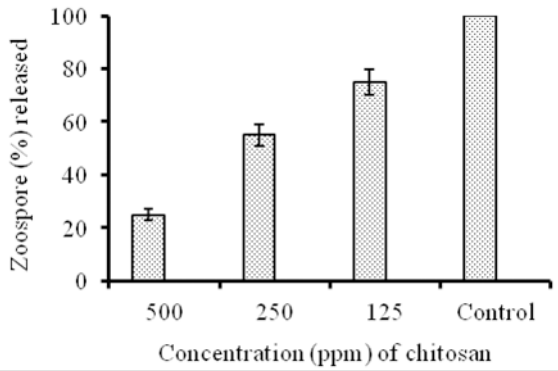

Fig. 2 Relative percentage of zoospores released from the sporangia of *P. capsici* in the presence of three different concentrations of chitosan along with control. The error bars represent the standard errors. Means within the bars followed by the same letter are not significantly different as assessed by Tukey's HSD post hoc test ($p \leq 0.05$).

Reduction of zoospore motility

To determine whether chitosan can affect motility and swimming behavior of *P. capsici* zoopores, we evaluated three doses (125, 250, and 500 ppm) of chitosan using the homogeneous solution method. Chitosan at 500 ppm remarkably impaired the motility of zoospores and most of the affected zoospores settled at the bottom of the multi-dish (Fig. 3). Microscopic observation revealed that the affected zoospores swam very slowly and finally stopped moving and transformed into round cystospores within 30 minutes of the treatment. However, *P. capsici* zoospores in nontreated control dishes displayed the characteristic swimming for several hours.

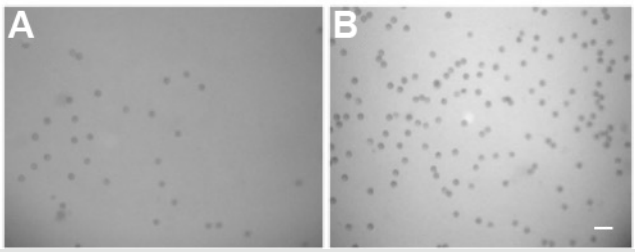

Fig. 3 Halting of zoospores by chitosan application: (**A**) control and (**B**) 500 ppm chitosan treatment.

Chitosan increased seed germination and enhanced growth of cucumber

To assess whether chitosan has any effect on seed germination and growth of cucumber seedlings, we evaluated four doses of chitosan including 0 ppm or nontreated control. Chitosan application significantly increased

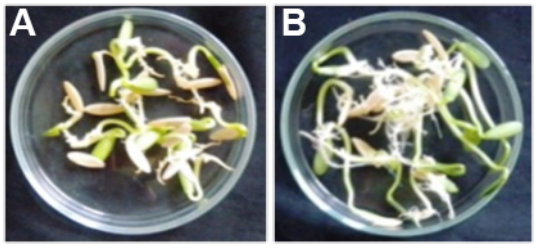

Fig. 4 Cucumber seed germination and seedling growth enhancement by chitosan treatment: (**A**) control, (**B**) seeds treated with 500 ppm chitosan.

seed germination and promoted growth of cucumber seedlings (Fig. 4). Hundred percent (100% ±0%) of cucumber seeds germinated when seeds were treated with chitosan at higher doses of 250 and 500 ppm (Fig. 4). A slightly lower rate of germination percentage (92% ±2%) of seeds was obtained at 125 ppm of chitosan. On the other hand, only 85% ±3.4% seeds germinated in untreated control. Both shoot and root growths of the seedlings were also significantly increased by the treatments of chitosan (Fig. 5). The highest shoot (2.06 g) weight was recorded when cucumber seeds were treated with 500 ppm chitosan, followed by treatment with 250 ppm (1.589 g) and control (1.535 g). The lowest shoot weight was recorded in 125 ppm (1.287 g) chitosan treatment. Similarly, the highest root (0.109 g) weight was recorded when cucumber seeds were treated with 500 ppm chitosan, followed by 250 ppm (0.030 g) and 125 ppm (0.0160 g) chitosan treatment. The lowest root weight was recorded in control (0.012 g) treatment.

