GABRIEL FERREIRA PAIVA

CHITOSAN OLIGOSACCHARIDE: USE TO CONTROL FUSARIUM HEAD BLIGHT AND TO REDUCE DEOXYNIVALENOL ACCUMULATION IN WHEAT GRAINS

Dissertation submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Franklin Jackson Machado

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Aos meus pais, Samantra e Wander (*in memorian*)

Aos meus irmãos, Beatriz, Rafael, Ana Carolina e Miguel

À minha vó, Maria

As minhas tias Débora e Wanderléia

A todos os familiares e amigos sempre presentes e que torceram por mim

dedico.

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BIOGRAPHY

GABRIEL FERREIRA PAIVA, son of Samantra Ferreira Mota and Wander de Oliveira Paiva Júnior (*in memorian*), was born in Barbacena, Minas Gerais, September 03, 1995. In 2021 he graduated in Agronomy from the Instituto Federal de Educação, Ciência e Tecnologia de Mato Grosso do Sul - Nova Andradina, Mato Grosso do Sul. In 2021 he started his masters in the Plant Pathology Department of Universidade Federal de Viçosa, Minas Gerais, mentored by Dr. Franklin Jackson Machado.

ABSTRACT

PAIVA, Gabriel Ferreira, M.Sc., Universidade Federal de Viçosa, August, 2022. **Chitosan oligosaccharide: use to control Fusarium head blight and to reduce deoxynivalenol accumulation in wheat grains.** Adviser: Franklin Jackson Machado.

Wheat is one of the most important cereal crops worldwide, but several fungal diseases can affect crop yield. Among them, Fusarium head blight (FHB), caused by members of the *Fusarium graminearum* species complex, is one of the most serious wheat diseases worldwide, resulting in lost ofin yield and grain quality due to the accumulation of mycotoxins. There are several strategies for FHB management, including cultural practices, sowing of resistant cultivars, chemical control, biological control, among others. The use of alternative products, such as chitosan and its derivatives, can be a viable alternative for disease control. The present study proposes to investigate the use of chitosan oligosaccharide as an alternative product to reduce FHB intensity and to reduce mycotoxin contamination in wheat grains. The trials were carried out in the 2021 and 2022. The experiments were divided into *in vitro* tests, seed treatment and foliar application on wheat plants under greenhouse and field conditions. In the *in vitro* trials, it was possible to observe that the chitosan oligosaccharide directly affected the growth of *F. graminearum*, depending on the concentration used. When applied in seed treatment, both tebuconazole and chitosan oligosaccharide reduced the incidence of *F. graminearum* and preserved the germination potential of seeds when inoculated with the fungus. In general, there was no significant reduction in disease severity between doses or times of chitosan oligosaccharide application in relation to the unsprayed check and the fungicide. However, an association between the reduction of disease intensity was observed, although not significant, with the reduction of the accumulation of DON in the grains. The results of this study suggest that chitosan oligosaccharide can potentially be used as a tool in the integrated management of the disease, establishing a basis for future studies to explore the use of this product associated with chemical products, aiming to increase the efficacy of FHB control in wheat.

Keywords: *Fusarium graminearum*. *Triticum aestivum*. Alternative control. Integrated management of plant diseases.

RESUMO

PAIVA, Gabriel Ferreira, M.Sc., Universidade Federal de Viçosa, agosto de 2022. **Oligossacarídeo de quitosana: uso para controlar a giberela e reduzir o acúmulo de desoxinivalenol em grãos de trigo.** Orientador: Franklin Jackson Machado.

O trigo é um dos cereais mais importantes mundialmente, porém diversas doenças fúngicas impactam a produtividade da cultura. Dentre elas, a giberela, causada por espécies do complexo *Fusarium graminearum*, é uma das doenças mais graves em todo o mundo, resultando em perda de produtividade e qualidade dos grãos pelo acúmulo de micotoxinas. Existem várias estratégias para o manejo da giberela, incluindo práticas culturais, plantio de cultivares resistentes, controle químico, controle biológico, entre outros. A utilização de produtos alternativos, como a quitosana e seus derivados, pode ser uma alternativa viável para o controle de doenças. O presente estudo propõe investigar o uso do oligossacarídeo de quitosana como um produto alternativo visando reduzir a intensidade da giberela e redução dos níveis de micotoxinas em grãos de trigo. Os ensaios foram conduzidos nas safras de 2021 e 2022. Os experimentos foram divididos em testes *in vitro*, tratamento de sementes e aplicação foliar em plantas de trigo sob condições de casa de vegetação e de campo. Nos ensaios *in vitro* foi possível observar que o oligossacarídeo de quitosana exerceu efeito direto sobre *F. graminearum*, apresentando um efeito dose-dependente. Quando aplicados no tratamento de sementes, tanto o tebuconazol quanto o oligossacarídeo de quitosana reduziram a incidência de *F. graminearum* e preservando o potencial de germinação das sementes quando inoculadas com o fungo. De maneira geral, não houve redução significativa na severidade da doença entre as doses ou as épocas de aplicação do oligossacarídeo de quitosana em relação a testemunha não pulverizada e o fungicida. Entretanto, foi possível observar uma associação da redução da intensidade da doença, embora não significativa, com a redução do acúmulo de DON nos grãos. Os resultados deste estudo sugerem que o oligossacarídeo de quitosana apresenta potencial de ser utilizado como uma ferramenta no manejo integrado da doença, estabelecendo uma base para estudos futuros para explorar o uso deste produto associado com algum produto químico, visando aumentar a eficácia de controle da giberela do trigo.

Palavras-chave: *Fusarium graminearum*. *Triticum aestivum*. Controle alternativo. Manejo integrado de doenças de plantas.

TABLE OF CONTENTS

1. Introduction

Wheat (*Triticum aestivum* L.) is one the most important crops worldwide (Igrejas; Branlard, 2020; FAO, 2021). Brazil is not self-sufficient in terms of wheat supply, rather, the country needs imported wheat to meet its needs (Costa et al., 2008). There are several diseases of major concern affecting wheat production. Fusarium head blight (FHB), caused by members of the *Fusarium graminearum* species complex, among which *Fusarium graminearum* s.s. is one of the most frequent species, being one of the important diseases of wheat worldwide. The disease has been responsible for significant losses in both yield and grain quality due to the accumulation of mycotoxins, such as the B-trichothecene deoxynivalenol (DON), nivalenol (NIV) and their acetyl-derivatives, that can be harmful to livestock and human health (Figueroa et al., 2018; Huang et al., 2020; Tralamazza et al., 2016).

The management of FHB aims to reduce disease intensity as well as micotoxin levels to meet the maximum tolerated limits wheat products as established elsewhere for each country, including Brazil (ANVISA 2011, 2017, 2022). There are several strategies for the management of FHB, including cultural practices, resistant cultivars, chemical control, biological control, and others, although the integration of all these practices has been shown to be more effective (Chen et al., 2019; Wegulo et al., 2015). Amongst them, chemical control is one of the most used measures for controlling FHB. Demethylation inhibitor fungicides (DMI) are the most widely used for the control of disease, and the reduction in the accumulation of mycotoxins (McMullen et al., 2012; Paul et al., 2008; Wegulo et al., 2015). Overall, the fungicides used to control FHB show variable efficacy. In a meta-analytic study in North America, it has been shown a large variation of control efficacy across the trials, with performances ranging from 32 to 50% for different the DMI fungicides tebuconazole, propiconazole, metconazole and prothioconazole (Paul et al., 2008) and in Brazil for triazole and benzimidazole (Machado et al., 2017b).

With the recurrent use of fungicides in the management of this disease, there is a risk to select resistant individuals within the *F. graminearum* population. In fact, in the state of New York and in Henan Province in China, tebuconazole less sensitivity isolates of *F. graminearum* have been reported (Spolti et al., 2014; Chen et al., 2021). Therefore, new control methods must be developed. The use of alternative products such as chitosan may be a viable alternative for FHB control and mycotoxin reduction in wheat grains. Chitosan is a polymer of D-glucosamine derived from the deacetylation of chitin which is found naturally in the cell wall of fungi and

also in the exoskeleton of crustaceans, being considered the second most abundant polysaccharide in nature (Almeida et al., 2019; Berger et al., 2011). The chitosan has been studied for its ability to increase plant tolerance to stress and to activate plant defense responses (Benhamou, 1998; Lee et al., 1999). The induction of resistance in plants by chitosan was shown in several crops, including wheat in seed treatment to control *F. graminearum* (Bhaskara Reddy et al., 1999). This study aims to investigate the use of chitosan oligosaccharide, a chitosan derivative, as an alternative product, sprayed alone, aiming to reduce FHB intensity as well as DON accumulation in wheat grains.

2. Literature review

2.1 The disease

Fusarium head blight is an important wheat disease, caused by members of the *Fusarium graminearum* species complex (FGSC). Based on the DNA sequences of twelve genes, fifteen species have been reported within FGSC, *F. acacia-mearnsii*, *F. aethiopicum*, *F. asiaticum*, *F. austroamericanum*, *F. boothii*, *F. brasilicum*, *F. cortaderiae*, *F. gerlachii*, *F. graminearum* sensu stricto, *F. meridionale*, *F. mesoamericanum*, *F. ussurianum*, *F. vorosii*, *F. nepalense* and *F. lousianense* (O'Donnell et al., 2000, 2004, 2008; Sarver et al., 2011; Starkey et al., 2007; Yli-Mattila et al., 2009). Fungal infection occurs in the flowering stage of wheat. The main symptoms are bleaching of spikelets with presence of pinkish or orange-ish signs of the pathogen in the base of the glumes, leading to kernel infection, yield reduction and contamination of tissues and grains with mycotoxins (Gilbert e Haber, 2013; Snijders, 2004). FGSC members produce B-trichothecene mycotoxins, including nivalenol (NIV) and its acetyl-derivatives; and deoxynivalenol (DON) and its acety-derivatives: 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) (Reynoso et al., 2013).

