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CHITOSAN, A RENEWABLE BIOPOLYMER, AS A MODULATOR OF PHYTOPATHOGENIC FUNGAL GROWTH

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Abstract

Fungal pathogens from genera such as *Alternaria, Aspergillus, Macrophomina*, and *Sclerotium* inflict significant annual losses on major crops globally. This study delved into the effects of varying concentrations (0.05 to 0.25%) of chitosan on the growth of four phytopathogenic fungi namely *Alternaria alternata, Aspergillus fumigatus, Macrophomina phaseolina,* and *Sclerotium rolfsii* using the poison food technique. Results unveiled a substantial decrease in fungal growth with increasing chitosan concentration, indicating a linear relationship between chitosan concentration and fungal growth inhibition. Fungal mycelium colonization of the chitosan-amended medium occurred within 7 days at lower concentrations (0.05%), while medium (0.10 and 0.15%) and higher concentrations (0.20 and 0.25%) necessitated a prolonged duration (18-22 days) compared to the control. Minimum inhibitory concentration (MIC) values for chitosan were determined at 0.20% for *A. alternata*, *A. fumigatus*, and *S. rolfsii*, whereas concentration exceeding 0.25% was required to completely inhibit the growth of *M. phaseolina*. Based on the principal component analysis (PCA), the fungi were grouped based on susceptibility, with *A. alternata* and *S. rolfsii* showing a stronger response followed by *A. fumigatus* and *M. phaseolina*, while 0.20 and 0.25% chitosan concentrations exhibited similar effects on growth inhibition. The findings of this study will contribute valuable insights into the potential utility of chitosan as an antimycotic agent against these pathogenic fungi.

Key words: Chitosan; Fungistatic; Fungicidal; Phytopathogenic fungi

Introduction

Phytopathogenic fungi, including those from the genera *Alternaria*, *Aspergillus*, *Macrophomina*, and *Sclerotium*, are formidable adversaries to global crop productivity. Their detrimental impact extends beyond mere yield reduction, as they disrupt the morphological growth and physiological-chemical attributes of crops, posing substantial challenges to agricultural sustainability and food security efforts worldwide (Shoaib *et al*., 2019, 2021, 2023; Riaz *et al.,* 2023). Among these fungi, *Alternaria alternata* stands out for its filamentous nature and spoilage capabilities have garnered significant attention, as this pathogen has been implicated in affecting over 380 host species of plants worldwide. Its pervasive presence across diverse crops has led to substantial financial losses and raised concerns regarding seed quality on a global scale (Costa *et al*., 2019). Studies have revealed that *A. alternata* employs a range of virulence mechanisms, including the utilization of cell wall-degrading enzymes and appressoria, to maximize its pathogenic impact (Ma *et al*., 2019). Moreover, *A. alternata* is notorious for its ability to produce secondary metabolites, which can serve as both phytotoxins and mycotoxins. This dual role raises significant concerns about food safety and underscores the importance of vigilant monitoring the agricultural production and food processing chain (Shoaib *et al*., 2021).

As a plant pathogen, *Aspergillus fumigatus* poses a unique challenge due to its versatile nature and remarkable adaptability. It is primarily known for its role as a human pathogen, particularly in causing respiratory infections, while it also has the ability to infect plants under certain conditions. This fungus can penetrate plant tissues break down plant defenses and make itself at home leading to various diseases such as root rot, seedling damping-off, and fruit rot. Its resilience to environmental stresses, including temperature fluctuations and acidic conditions, further enhances its effectiveness as a plant pathogen (Paulussen *et al*., 2017).

Furthermore, soil borne pathogens viz., *Macrophomina phaseolina*, threatens over 750 plant species, causing stalk rot and charcoal rot diseases (Shoaib *et al*., 2022), while *Sclerotium rolfsii,* ubiquitously distributed globally is known to infect more than 500 species (Yousaf *et al*., 2023). Both fungal pathogens forms heat-tolerant sclerotia capable of enduring in soil or root debris for 2–15 years (Khan *et al*., 2018). Upon decomposition of host tissues, these sclerotia are released in clusters near the soil surface, effectively colonizing both living and dead tissues. The remarkable durability of its sclerotia against biological and chemical degradation is attributed to their melanized cuticle (Bidima *et al*., 2021). Thriving in moist soil within a temperature range of 25 to 30 °C, this pathogen poses a formidable challenge for control due to its prolific growth and the extensive production of numerous sclerotia (Shoaib *et al*., 2023).