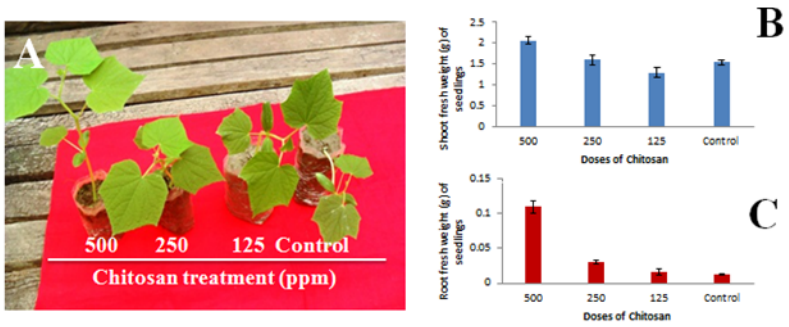

Fig. 5 Effect of varying doses (0, 125, 250, 500 ppm) of chitosan on vegetative growth of cucumber: (**A**) whole plant growth, (**B**) shoot fresh weight (g), and (**C**) root fresh weight (g). The error bars represent the standard errors of the means. Means within the bars followed by the same letters are not significantly different as assessed by Tukey's HSD post hoc test ($p \leq 0.05$).

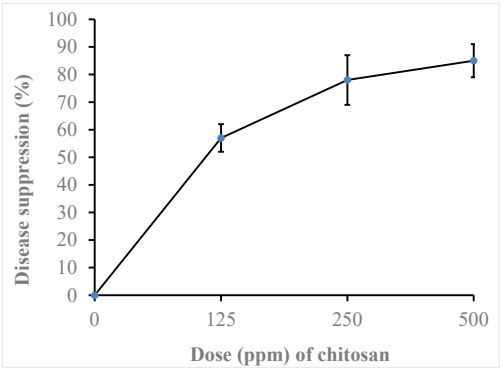

Fig. 6 The suppression of disease caused by *Phytophthora capsici* depending on chitosan dose. The error bars represent the standard errors of the mean. Each treatment was replicated three times.

Phytophthora capsici disease suppression in cucumber by chitosan in vivo

Chitosan application significantly suppressed *P. capsici* infection and disease of cucumber in a dose-dependent manner (Fig. 6). The highest disease suppression (85%) was recorded in the seedlings treated with 500 ppm of chitosan followed by 250 ppm (78%) and 125 ppm (57%). Hundred percent (100%) seedlings died when chitosan untreated cucumber seedlings were inoculated with an equal volume of zoospore suspension. On the other hand, no disease (0%) incidence was observed in control (noninoculated) seedlings not treated with *P. capsici* zoospore suspension.

Discussion

Chitosan is regarded as an eco-friendly natural plant growth promoter and a potential plant protection product due to its unique efficiency in plant growth promotion and plant disease suppression [5,7,18,19]. Chitosan is a low risk plant growth promotion and protection product that does not pose any threat to the environment, humans and health of other mammalians [8]. Thus, any positive effect of chitosan on a specific plant species as growth enhancement or biotic stress alleviator should be considered as a component of integrated pest management and sustainable agriculture. To infect host tissue, *P. capsici* produces biflagellate motile zoospores from

multinucleated sporangia when a favorable environment exists for the infection [16]. In the current study, we demonstrated that chitosan suppressed development of sporangia, release of zoospores (zoosporogenesis), and impaired the motility of *P. capsici* zoospores in a dose-dependent manner. In vivo assay revealed that application of low doses of chitosan as seed treatment also suppressed damping-off disease in cucumber incited by *P. capsici* inoculation on seedlings. Furthermore, chitosan treatments enhanced seed germination and seedling growth of cucumber. Our results indicate that biostimulant chitosan exerts *P. capsici* disease suppression in cucumber through suppression of asexual development and motility of the inoculum. This study for the first time demonstrated that in addition to growth promotion, chitosan biopolymer is a potential natural product for sustainable management of a notorious phytopathogen *P. capsici* in cucumber. In an earlier study, researchers found that chitosan caused disruption of the endomembrane systems of zoospores, especially the integrity of the vacuoles of *P. capsici* [19]. Oligochitosan-coated silver nanoparticles (OCAgNPs) (9 ppm) strongly inhibited mycelial growth, sporangium production, zoospore release and zoospore germination of *P. capsici*, *P. nicotianae*, and *P. colocasiae* [20]. Earlier investigators also suggested that mycelia growth, sporulation, elongation, and germination of spores of *P. capsici* were effectively suppressed by the application of chitosan [5,21,22]. Reports also revealed that inhibition of mycelial growth decreased with the increase of chitosan concentration [23]. In our study, low to moderate doses (125–500 ppm) of chitosan remarkably affected the asexual life stages of the *P. capsici* and suppression of the disease in cucumber. Suppression of damping-off disease in cucumber by 500 ppm of chitosan treatment was 85% on inoculated plants, which is highly significant as 100% nontreated plants damped-off. Interestingly, when we primed cucumber seeds with plant probiotic bacteria such as *Lysobacter oryzae*, *L. capsici* or *Pseudomonas* spp. before chitosan treatment, the efficiency of disease suppression by chitosan at only 250 ppm rose to 100% (data not shown). These findings indicate that chitosan could be used with biocontrol bacteria and an additive and consistent biocontrol of the disease could be achieved.