2.2 Importance of the disease

Wheat is one of the most important crops, as it is largely consumed worldwide, due to its many uses, as human food, food additives, fermentation, animal food, and others. In Brazil in 2020, wheat production was 6.347.987 tons in an area harvested of 2.434.703 ha (Igrejas; Branlard, 2020; FAO, 2021). In 1917, surveys were carried out across the United States with

FHB reported in 31 out of 40 surveyed states with estimated losses of 288.000 ton (Atanasoff, 1920). In the USA, it has been estimated that FHB caused billions of dollars of losses in wheat and barley yield and quality in the 1990s and early 2000s (Nganje et al., 2004). In Brazil, the increase in the frequency of epidemics may be related to the climatic factors, and also to changes that occurred in cultural practices, especially the growth of no-till acreage which may have contributed to the maintenance of the inoculum within the area (Del Ponte et al., 2005, 2009; Duffeck et al., 2020). Nowadays, FHB, and its main causal agent *Fusarium graminearum,* is widely distributed in all the main wheat producing regions worldwide (Figure 1) (Del Ponte et al., 2022).

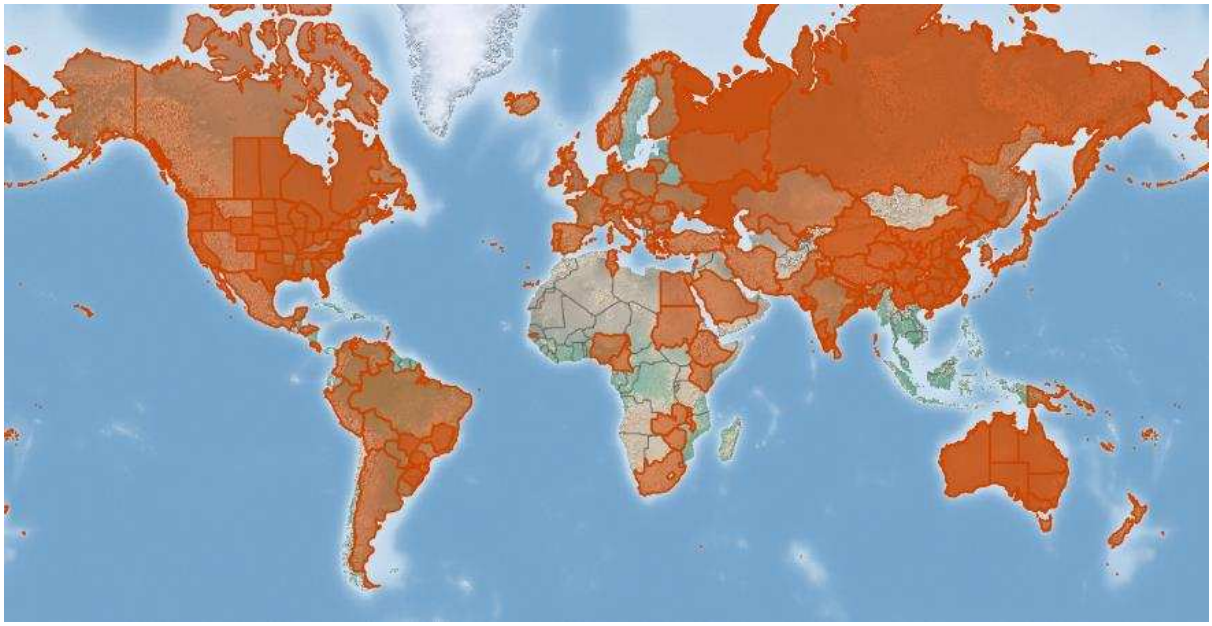

Figure 1. Global distribution of Fusarium head blight prevalence in the world. CABI, 2022.

In addition to the damage caused in the grains, the mycotoxins produced by *F. graminearum* are a major concern to the small grain production. Among the mycotoxins reported, several studies indicated the predominance of DON (Marasas, 1991; Pinto et al., 2013). A study investigated mycotoxin contamination in 66 wheat samples obtained in 38 municipalities in Rio Grande do Sul state in Brazil. The authors reported that DON was found in 65 samples and NIV was detected in 57 samples, with concentrations ranging from <250 µg/kg to >1250 µg/kg for DON, and <250 µg/kg to 1000 µg/kg for NIV (Del Ponte et al., 2012). Maximum tolerable levels of DON have been established for grains and foods in many countries including Brazil (Table 1) (ANVISA 2017).

Table 1. Maximum tolerable levels (MLT) of deoxynivalenol (DON) in wheat grains and foods proposed in 2017 (ANVISA 2017).

Mycotoxin	Food	MTL (µg/kg)
Deoxynivalenol (DON)	Wheat and corn in grains for further processing	3000
	Whole wheat, kibbeh wheat, whole wheat flour, wheat bran, rice bran, barley grain	1250
	Wheat flour, pasta, crackers, and baked goods, cereals, and cereal products other than wheat and including malted barley	1000

2.3 Pathogen life cycle and epidemiology

F. graminearum is an ascomycete belonging to the Sordariomycetes class, Hypocreales order, Nectriaceae family, and *Fusarium* genus (NCBI:txid5518). It is known that some *Fusarium* species can survive in alternative hosts, contributing to the maintenance of the inoculum for epidemics (Landschoot et al., 2011; Machado et al., 2015). In wheat, FHB is considered a floral and monocyclic disease, where primary infection occurs from inoculum surviving in plant debris. Warm and humid conditions are conducive for the production of macroconidia and perithecia formation, the latter will produce and release ascospores during the wheat anthesis (Markell; Francl, 2003). Perithecia are formed on crop debris, when mature ascospores are forcibly discharged and are dispersed, thus ensuring the availability of annual inoculum, being considered the primary inoculum of the disease. In addition, the pathogen can also survive as latent mycelium or by the formation of chlamydospores in soil (Guenther; Trail, 2005; Leplat et al., 2013; Pereyra et al., 2004; Trail et al., 2002).

Macroconidia is produced in sporodochia on the surface of infected plants or in crop residues and can be dispersed over short distances through rain splashes during wet periods. (Maguiano; Kikot, 2013). After ascospores or macroconidia come into contact with wheat spikelets through wind or rain, the fungus initiates the pathogenic phase, which can occur from the time of anthesis to the soft dough stage of grain development (Del Ponte et al., 2007; McMullen et al., 1997). Macroconidia of *F. graminearum* adhere and germinate on the host

tissue and once inside of plant tissue, the fungus is able to colonize systemically. Hyphae growth on the infected spikelet spreads to adjacent spikelets throughout the rachis, and symptoms can be observed after 4-6 days in the form of necrotic areas and premature senescence of infected spikelets (Kang et al., 2005). As colonization continues, it becomes chlorotic and tissue death later occurs (Figure 2). Infected grains are deformed and hollow. Yield losses may be greater when infection occurs at flowering (anthesis) as in this period there is a large reduction in grain weight (Salgado et al., 2011).

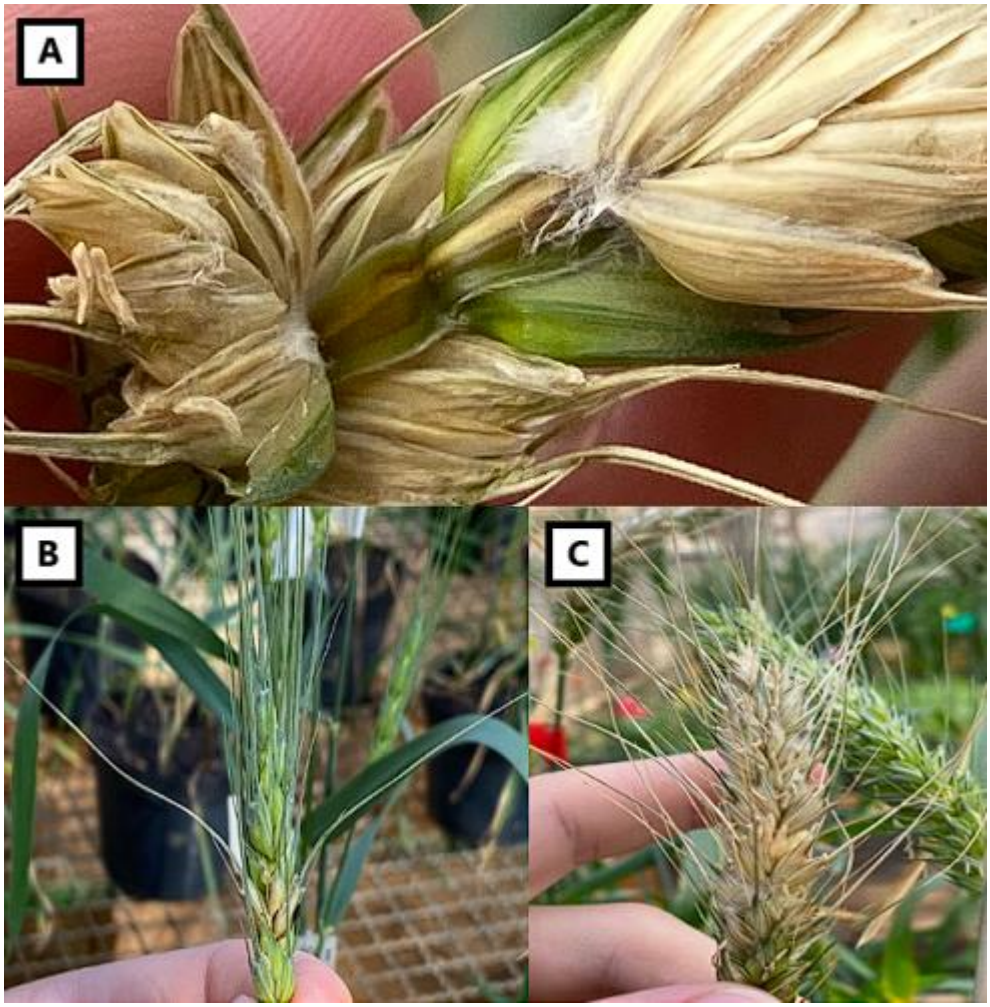

Figure 2. (A) Evolution of symptoms to other spikelets through the rachis. (B and C) Premature senescence of infected spikelets. Source: Paiva, 2022.