Despite efforts in cultural, chemical, or biological management, these fungi have proven to be highly resistant (Khan *et al*., 2018). Still chemical fungicides have traditionally been used for disease control (Yuan *et al*., 2023), yet their widespread application has sparked worries about their effects on human health and the environment (Liu *et al*., 2020). In this regard, chitosan, derived from deacetylation of chitin (polysaccharide) is gaining attraction as an eco-friendly alternative to chemical fungicides offering effective solutions while minimizing environmental impact (Maliki *et al*., 2022). Apart from its commendable biodegradability, biocompatibility, cell affinity, blood compatibility, safety, and non-toxic nature, chitosan contributes to various biological activities (Azmana *et al*., 2021). These include antibacterial properties, hemostatic capabilities, antitumor effects, anti-Alzheimer's disease potential, immune system support, wound healing, and other bioactive attributes. Collectively, these features position chitosan as an excellent option for microbial control (Chen *et al*., 2022).

Chitosan has been extensively employed in agriculture on pre- and postharvest treatments of crops to control microbial infections (Lopez-Moya *et al*., 2019). Various studies revealed that chitosan exhibited a profound impact on fungal pathogens such as *Rhizopus stolonifera*, *Botrytis cinerea*, *Fusarium oxysporum* f.sp. *radicis lycopersici*, *Verticillium dahliae* as well as various species of *Alternaria*, *Colletotrichium*, and *Trichoderma* (Zavala-González *et al*., 2016; Verlee *et al*., 2017; Lopez-Moya *et al*., 2019). It disrupts their germination and hyphal morphology, setting the stage for inhibiting the growth, while the sensitive fungi experience plasma membrane permeabilization through chitosan-induced oxidative stress leading to cell demise (Huang *et al*., 2021). Thus, the in the current study, effect of varying concentrations of chitosan was checked on mycelial growth and tolerance limits of four notorious phytopathgens including *A. alternata*, *A. fumigatus*, *M. phaseolina*, and *S. rolfsii*.

Materials and Methods

Chitosan powder with a deacetylation degree exceeding 90% was procured from Chemsavers. Acetic acid and sodium hydroxide were obtained from Sigma-Aldrich, St. Louis, USA. Double distilled water was consistently employed as the preferred solvent during the experimental procedures.

Pure cultures of phytopathogenic fungal strains, including *A. alternata* (FCBP-0188), *A. fumigatus* (FCBP-1254), *M. phaseolina* (MPSS-01), and *S. rolfsii* (MPSS-03) were sub-cultured on 2% malt extract agar (MEA) medium, composed of 20 g of Malt extract and 20 g of agar in 1 L of distilled water. This nutrient-rich medium served as the growth substrate for the fungi, and the cultures were stored at 4 °C until further use.

To prepare 100 mL of chitosan solution, a 3% acetic acid solution was initially made by dissolving 2 mL of acetic acid in 98 mL of distilled water. Subsequently, 0.2 g of chitosan powder was added to the solution, which was stirred on a magnetic stirrer at 60 °C for 24 h. The pH was then adjusted to 5 using a 0.5 M NaOH solution, followed by additional stirring for 1 h. The resulting homogeneous mixture was allowed to cool at room temperature before proceeding to experimentation (Vanti *et al*., 2020).

Antifungal assays were conducted to evaluate the efficacy of chitosan using the poison food technique with some modifications (Reyna *et al*., 2022). Various concentrations (0.05, 0.10, 0.15, 0.20, and 0.25%) of chitosan were prepared by adjusting the volume to 15 mL with 2% (w/v) MEA. To prevent bacterial contamination, an antibacterial capsule, streptomycin was introduced. The control group consisted of MEA only. For the experiment, a 6 mm mycelial disk of the fungus was carefully excised from a 7-day-old fungal culture and placed at the center of each Petri plate. Three sets of replicates were arranged for each of the five treatments including control, and the Petri plates were incubated for 7 days at 30 °C (Shoaib *et al*., 2021). The same procedure was followed for the remaining three fungi.

Throughout the incubation period, the diameter of the fungal mycelial growth was precisely measured, and the percentage of mycelial growth inhibition (MGI) was calculated using the formula:

$$\text{MGI}\ \% = {}^{\text{Mc} - \text{Mt}}/_{\text{Mc}}\ \text{X}\ 100$$

Where, *Mc* represents mycelial growth in the control and *Mt* stands for mycelial growth in the treatment.