In the current study, the highest germination and seedling growth of cucumber were obtained when seeds were treated with 500 ppm chitosan solution followed by 250 ppm and 125 ppm. Seed priming with chitosan alters the permeability of seeds' plasma membrane, enhances peroxidase, catalase, tyrosine ammonia lyase activity, and increases antioxidant activity [1,24]. Germination and growth rate of maize, nuts, fruits, and many other vegetable seeds increased by the application of chitosan [7,18,24–26]. Earlier studies also reported that foliar application of chitosan enhanced growth and development as well as yield of strawberry [7,18]. Development of diseases caused by *P. infestans* [23,27,28], *P. cactorum* [29], and *P. capsici* [29] on other crop plants has been suppressed by soil amendment and/or spraying chitosan, corroborating the findings of our current study. We recently demonstrated that chitosan not only can boost growth and development of plant but also enhances the content of antioxidants in strawberry [7].

Taken together, our results indicate that application of low doses of chitosan suppressed damping-off disease in cucumber caused by *P. capsici* likely through suppression of asexual development of the pathogen. To the best of our knowledge, this study also for the first time demonstrated an effective concentration of chitosan that might be used as a biopesticide for controlling the devastating cucumber damping-off disease caused by *P. capsici*. Further studies are needed to elucidate precise underlying mechanisms of disease suppression and growth promotion of cucumber by chitosan before recommending this biopolymer as an agent for promoting eco-friendly and sustainable production of cucumber.

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Biostymulant chitozan kontroluje zakażenie ogórka przez *Phytophthora capsici* poprzez hamowanie bezpłciowego rozmnażania patogenu

Streszczenie

Biopolimer chitozan jest pochodną chityny, może on stymulować wzrost roślin i chronić je przed fitopatogenami. Celem badań była ocena skuteczności chitozanu jako biostymulatora i „bioracjonalnego" środka ochrony ogórka przed zgorzelą wywołaną przez *Phytophthora capsici*. Nasiona ogórka poddano działaniu różnych stężeń chitozanu tj. 0, 125, 250 i 500 ppm w celu oceny ich wpływu na kiełkowanie nasion oraz świeżą masę korzeni i pędów siewek. Chitozan istotnie ($p \leq 0{,}05$) stymulował kiełkowanie nasion oraz wzrost korzeni i pędów ogórka w sposób zależny od dawki do 500 ppm. Zastosowanie zawiesiny chitozanu w zakresie stężeń 125–500 ppm w warunkach in vitro hamowało wzrost grzybni *P. capsici*, tworzenie sporangiów i uwalnianie zoospor *P. capsici*. Siewki roślin uzyskanych z nasion traktowanych chitozanem charakteryzowały się zwiększoną odpornością na zgorzel wywołaną przez *P. capsici* w porównaniu z nietraktowanych chitozanem. Siewki ogórków z nasion traktowanych 500 ppm chitozanu wykazywały 100% odporność na zgorzel powodowaną przez *P. capsici*. Wyniki te sugerują, że chitozan może być stosowany jako naturalna i bezpieczna dla środowiska alternatywa dla syntetycznych stymulatorów wzrostu oraz pestycydów w zrównoważonej produkcji ogórka.