2.4 Disease management

Due to the variety of hosts, even alternative hosts, the genetic diversity, and the existence of different inoculum sources, one of the management measures used to control and

reduce the inoculum sources that can cause FHB epidemics is the use of resistant wheat cultivars (Buerstmayr et al., 2002; Gilbert; Fernando, 2004; Yan et al., 2011). Other measures that are used includes cultural practices (irrigation management or crop rotation), chemical control, biological control, and other strategies for minimizing FHB intensity (Chen et al., 2019; Wegulo et al., 2015). For example, crop rotation with host grasses, such as corn and barley, has contributed to inoculum buildup and consequently to the increase in the risk of FHB epidemics. Previous study have shown that after three years, the incidence of FHB was higher when rotating wheat with corn and lower in the soybean-wheat system, showing that corn, as a host of *F graminearum*, produces a higher volume of crop residue with a lower rate of decomposition (Dill-Macky & Jones, 2000). Some post-harvest practices such as reducing humidity and temperature in silos and sorting and discarding broken and damaged grains, increasing the wind force of harvester fans; are described as effective in reducing *Fusarium* damaged kernels and consequently DON levels in stored grains (Jouany, 2007; Salgado et al., 2011).

Wheat growers often rely on fungicides applied at early anthesis to control FHB and reduce mycotoxin levels in grains. The increased use of fungicides to manage FHB raises concerns about fungicide resistance, especially systemic single-site fungicides are used (Freije; Wise, 2015; Gilbert e Haber 2013; Machado et al., 2017a; Wegulo et al., 2015). *F. graminearum* isolates with reduced sensitivity to triazoles have been found in wheat fields in Brazil (Machado et al., 2017b; Spolti et al., 2012), the eastern United States (Spolti et al. 2014), China (Chen et al., 2021) and Germany and the United Kingdom (Talas; McDonald, 2015) and to benzimidazoles in China (Chen et al., 2019), and in Brazil (Machado et al., 2017b).

2.5 Chitosan and its derivatives

Chitosan is a natural polysaccharide produced by the partial alkaline deacetylation of chitin (Figure 3), extracted from shrimp and crab shells, but can also be found in the cell walls of fungi and in the cuticles of insects (Kean; Thanou, 2010; Raafat et al., 2008). Chitosan oligosaccharides are the degraded products of chitin or chitosan by acid hydrolysis and/or enzymatic degradation, and it is considered a chitosan oligomer (Figure 4, Muanprasat; Chatsudthipong, 2017; Naveed et al., 2019). Chitosan oligosaccharide, compared to chitosan, has a higher water solubility and lower viscosity (Muanprasat; Chatsudthipong, 2017; Yin et al., 2016).

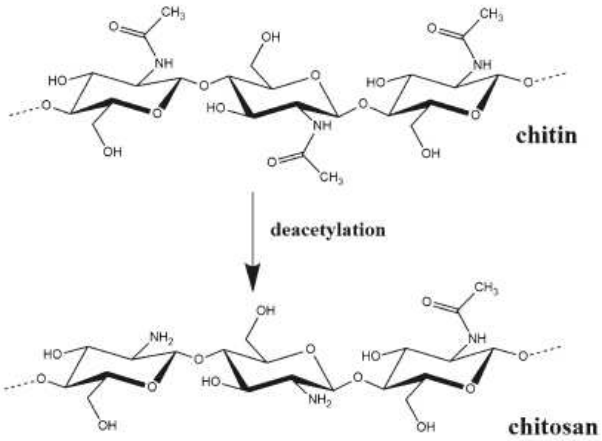

Figure 3. Process of chitosan formation by partial deacetylation of chitin (Adapted from: QIN; ZHAO, 2019)

The chitosan oligosaccharide, after coming into contact with the plant, is recognized by a receptor on the plant cell membrane, followed by signal transfer and amplification, activation of responsive genes, accumulation of responsive proteins, induction of defense-related secondary metabolites, and finally the plant response, such as hypersensitivity responses, production of phytoalexins and reinforcement of cell walls (Yin et al., 2010). The induction of plant defense mechanisms with the use of chitosan oligosaccharide and its derivatives has been increasingly studied, aiming to control diseases in several plant species and plant pathogens *in vitro* and *in vivo*, as shown in the Figure 5.

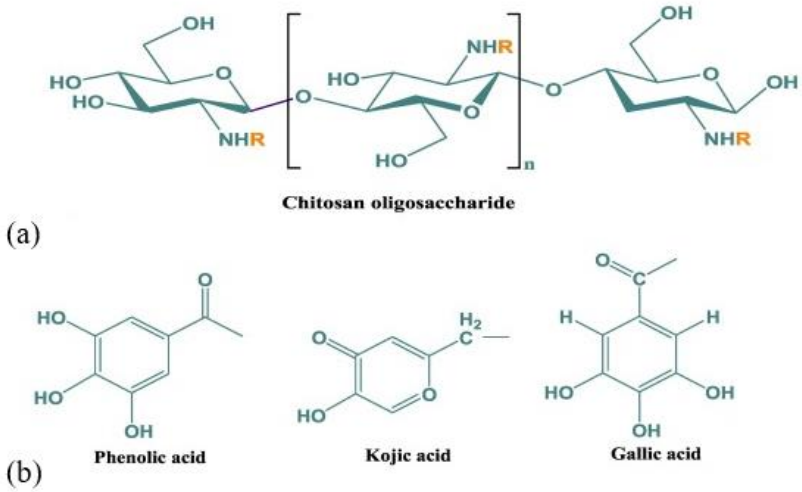

Figure 4. (A) Forming of chitosan oligosaccharide and (B) possible derivatives of COS, replacing the 'R' (Adapted from: Naveed et al., 2019).

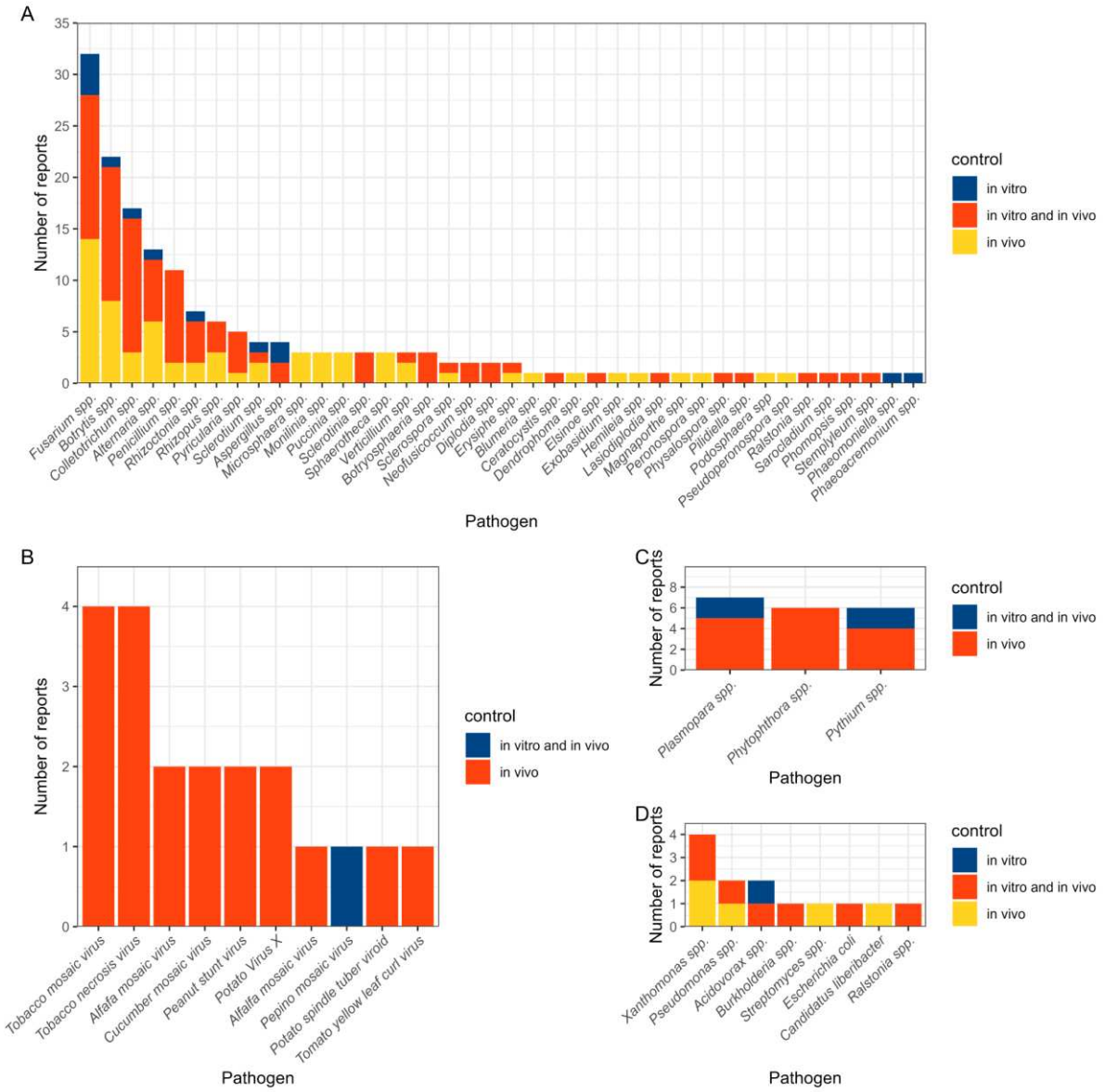

Figure 5. (A) Number of reports in the literature using chitosan to control plant pathogenic fungi; (B) virus; (C) oomycetes and (D) bacteria *in vitro* or *in vivo*. Source: Paiva, *unpublished data.*

Based on a systematic review of the available literature, studies investigating the use of chitosan and its derivatives to control plant diseases emerged in the 1980s, increasing the number of studies published over the last few decades (Figure 6).