Minimum inhibitory concentration and tolerance index were also calculated. Minimum inhibitory concentration (MIC) of the chitosan is that concentration where visible growth of the fungus is inhibited, and tolerance index (TI) is the tolerance potential of the fungal species in the test medium and it was calculated in relation to control radial growth (Shoaib *et al*., 2021).

An experimental design employing a completely randomized setup was executed, involving three replicates and three repetitions. The data underwent analysis of variance using Statistix Software (version 8.1). Mean comparisons were conducted using the Tukey's test with a significance level set at $p \leq 0.05$. Regression analysis and Principal components analysis (PCA) were built to summarize the variability of the treatments, and to determine the association among the measured trait (Shoaib *et al*., 2022).

Result and Discussion

The investigation centered on evaluating the impact of chitosan, administered at diverse

concentrations, on the mycelial diameter growth of fungi in a solid medium containing 2% MEA. Concentrations of chitosan ranging from 0.05 to 0.25% were tested, and a control group devoid of chitosan was included for comparison. The response variable was the mycelial diameter of *A. alternata*, *A. fumigatus, M. phaseolina*, and *S. rolfsii*.

The outcomes demonstrated a discernible reduction in fungal growth proportionate to the elevation in chitosan concentration, indicating a linear correlation (Fig. 1 & 2 A). At the lowest concentration (0.05%), there was a 20-40% decline in fungal growth, accompanied by a higher tolerance index (TI) ranging from 0.61 to 0.80. Moderate concentrations (0.10 and 0.15%) induced a 40-50% reduction in growth, with a TI ranging from 0.52 to 0.62. *A. alternata* and *S. rolfsii* displayed low TI at concentrations ≥ 0.20%, with growth cessation observed beyond 0.25%. *A. fumigatus* exhibited a TI of 0.10, experiencing a 90% inhibition at the 0.20% concentration. *M. phaseolina* growth was suppressed by 85% up to a 0.25% concentration. The minimum inhibitory concentration (MIC) values for chitosan were established at 0.25% for *A. alternata*, *A. fumigatus*, and *S. rolfsii*, while for *M. phaseolina* concentration greater than 0.25% was required for complete growth inhibition.

The temporal dynamics of fungal mycelium coverage on the chitosan-amended medium indicated more rapid coverage at lower chitosan concentrations (within 7 days) compared to extended durations (18-22 days) required for growth at medium and higher concentrations, in contrast to the control (Table 1).

Principal component analysis (PCA) was applied to assess fungal growth inhibition at distinct chitosan concentrations. Principal component 1 elucidated 97% of the overall variation, while principal component 2 explained 21%. The cumulative explanatory power of PC1 and PC2 was 99.608%. Fungi were collectively grouped with positive loadings on the upper and lower right side of the biplot, reflecting their susceptibility to varying chitosan concentrations. *A. alternata* and *S. rolfsii* exhibited a more pronounced influence of chitosan concentration, as evidenced by a lengthier projection compared to *A. fumigatus* and *M. phaseolina*. The alignment of 0.20 and 0.25% chitosan concentrations in a similar direction suggested a robust and analogous effect on fungal growth inhibition (Fig. 2 B).

This investigation into the influence of chitosan on the mycelial diameter growth of diverse fungi in a solid medium has yielded valuable insights, shedding light on its potential as a fungistatic agent. As previously discussed by Meng *et al*. (2020), the findings of this study consistently demonstrated a reduction in fungal growth with increase in concentrations of chitosan, indicating its inhibitory effects on the selected fungal species. This observed linear correlation between chitosan concentration (0.05 to 0.25%) and fungal growth reduction implied a dose-dependent relationship as emphasized by Sampedro *et al*. (2022), with higher concentrations leading to a more substantial inhibition of mycelial diameter growth.

The sensitivity of the fungi to chitosan was further underscored by the tolerance index (TI) values, referencing Kim *et al*. (2023). Notably, *A. alternata*, *A. fumigatus*, and *S. rolfsii* exhibited negligible TI at concentrations ≥ 0.20%, with growth cessation beyond 0.25%, highlighting the potent inhibitory effects of higher chitosan concentrations on these fungi. The determination of MIC values, as explained by Oliveira *et al*. (2012) proved crucial in understanding the concentration at which chitosan effectively inhibited the growth of the mentioned fungi. The established

MIC values revealed varying sensitivities among the tested fungi. Temporal dynamics in fungal mycelium coverage on chitosan-amended medium added an intriguing dimension to the study (Crognale *et al*., 2022). The more rapid coverage at lower and medium chitosan concentrations within 7 days, in contrast to the extended durations (18-22 days) required for growth at higher concentrations, suggested a potential time-dependent aspect to the inhibitory effects of chitosan.