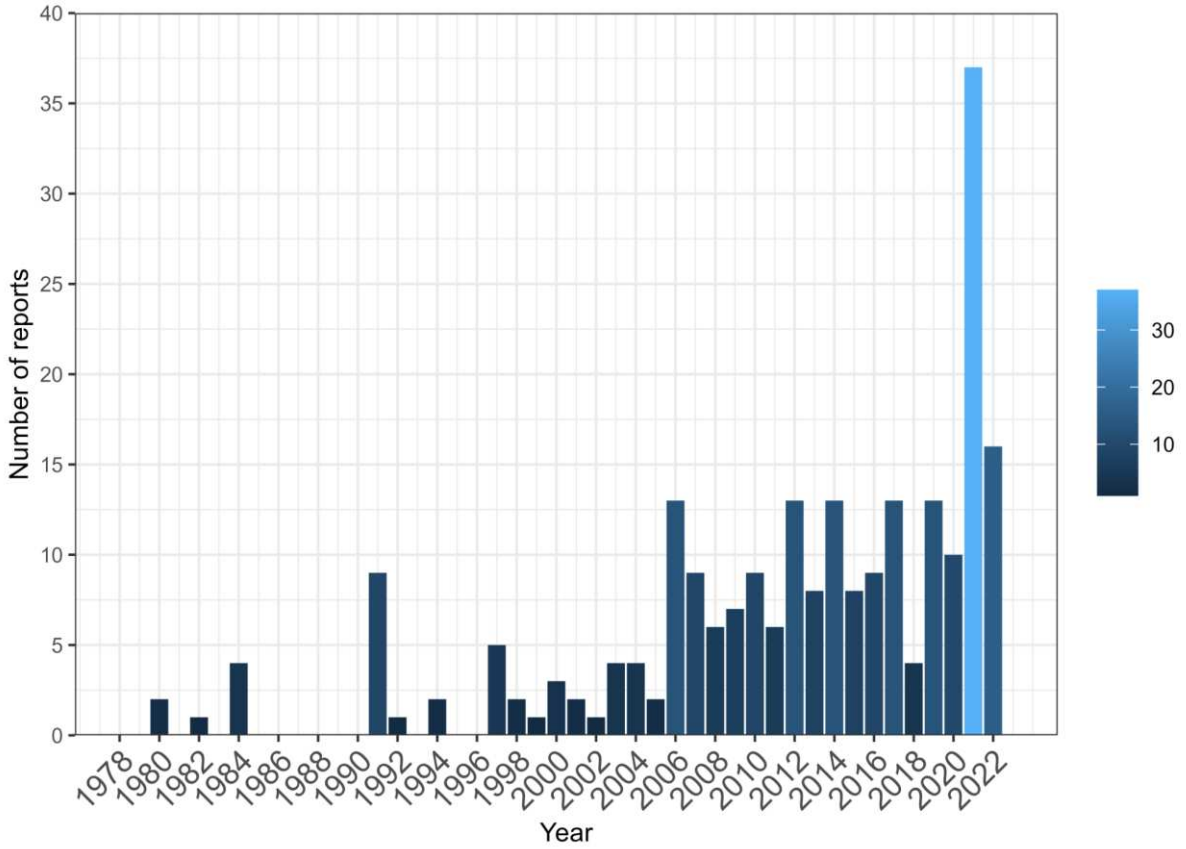

Figure 6. Number of studies published per year reporting the use of chitosan and its derivatives in the control plant pathogens in the last decades. Source: Paiva, *unpublished data.*

2.6 Chitosan vs FHB

Chitosan oligosaccharide and its derivatives have been reported to induce plant defense mechanisms against the infection by plant pathogens. Several studies have investigated the effects of chitosan oligosaccharide in the growth of *F. graminearum* and progress of FHB. Kheiri et al. (2016) showed the effects of chitosan and chitosan nanoparticles in reducing mycelial growth, number of colonies formed, conidia germination, and controlling the disease under greenhouse conditions. The authors have concluded that chitosan and chitosan nanoparticles were effective to control FHB. Francesconi et al. (2020) showed that chitosan hydrochloride effectively reduced mycelial growth of *F. graminearum in vitro* (ranging from 53 to 87%) and controlled FHB in the greenhouse (40% after 21 days inoculation). The study demonstrated that chitosan hydrochloride boosted the growth and the development of wheat plants, reduced the severity of FHB and the accumulation of mycotoxins and FHB-related compounds in the wheat heads. More recently, Deshaies et al. (2022) showed the effects of chitosan hydrochloride in reducing the mycelial growth 85%), and controlling the disease in the

greenhouse (around 30% in treatments with chitosan in relation to the control), metabolite production regulated by either chitosan or *F. graminearum*. The authors showed that chitosan hydrochloride inhibited spore germination and hyphae development of *F. graminearum in vitro*, also decreasing the severity of the disease.

3. Materials and Methods

3.1 Fungal isolate and inoculum preparation

One representative *F. graminearum* DON/15ADON-producing isolate (CML 3066) was selected from a collection obtained from symptomatic wheat heads (Del Ponte et al., 2015; Wood et al., 2020) was used in all assays. The isolate was grown on potato dextrose agar (PDA) at 25°C with a 12-h dark/light cycle for 7 days. After this period, mycelial plugs were transferred to plates containing *Spezieller Nahrstoffarmer* Agar (SNA) culture medium and incubated for seven days. SNA plates were scraped with sterile distilled water and the spore suspension was evenly distributed onto new SNA plates and incubated under the same conditions mentioned above. Spore suspension was prepared by scraping 7-days-old SNA plates. The macroconidia were filtered through two layers of cheesecloth to remove mycelial fragments. The concentration of macroconidial suspensions were quantified using a hemocytometer and diluted properly in each assay.

3.2 Chitosan oligosaccharide sensitivity *in vitro*

3.2.1 Inhibition of mycelial growth

Chitosan oligosaccharide (Concentration = 95% and N = 5%; pH = 4-6; MO = 80%, molecular weight ≤1.500 kDa and water-soluble) was dissolved in 60 mL of sterile distilled water to obtain 200.000 mg/L for the stock solution. The stock solution was added to molten PDA (45-55°C) to obtain the final concentrations of 0 (non-amended agar - PDA), 1000, 2000, 4000, 8000, 16000 and 32000 mg/L. The commercial formulation of tebuconazole (Tebufort® 200 g/L, UPL) was used as a control. The tested concentrations of tebuconazole were: 0, 0.5, 1, 2, 4 and 8 mg/L. A mycelial agar plug (5 mm) from the edge of a 7-day-old culture on PDA was placed in the center of a 90-mm-diameter Petri dish containing 15-ml of amended PDA.

After 4 days of incubation at 25°C with a 12-h dark/light cycle, radial growth was measured in two perpendicular directions using a digital caliper. Three replicates were used for each concentration and the experiment was repeated once.

3.2.2 Inhibition of macroconidia germination

The tested concentrations for chitosan oligosaccharide were: 0 (non-amended agar - PDA), 62.5, 125, 250, 500, 1000, and 2000 mg/L. The inhibition of macroconidia germination assay was performed using the glass drop technique (Dhingra and Sinclair 1995). Macroconidial suspensions were obtained as described previously with some modifications. SNA plates (7-days-old cultures) were scraped with sterile distilled water with Tween 20 (0.01%) and gelatin (6%). Suspensions were diluted to obtain a final concentration of 1 x 10^7 macroconidia/mL. The commercial formulation of pyraclostrobin (Comet® 250 g/L, BASF) was used as a control. The tested concentrations of pyraclostrobin were: 0, 0.01, 0.1 and 1 mg/L. A 30-µl drop of macroconidial suspension was transferred to a glass slide and mixed to a 30-µl drop of chitosan oligosaccharide or pyraclostrobin stock solutions. The glass slides were aligned on a moistened sterile paper towel and placed inside a closed plastic box. The boxes were incubated in the dark at 25°C for 7 hours (Duan et al., 2020). Macroconidia germination was assessed by randomly counting fifty macroconidia under a light compound microscope (germinated macroconidia was considered when the germination tube grows to at least half the total length of the conidia). Three replicates (slides) were prepared for each isolate and the experiment was repeated once.

3.3 Chitosan oligosaccharide in seed treatment

Wheat seeds (BR18 Terena) were treated with chitosan oligosaccharide (2000 mg/L) and tebuconazole (2.5 mg/L). A sample of 1200 seeds were separated and surface-sterilized (30 s in 70% alcohol, 2 min in 2% sodium hypochlorite, followed by 3 washes in sterile distilled water) to remove the presence of background contaminants that may be present on the seed surface. Seed sample was divided into two sub-samples. The first sub-sample was inoculated by soaking the seeds in a spore suspension (1 x 10^4 macroconidia/mL) for 2 min and allowed to dry on sterile paper towels for 4.5 h. The other subsample was mock-inoculated using the same approach. Half of the inoculated and the mock-inoculated seeds were surface sterilized

before seed treatment. After inoculation, 400 seeds (200 inoculated and 200 mock-inoculated) were treated with tebuconazole and 400 seeds were treated with chitosan oligosaccharide. Treated seeds were immersed into the stock solutions for 5 min and allowed to dry on sterile paper towels for 4 h. The control treatments consist of 400 seeds, of which 200 were inoculated (100 disinfested and 100 non-disinfested) and 200 non-inoculated (100 disinfested and 100 non-disinfested).

Each treatment consisted of 100 seeds divided into five plastic boxes (replicates) with four sheets of germitest paper, previously moistened with sterile saline solution (12 g/L) and containing 20 seeds each. Plastic boxes were incubated at 25°C with a 12-h light/dark cycle for 6 days. The incidence of affected seeds was assessed as the percentage of seeds within each box showing *F. graminearum*-like growth. The experiment was repeated once.

Another assay was performed to investigate the effect of seed treatment in seed germination. For such, the same procedures described above were used with only one modification. The seeds for each treatment were also divided into five plastic boxes with four sheets of germitest paper, previously moistened with sterile water instead of saline solution to allow the seeds to absorb water and to germinate. Plastic boxes were also incubated at 25°C with a 12-h light/dark cycle for 6 days. Five replicates were prepared for each treatment (plastic box) with 20 seeds each. The incidence of affected seeds was assessed as the percentage of seeds within each box showing *F. graminearum*-like growth. Seed germination was assessed by counting the number of germinated seeds out of the twenty within each plastic box. Seed was considered germinated when the coleoptile was larger than twice the length of the seed. The experiment was repeated once.

3.4 Chitosan oligosaccharide in the whole plant applications

3.4.1 Plant growth conditions

Sowing was carried out in a greenhouse at Universidade Federal de Viçosa, MG, Brazil (20°45'29.12" S, 42°52'11.92" W, 660 m above sea level) during the April 2021 - August 2021 and April 2022 - August 2022 harvest. Ten seeds of the cultivar BR 18 Terena, susceptible to *F, graminearum*, were sown in 1-liter pots containing commercial substrate (Sousa, 2002). After two weeks, the plants were thinned to keep 5 plants/pot and were staked with wire tutors to prevent the plants from tipping over. After that, the plants receive weekly fertilization in the

form of a nutrient solution. Each pot received 50 mL of the solution prepared with 6.4mg/L KCl, 3.48mg/L K2SO4, 5.01mg/L MgSO4.7H2O, 2.03mg/L (NH2)2CO, 0.009mg/L NH4MO7O24.4H2O, 0.054mg/L H3BO3, 0.222mg/L ZnSO4.7H2O, 0.058mg/L CuSO4.5H2O, 0.137mg/L MnCl2.4H2O, 0.27g/L FeSO4.7H2O, and 0.37g/L disodium-EDTA prepared with distilled water (Xavier Filha et al., 2011). Each treatment Four replicates were prepared for each treatment (pots with five plants each). The experiment was repeated once. Assessments include counting symptomatic spikelets at 8, 11, 13, and 21 days after inoculation. The disease severity (%) was determined as ([number of symptomatic spikelets / total number of spikelets] x 100).

3.4.2 Plant inoculation procedures

Spore suspensions were prepared as described previously. The macroconidia suspension concentration was adjusted to a final concentration of 1 x 10^4 macroconidia/mL. Inoculations were carried out when the plants reached the mid-anthesis stage (Feeks growth stage 10.5.2 (Miller, 1999), since this stage favors the development of *F. graminearum*, as the anthers have choline and betaine, which promote faster fungus growth, aiding in the subsequent invasion of head tissues (Strange & Smith, 1971; Strange et al., 1974). Plants were spray-inoculated (1 ml/head) using a 0.5 L manual plastic sprayer (Vonder®). After the inoculation, the heads were covered with a transparent plastic bag and kept in an incubator at 25°C with a 12-h dark/light cycle for 24 hours.

3.4.3 Timing of chitosan oligosaccharide sprays

For this assay, the concentration of 2000 mg/L of chitosan oligosaccharide and 2.5 mg/L of the commercial formulation of tebuconazole were used. The products were sprayed using a 0.5 L manual plastic sprayer (Vonder®) at different times, 5 and 2 days before and after inoculation (-5, -2, +2, +5 days) and on the day of the inoculation one hour before to allow plant to dry out (0 days). Nonsprayed plants and noninoculated plants were used as controls.

3.4.4 Concentration of chitosan oligosaccharide

For this assay, the concentrations of 500, 1000, 2000 and 3000 mg/L of chitosan oligosaccharide and 2.5 mg/L of the commercial formulation of tebuconazole were used. Plants were sprayed with each concentration individually using a 0.5 L manual plastic sprayer (Vonder®), 5 days before inoculation at mid-anthesis. Nonsprayed plants and noninoculated plants were used as controls.

3.4.5 Biostimulant effect

To evaluate whether the chitosan oligosaccharide had any biostimulant effect, at the end of the cycle, before harvesting heads, the height of the plants was measured using a tape measure. After harvesting, wheat heads were hand threshed and the grains were weighed, to obtain the thousand-kernel weight (TKW), and visual analysis of these grains was also performed to determine the percentage of *Fusarium*-damaged kernels (FDK).

3.5 Field assay

The field trial was conducted in the experimental station at Universidade Federal de Viçosa (20°44'48.11" S, 42°50'57.51" W, 679 m above sea level) during the (April - August) in the 2021 growing season. The cultivar BR 18 Terena was also used in this trial. The treatments used in the field trial were 5 and 2 days before and after inoculation (-5, -2, +2, +5 days) and on the day of the inoculation one hour before to allow plant to dry out (0 days). After inoculation, wheat heads were covered with a transparent plastic bag for 24 hours. Experimental units consisted of microplots (1 x 1m). Five to six plants were inoculated per plot.

3.6 Mycotoxins analysis

Deoxynivalenol concentrations were determined from wheat kernels harvested from the greenhouse and field trials. Dried wheat heads were harvested at the end of the season, threshed and the kernels were stored at -20 °C until analysis. DON were determined by bulking the grains

from the individual heads (n = 5-6 heads per pot) and replicates (n = 3-4 pots). A 10-g subsample of the pooled kernels was sent to the Laboratory SAMITEC - Soluções Análiticas Microbiológicas e Tecnológicas Ltda, in Santa Maria, for analysis of DON. The amount of DON was quantified in the samples using a gas chromatography-mass spectrometry method as described previously (Fuentes et al., 2005; Mirocha et al., 1998).

3.7 Experimental design and data analysis

All experiments were conducted as a completely randomized design. Data from the replication of the assays in time were combined for the analysis when the experiment (added as a fixed effect in the model) was not significant. Effective concentration leading to a 50% reduction of mycelial growth or macroconidia germination (EC_{50}) and the respective standard error (SE) was estimated using the 'ec50estimator' and 'drc' packages (Ritz et al. 2015; Alves 2020). The data of seed treatment and foliar application assays were previously tested for normality and homoscedasticity and then subjected to the analysis of variance (ANOVA). Thus, the effect of main factors and the interaction between them were evaluated by the F test (α = 5%). Post hoc analyses were performed with the 'emmeans' package to obtain the estimates of the marginal means and the respective confidence intervals (Lenth, 2019). The ‘cld’ function from ‘multicomp’ R package (Hothorn et al. 2008) was used for multiple comparison of treatment means at 5% significance using Tukey test. Whenever there was a significant effect of the interaction between the factors, the multiple comparisons were conducted, unfolding all combinations of the levels of each factor within the other factors. All analyzes and plots were produced using R software (R Core Team 2022).

4. Results

4.1 Chitosan oligosaccharide sensitivity *in vitro*

Overall, the mycelial growth and conidia germination of *F. graminearum* were reduced with the increase of concentrations of tebuconazole and pyraclostrobin, respectively, and also for the chitosan oligosaccharide (Figure 7). For the mycelial growth assays using chitosan oligosaccharide and tebuconazole, the estimated EC_{50} values were 7,155.9 mg/L (± 1,344.69 standard error [SE]) and 0.11 mg/L (± 0.05 SE) respectively and for conidia germination assay

using chitosan oligosaccharide and pyraclostrobin the estimated EC_{50} were 519.31 mg/L (± 28.07 SE) and 0.0002 mg/L (± 0.21 SE), respectively.

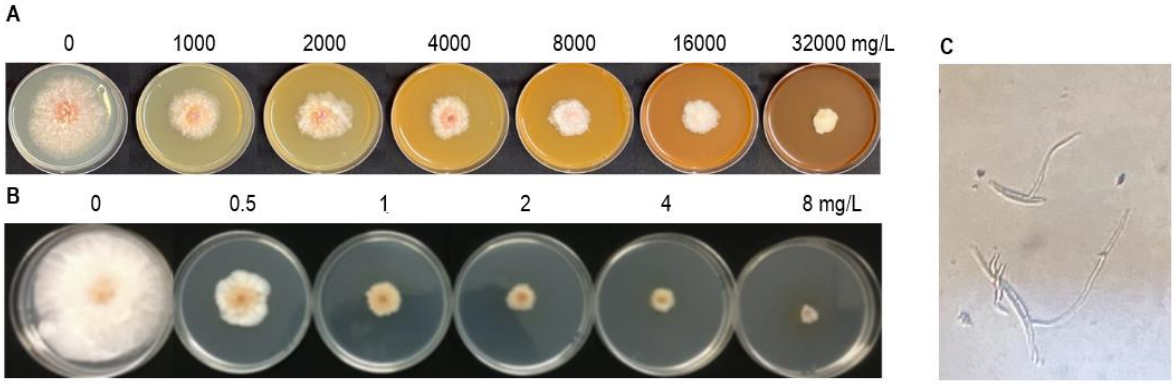

Figure 7. (A) Mycelial growth test for chitosan oligosaccharide **(B)** for tebuconazole and **(C)** conidia germination assay using chitosan oligosaccharide and pyraclostrobin.

4.2 Chitosan oligosaccharide in seed treatment

There was a significant effect for the triple interaction between the factors (treatment, inoculation, and surface sterilization; $P \leq 0.05$), but not for the experiment replication as a fixed effect ($P = 0.808$). For this reason, data from the two experiments were combined (Table 2). The incidence of *F. graminearum* in the inoculated and not desinfested control significantly higher than in the other controls (31.5%) ensuring that both the inoculation and the surface sterilization method were effective. The incidence of the pathogen in seeds treated with tebuconazole and chitosan oligosaccharide, disinfested and not disinfested, ranged from 6 to 7.5% and 8.5 to 15.5%, respectively. Tebuconazole and chitosan oligosaccharide treatments were able to reduce the incidence of *F. graminearum* in the seeds, differing statistically from the control, when the seeds were inoculated but not surface sterilized. On the other hand, there was no significant difference between the treatments when the seeds were not inoculated, regardless of surface sterilization.

Table 2. Estimates for the mean values (data from two experiments combined) of *F. graminearum* incidence in wheat seeds.

		Treatments		
Surface	**Inoculation**	**Tebuconazole**	**Chitosan**	**Check**

sterilization		oligosaccharide		
Disinfested	Inoculated	7.5 Aa	18 Bb*	13.5 ABa
Not disinfested		6 Aa	10 Aa	31.5 Bb*
Disinfested	Not inoculated	6.5 Aa	8.5 Aa	12 Aa
Not disinfested		7.5 Aa	15.5 Aa	13.5 Aa

The means followed by the lowercase letters in the columns and not followed by an asterisk in the columns did not differ from each other at 5% of significance by Tukey test.

Similar results were observed in the assay aiming to investigate the effect of seed treatment in seed germination. There was a significant effect for the triple interaction between the factors (treatment, inoculation, and surface sterilization; $P \leq 0.05$), but not for the experiment replication as a fixed effect ($P = 0.465$). For this reason, data from the two experiments were also combined (Table 3). The highest incidence of *F. graminearum* were observed in the inoculated and non-surface sterilized control. Consequently, the highest reduction in the percentage of seed germination was also observed for this treatment, showing a direct association between the presence of the fungus in the seeds and the reduction of the germination. The incidence of the pathogen in seeds treated with tebuconazole and chitosan oligosaccharide disinfested and not disinfested, ranged from 0 to 0.993% and from 1.49 and 6.33%, respectively. Seed germination for these treatments ranged from 65 to 80% and from 62 to 85%, respectively.

Table 3. Combined data from the two experiments, estimating the mean values of *F. graminearum* incidence in the germination assay in wheat seeds.