Karamchandani *et al*. (2022) applied principal component analysis (PCA) to overview of the data, uncovering distinct groupings of fungi based on their susceptibility to varying chitosan concentrations. The high explanatory power of the principal components (97% for PC1 and 21% for PC2) attested to the robustness of the analysis. The biplot visually represented the relationships between fungi and chitosan concentrations *A. alternata*, *A. fumigatus*, and *S. rolfsii* exerting a more pronounced influence and displayed desired responses. The alignment of 0.2% and 0.25% chitosan concentrations in a similar direction suggested a consistent and potent effect on fungal growth inhibition, providing further support for the observed dose-dependent relationship. In conclusion, this comprehensive study delved into the inhibitory effects of chitosan on fungal mycelial diameter growth, elucidating dose-dependent responses, temporal dynamics, and varying sensitivities among the tested fungi. These findings combined with those by Sun *et al*. (2021), significantly contribute to our understanding of chitosan as a potential antifungal agent, laying the groundwork for further exploration of its applications in agriculture and related fields.

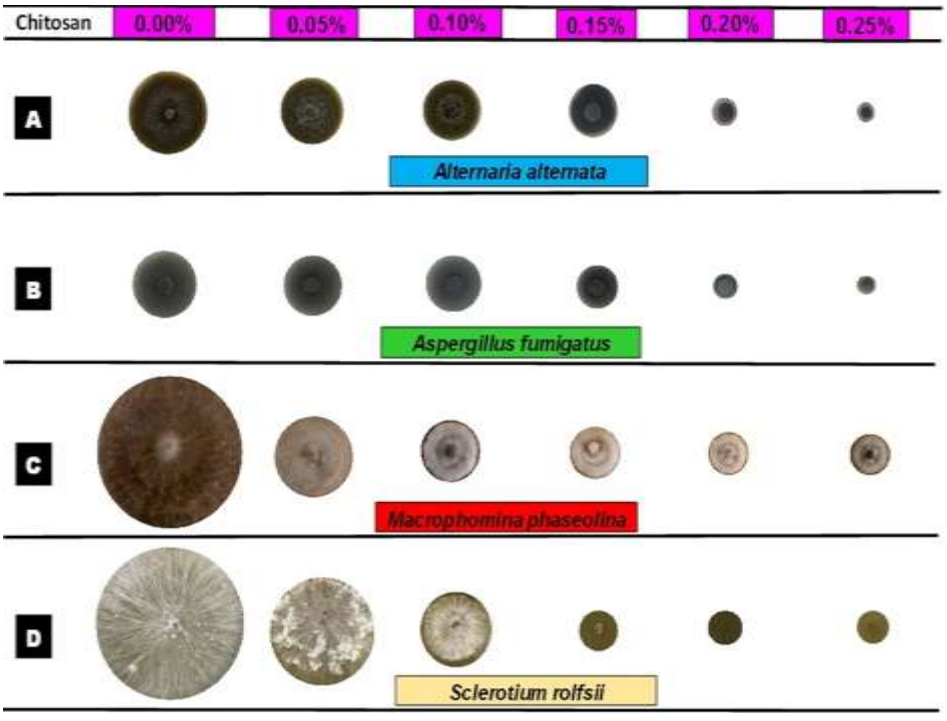

Fig. 1: Effect of different concentrations (0.05-0.25%) of chitosan on growth magnitude of phytopathogenic fungi on 2% malt extract agar (MEA) incubated at 30 °C for 25 days

Table 1: Colony diameter (cm) and tolerance indices of phytopathogenic fungi at different concentrations (0.05-0.25%) of chitosan on 2% malt extract agar (MEA) incubated at 25 °C for 7-25 days.