		Incidence (%)			Seed germination (%)		
Surface sterilized	**Inoculation**	**Tebuconazole**	**Chitosan oligosaccharide**	**Check**	**Tebuconazole**	**Chitosan oligosaccharide**	**Check**
Disinfested	Inoculated	0.993 Aa	1.49 Aa	3.98 Aa	65 Aa	74 Aa	69 Ab
Not disinfested		0 Aa	6.33 Bb	28.52 Cb	77 Bb	77 Ba	55 Aa
Disinfested	Not inoculated	0.457 Aa	3.26 Aa	0.299 Aa*	80 Aa	72 Aa	80 Aa*
Not disinfested		0.103 Aa	2.27 Aa	0.457 Aa*	80 Aa	85 Ab	81 Aa*

The means followed by the lowercase letters in the columns and not followed by an asterisk in the columns did not differ from each other at 5% of significance by Tukey test.

4.3 Timing of chitosan oligosaccharide sprays

Overall, there was a higher FHB severity in the second trial compared to the first trial. Disease severity at last evaluation, were reduced up to 8 times in wheat heads treated with chitosan oligosaccharide in the first assay compared to the control (Figure 8A), but no reduction was observed in the second trial (Figure 8B). For tebuconazole, in both trials, there was a reduction in disease severity (Figure 8).

Means values for AUDPC in plants treated with chitosan oligosaccharide ranged from 118.92 to 276.91, with the highest value presented in the treatment chi -2 in the first trial and chi -5 in the second, and for tebuconazole in inoculated plants, mean AUDPC values ranged from 37.20 to 89.60 (Figure 9). In general, both fungicide and chitosan oligosaccharide performed best in the first trial but only fungicide significantly reduced AUDPC compared to the NTC.

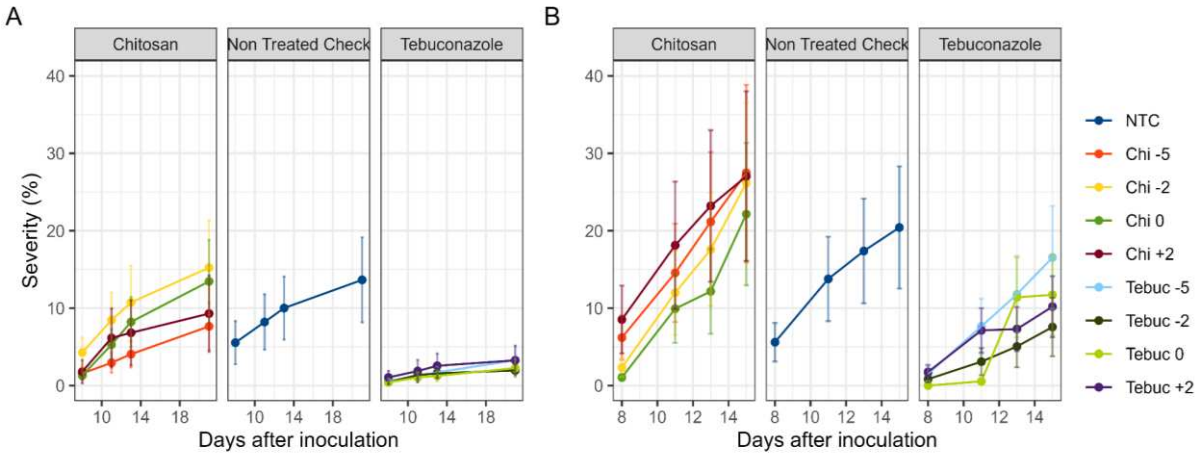

Figure 8. (A) Progress of mean Fusarium head blight severity and respective standard error in wheat heads sprayed at different timings in the first and (B) second trial. Treatments: NTC = Nontreated check, Chi = chitosan oligosaccharide and Tebuc = tebuconazole. Chi or Tebuc followed by: -5 = applied 5 days before inoculation, -2 = applied 2 days before inoculation, 0 = chitosan oligosaccharide applied on the day of inoculation, and +2 applied 2 days after inoculation.

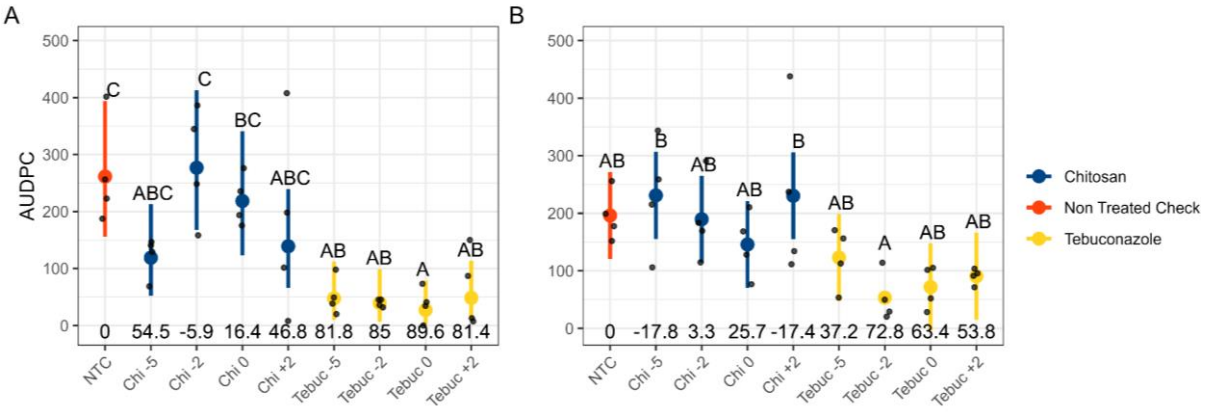

Figure 9. (A) Estimated mean area under disease progress curve (AUDPC) and respective 95% confidence intervals of Fusarium head blight severity in wheat heads sprayed at different timings in the first and (B) second trial. Treatments: NTC = Nontreated check, Chi = chitosan oligosaccharide and Tebuc = tebuconazole. Chi or Tebuc followed by: -5 = applied 5 days before inoculation, -2 = applied 2 days before inoculation, 0 = chitosan oligosaccharide applied on the day of inoculation, and +2 applied 2 days after inoculation. NTC = Nontreated check. The values below the points represent the percentage reduction compared to the NTC. Means followed by the same uppercase letters did not differ from each other at 5% significance by Tukey test.

4.3.1 Mycotoxin

DON was detected in all samples of bulked kernels, except in the mock not inoculated control (*data not shown*). There was an association between the reduction of mean FHB severity and mean AUDPC (Figure 8 and 9) and reduction in DON accumulation wheat grains (Figure 10). In general, some treatments with chitosan oligosaccharide have an effect, suppressing DON accumulation compared to the NTC. For example, the treatments Chi -5 in the first trial and Chi 0 and Chi +2 in the second trial, with reduction ranged from from 13.6 to 42.3%. Additionally, fungicide treatments were able to suppress DON accumulation from 49 to up to 97.1%.

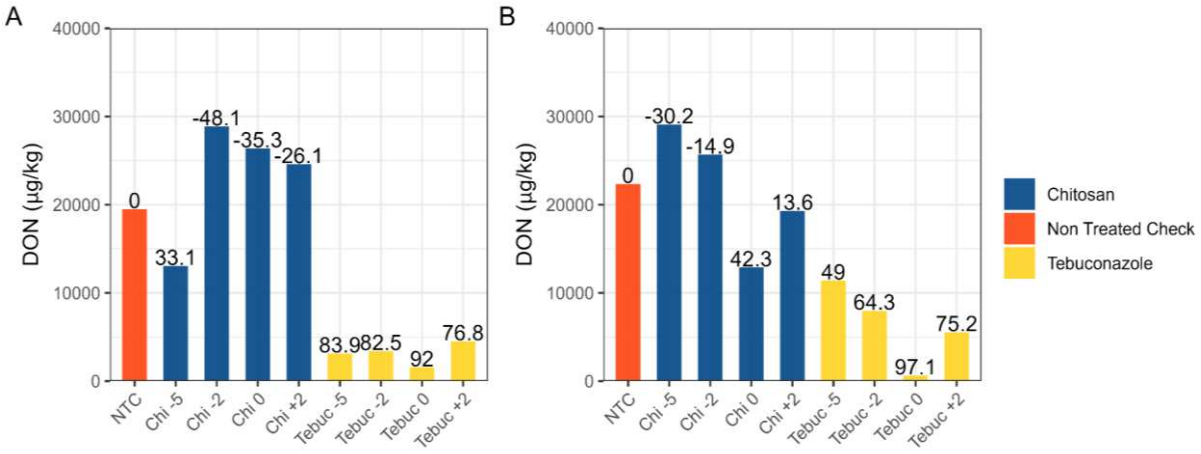

Figure 10. (A) Deoxynivalenol (DON) production in bulked kernel samples (n = 20 heads from all replicates) from inoculated wheat heads sprayed at different timings in the first and (B) second trial. Treatments: NTC = Nontreated Check, Chi = chitosan oligosaccharide and Tebuc = tebuconazole. Chi or Tebuc followed by: -5 = applied 5 days before inoculation, -2 = applied 2 days before inoculation, 0 = chitosan oligosaccharide applied on the day of inoculation, and +2 applied 2 days after inoculation. The values above the bars represent the percentage reduction in DON accumulation in relation to the NTC.

4.4 Concentration of chitosan oligosaccharide

Overall, wheat heads treated with different concentrations of chitosan oligosaccharide showed reduced disease severity compared to the control, in the first trial (Figure 11A), but not in the second trial (Figure 11B). For tebuconazole, there was a much higher reduction in FHB severity compared to the nontreated control.

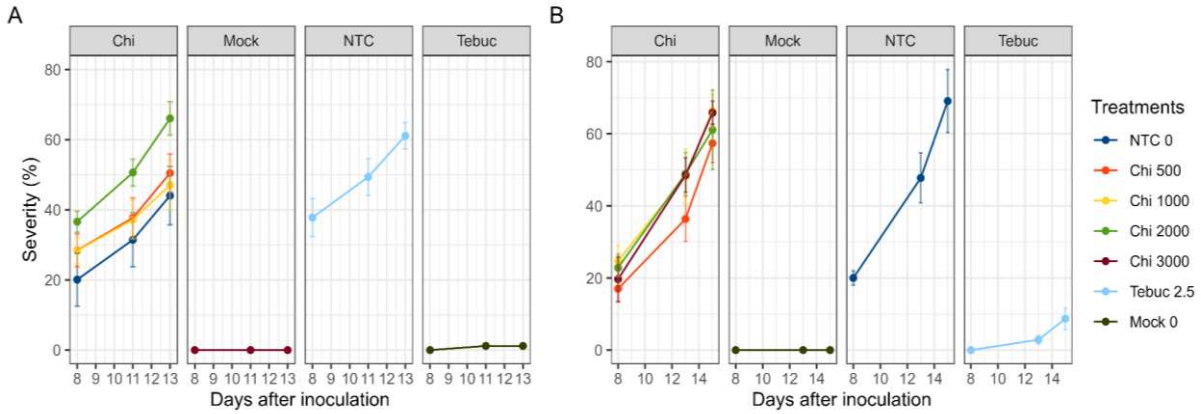

Figure 11. (A) Progress of mean Fusarium head blight severity and respective standard error in wheat heads sprayed at different chitosan oligosaccharide concentrations in the first and (B)

second trial. Treatments: NTC 0 = Nontreated check, Chi 500 = 500 mg/L chitosan oligosaccharide, Chi 1000 = 1000 mg/L chitosan oligosaccharide, Chi 2000 = 2000 mg/L chitosan oligosaccharide, Chi 3000 = 3000 mg/L chitosan oligosaccharide, Tebuc 2.5 = 2.5 mg/L tebuconazole an Mock 0 = mock inoculated control.

The AUDPC values in the first and second trials, using different doses of chitosan oligosaccharide, did not differ significantly from the NTC, with mean values ranging from 152.8 to 296.7 (Figure 12). Even without showing significant differences, a variation in the percentage of AUDPC reduction can be observed in the two assays. There was a significant difference for tebuconazole treatment compared to the NTC in both trials, with the mean AUDPC of 4.13 and 18.8 in the first and second trial, respectively. In both trials, mean AUDPC for chitosan oligosaccharide and for tebuconazole were significantly different from each other with the fungicide performing best.

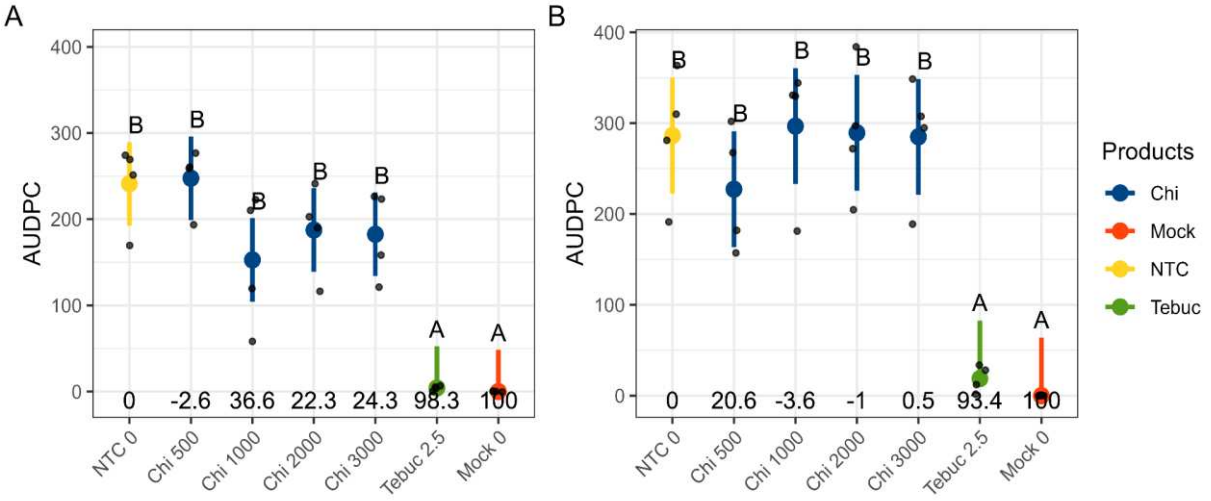

Figure 12. (A) Estimated mean area under disease progress curve (AUDPC) and respective 95% confidence intervals of Fusarium head blight severity in wheat heads sprayed at different concentrations in the first and (B) second trial. NTC 0 = Nontreated check, Chi 500 = 500 mg/L chitosan oligosaccharide, Chi 1000 = 1000 mg/L chitosan oligosaccharide, Chi 2000 = 2000 mg/L chitosan oligosaccharide, Chi 3000 = 3000 mg/L chitosan oligosaccharide, Tebuc 2.5 = 2.5 mg/L tebuconazole an Mock 0 = mock inoculated control. The values below the points represent the percentage reduction compared to the NTC. Means followed by the same uppercase letters did not differ from each other at 5% significance by Tukey test.

4.4.1 Mycotoxin

Similarly to the previous trial, DON was detected in all samples of bulked kernels, except in the mock inoculated control. Overall, there was an association between the reduction of mean FHB severity and mean AUDPC (Figure 11 and 12) and reduction in DON accumulation wheat grains (Figure 13). Similarly, some treatments with chitosan oligosaccharide have an effect, reducing both disease (AUDPC) and suppressing DON accumulation compared to the NTC. The treatments Chi 1000 in the first trial and Chi 500 in the second trial, reduced AUDPC in 36.6 and 20.6% and DON in 47.6 and 42.4% respectively. Consistently, fungicide treatment in both experiments was able to suppress DON accumulation from 78.6 up to 91.4% (Figure 13).

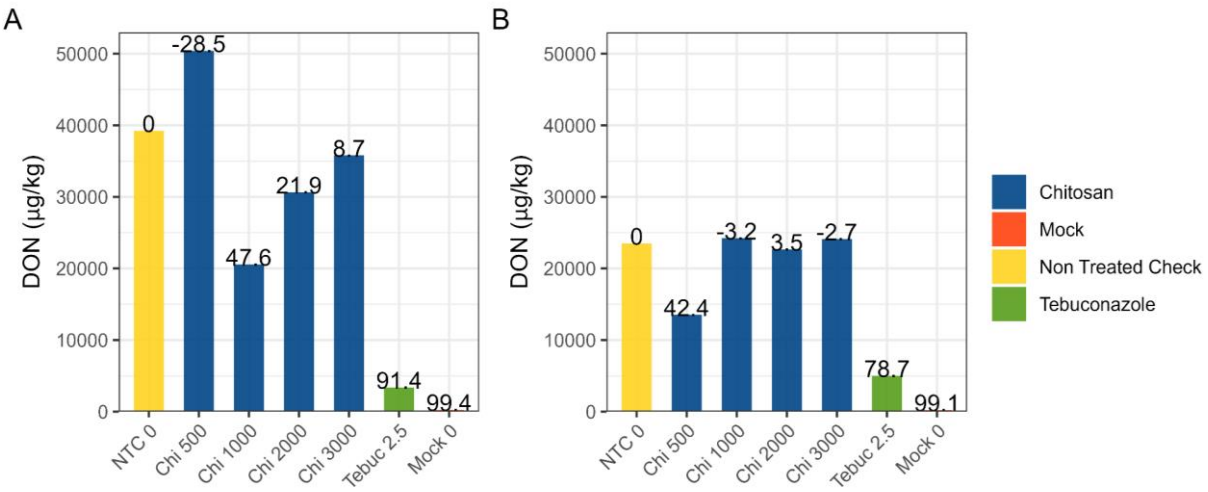

Figure 13. (A) Deoxynivalenol (DON) production in bulked kernel samples from inoculated wheat heads sprayed with different concentrations of chitosan oligosaccharide in the first and (B) second trial. NTC 0 = Nontreated check, Chi 500 = 500 mg/L chitosan oligosaccharide, Chi 1000 = 1000 mg/L chitosan oligosaccharide, Chi 2000 = 2000 mg/L chitosan oligosaccharide, Chi 3000 = 3000 mg/L chitosan oligosaccharide, Tebuc 2.5 = 2.5 mg/L tebuconazole an Mock 0 = mock inoculated control. The values above the bars represent the percentage reduction in DON accumulation in relation to the NTC.

4.5 Field assay

Overall, there was a large variation in the data which may have prevented us from detecting any significant difference between the treatments. Although not significant, there was a slight reduction in FHB severity on inoculated wheat heads treated with chitosan

oligosaccharide or tebuconazole. The highest reductions in disease severity were observed in plants treated with fungicide (44.5 to 100%) regardless of when it was applied. For plants treated with chitosan oligosaccharide, highest reduction in FHB severity was observed in the plants sprayed two days before inoculation (49.70%) followed by treatments applied on the day of inoculation (39.30%). On average, all treatments were able to reduce in relation to the nontreated check, ranging from 12.0 to 100%, except for chitosan oligosaccharide applied five days before inoculation (Figure 14).