#	Fungi	Chitosan concentration (%)	Growth (cm)	Tolerance Index (TI)	Days required for growth
1	*Alternaria Alternata*	0.00	5.50^{c}	0.64	7
2		0.05	3.50^{e}	0.56	7
3		0.10	3.10^{e}	0.45	7
4		0.15	2.50^{f}	0.04	15
5		0.20	0.20^{hi}	0.00	18
6		0.25	0.00^{i}	0.00	-
1	*Aspergillus Fumigatus*	0.00	4.00^{cd}	0.83	7
2		0.05	3.30^{e}	0.63	7
3		0.10	2.50^{f}	0.35	7
4		0.15	1.40^{g}	0.10	15
5		0.20	0.40^{h}	0.00	18
6		0.25	0.00^{i}	0.00	-
1	*Macrophomina phaseolina*	0.00	9.00^{a}	0.64	7
2		0.05	5.80^{c}	0.50	7
3		0.10	4.50^{d}	0.40	7
4		0.15	3.60^{e}	0.22	18
5		0.20	2.00^{fg}	0.16	19
6		0.25	1.40^{g}	0.00	22
1	*Sclerotium Rolfsii*	0.00	9.00^{a}	0.83	7
2		0.05	7.50^{b}	0.62	7
3		0.10	5.60^{c}	0.22	10
4		0.15	2.00^{fg}	0.01	18
5		0.20	0.10^{hi}	0.00	18
6		0.25	0.00^{i}	0.00	22

Values with different letters in superscript show significant difference ($p \leq 0.05$) as determined by Tukey's test.

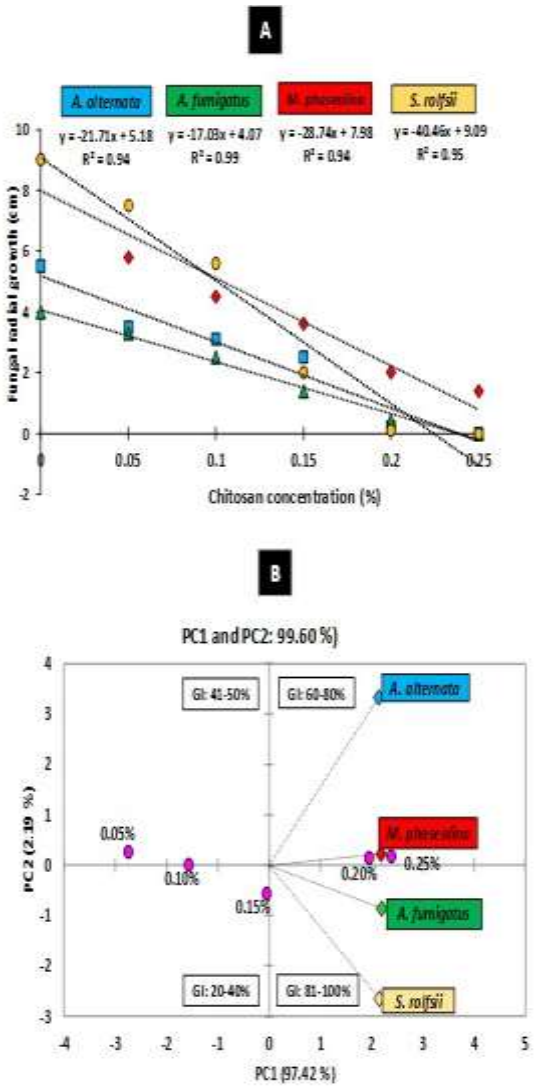

Fig. 2 (A & B): Regression analysis (A) and Biplot of the principal component analysis (B) of the growth inhibition (GI: %) response in phytopathogenic fungi to different concentrations (0.00-0.25%) of the chitosan. The colors depict as follows: *A. alternata* (blue), *A. fumigatus* (red), *M. phaseolina* (green), *S. rolfsii* (orange).

Conclusions

The demonstrated that chitosan exhibited a dose-dependent inhibitory influence on the mycelial diameter growth of diverse fungi in solid medium. The identified linear correlation between chitosan concentration and the reduction in fungal growth, along with specific the TI and MIC values, underscored the distinct sensitivities and susceptibilities among the tested fungi. The temporal dynamics in mycelium coverage suggested a time-dependent dimension to the inhibitory effects of chitosan. These findings provided valuable insights

into the fungistatic potential of chitosan, paving the way for future applications in agriculture and related fields.

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Conflict of Interest

Authors showed no conflict of interest.

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Author's contribution

Amna Shoaib: Conceptualization, supervision, data analysis & manuscript drafting; Nimra Iqbal: Experiment, data curation & Interpretation; Qudsia Fatima: Interpretation, acquisition & analysis; Nafia Gull: Analysis & Interpretation; Barizah Malik: Acquisition

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