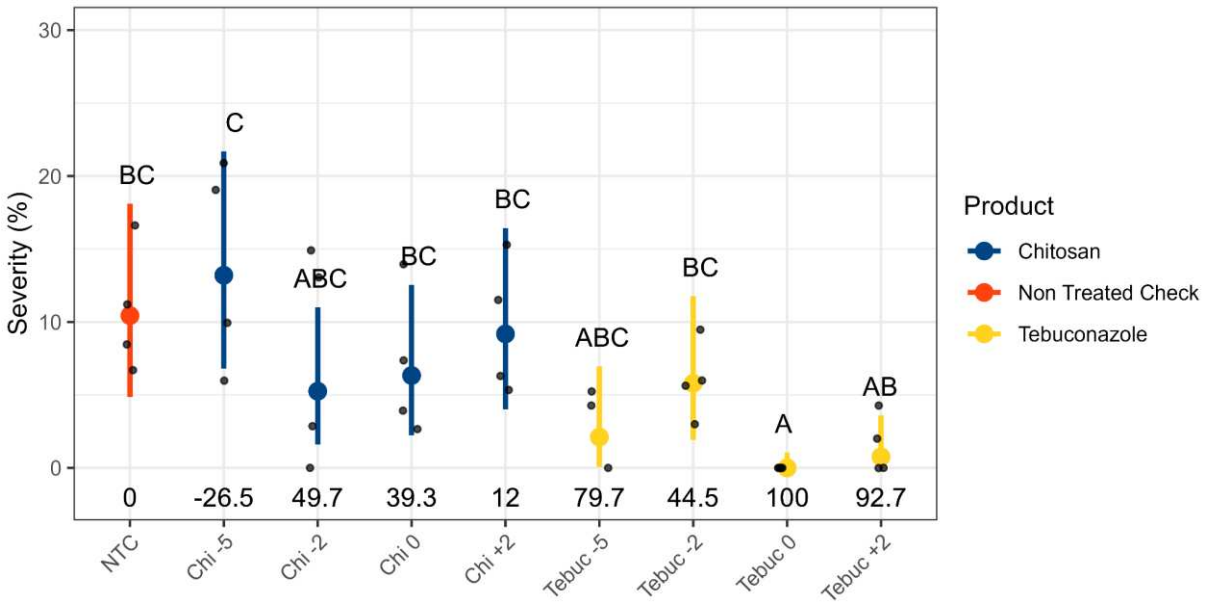

Figure 14. (A) Mean Fusarium head blight severity and respective 95% confidence intervals in wheat heads 21 days after inoculation sprayed at different timings in the first and (B) second trial. Treatments: NTC = Nontreated check, Chi = chitosan oligosaccharide and Tebuc = tebuconazole. Chi or Tebuc followed by: -5 = applied 5 days before inoculation, -2 = applied 2 days before inoculation, 0 = chitosan oligosaccharide applied on the day of inoculation, and +2 applied 2 days after inoculation. Means followed by the same uppercase letters did not differ from each other at 5% significance by Tukey test.

4.6 Biostimulant effect

There was no significant effect of chitosan oligosaccharide or even fungicide sprays in increasing treated plants height ($P > 0.05$), regardless of the timing of those sprays (Table S1) or concentration tested (Table S4). The percentage of *Fusarium*-damaged kernels ranged from 0 to 48.5% across all trials. Tebuconazole treatments significantly reduce FDK in inoculated plants across all trials (Table S2 and S5) compared to the NTC, except for the field trial (Table

S7). Conversely, chitosan oligosaccharide regardless of the timing of those sprays (Table S2) or concentration tested (Table S5) did not significantly reduce FDK compared to the NTC.

Thousand-kernel weight ranged between 25.80 and 53.20% across all trials (Tables S3, S6 and S8). Mean TKW was highest in plants treated with tebuconazole sprayed in different timings (Table S3). For chitosan oligosaccharide, some timings and concentrations were able to prevent TKW reduction of up to 50% under greenhouse conditions. There was no significant reduction in TKW in inoculated plants either sprayed with chitosan oligosaccharide or tebuconazole in the field (Table S8). No significant differences were also observed between inoculated or non-inoculated plants sprayed with either product (Table S8).

5. Discussion

In this study the potential use of the chitosan oligosaccharide in FHB management as well as its effect in suppressing DON accumulation in grains were investigated. In the literature, the use of chitosan and its derivatives is well reported for the control of pathogens and diseases. Several studies have investigated the potential use of chitosan in a wide range of plant pathogens, either the direct effect during fungal cultivation *in vitro*, for example *Sclerotium rolfsii* in carrots (Ahmed et al., 2019), *Botrytis cinerea* in kiwi (Hua et al., 2019), *Pseudoperonospora cubensis* in cucumber (Díaz et al., 2020), *Magnaporthe oryzae* in rice (Lopez-Moya et al., 2021), or to be use in post-harvest treatment of *Colletotrichum gloeosporioides, Colletotrichum acutatum, Fusarium oxysporum* and *Phytophthora* sp. in strawberry (Martínez et al., 2019), *Penicillium citrinum* and *Penicillium mallochii* in orange (Coutinho et al., 2020), *Lasiodiplodia theobromae* and *Colletotrichum gloeosporioides* avocado (Obianom et al., 2019), and others.

The present study was the first where the effect of chitosan oligosaccharide directly on *F. graminearum* was investigated. Although the estimated EC_{50} values were high compared to the fungicides included as controls, CML3066 isolate was sensitive to chitosan oligosaccharide as concentration was increased. These findings show that chitosan oligosaccharide can directly reduce both mycelial growth and macroconidia germination of *F. graminearum*. The direct effect of chitosan in some pathogens, interfering with mycelial growth, have already been reported (Berger et al. 2011). Previous studies have already reported the effect of chitosan inhibiting *F. graminearum* mycelial growth and conidia germination *in vitro* (Kheiri et al. 2016) and the effect of oligochitosan and chitosan hydrochloride, other chitosan derivatives, to inhibit

mycelial growth for *F. graminearum* (Xu et al. 2007; Deshaies et al. 2022) showing that as the concentration increased, the growth of the pathogen was affected.

The incidence of *F. graminearum* in seeds treated with tebuconazole and chitosan oligosaccharide, disinfested and not disinfested, were consistently reduced. In our study, the effect of chitosan oligosaccharide on the reduction of *F graminearum* incidence may be caused by a direct effect on the pathogen infecting seed surface. The direct effect of chitosan in pathogens infecting plant tissue surfaces have already been reported in other pathosystems, for example on gray mold and blue mold caused by *Botrytis cinerea* and *Penicillium expansum* in tomato fruit (Liu et al. 2007). More studies are needed to investigate the potential of oligosaccharide to reduce the incidence of already established infections, either by direct effect or inducing plant resistance.

In addition to controlling the pathogen, the chitosan oligosaccharide did not interfere with seed germination and, even if not significant, the chitosan oligosaccharide showed a slight increase in seed germination. Some studies in the literature mention that compounds derived from chitosan present can increase seed germination in several crops. Zohara et al. (2019) tested the use of chitosan as a biostimulant to control infection of cucumber by *Phytophthora capsici* and observed that in addition to controlling the pathogens, chitosan improve the germination rate and some traits such as shoot and root weight. Mazaro et al. (2009) evaluated the effect of treating sugar-beet and tomato seeds with chitosan to control of damping-off in substrate infested with *Rhizoctonia* sp., as a result, they reported that there was a reduction in the incidence of the pathogen, reducing the number of plants felled, which may be related to the increase in phenylalanine ammonia-lyase, since it acts in the formation of many plant defense mechanisms. Further studies can be performed to better understand the effect of the seed treatment with chitosan oligosaccharide in the development of the wheat plants.

In the greenhouse assays, we investigated if in addition to the direct effect on *F. graminearum*, chitosan oligosaccharide would be able to potentially induce host resistance. For such, chitosan oligosaccharide was sprayed in different timings around anthesis when the inoculation was performed. Kheiri et al. (2016) applied chitosan on wheat plants in 0, 3 and 5 days by spraying after inoculation. They observed that there was a reduction in severity of around 30% in treatments with chitosan in relation to the control also failing to differentiate the influence of the application period on disease control. Deshaies et al. (2022) observed that even when chitosan was applied pre or post inoculation in wheat plants for *F. graminearum*, the control was effective, reducing AUDPC. Unfortunately, our data did not provide enough

evidence to test this hypothesis. Although not significantly different from the NTC, there was a reduction in FHB severity and AUDPC in the plants sprayed five days before inoculation in the first trial and on the day of the inoculation in the second trial. These inconsistencies between them may be explained, at least in parts, by weather conditions during each trial. The disease progress rate was slower in the nontreated check in the first trial compared with the second, when the conditions were more conducive for the development of the disease. Further studies are needed to understand the best time of application of chitosan oligosaccharide to control FHB in wheat.

Our results suggest that some treatments were effective for the reduction of deoxynivalenol. In the first trial, in the treatment Chi -5 there was a reduction of 33.1% and in the second, in the treatment Chi 0 of 42.3% and +2 of 13.6% in the treatments that were applied to the chitosan oligosaccharide. Silva et al. (2015) used a chitosan film to reduce the aflatoxin produced by *Aspergillus parasiticus* in peanuts. The authors reported that the reduction of aflatoxin in the grains was greater than 80%. Zachetti et al. (2019) tested the use of chitosan as an alternative to control the growth and mycotoxin of *Fusarium verticilioides, F. proliferatum* (fumonisin) and *F. graminearum* (deoxynivalenol) production in corn and wheat and showed that chitosan had an effect on the reduction of these mycotoxins, as demonstrated in this study.

Chitosan oligosaccharide did not increase control efficacy with the increase of the concentration sprayed in wheat plants. The plants treated with different doses of chitosan oligosaccharide, applied five days before inoculation some doses were able to reduce the severity of *F. graminearum* in relation to the control, in the different days of evaluation and consequently reduce the AUDPC, but there were no significant differences between them. Zachetti et al. (2019) conducted an *in vitro* study using different doses of chitosan to inhibit the mycelial growth of different *Fusarium* species. In their results, for *Fusarium graminearum*, they observed that, regardless of the dose used, there was no interference in the growth of the isolate. In our study, as discussed above, in the *in vitro* tests we were able to observe a decrease in mycelial growth and macroconidia germination with the increase of chitosan oligosaccharide concentrations, which were not observed *in planta*.

Additionally, chitosan oligosaccharide did not present a biostimulant effect on plant heights in all assays. Cancio et al. (2014) included in their study that chitosan-treated bean seeds did not show significant differences in plant height when compared to the control. The weight of 1000 grains was also evaluated. The treatments with chitosan oligosaccharide applied 5 days before inoculation showed an increase of 11.8% in the final weight of the grains. In the second

trial, the treatments applied on the day of inoculation and 2 days after inoculation showed an increase of 14.76 and 7.05%. He et al. (2018) using chitosan oligosaccharide for pre-harvest treatment of strawberry and concluded that the product brought an increase in viscosity, lignin, sugar, protein, total soluble solids and titratable acidity in the fruits.

6. Conclusions

1. In *in vitro* tests, chitosan oligosaccharide was able to reduce mycelial growth and conidia germination at higher doses of the product.
2. In the greenhouse and field trials, chitosan oligosaccharide was able to reduce the FHB intensity, although it did not differ from the control.
3. The amount of deoxynivalenol accumulated in wheat grains inoculated under greenhouse conditions was reduced in some greenhouse treatments.
4. The results of this study suggest that chitosan oligosaccharide has some direct effect on *F. graminearum*, but further studies are needed to better understand the *in vivo* efficacy of this product in combination with fungicides.

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SUPPLEMENTARY MATERIAL

Table S1. Mean values of plant height (cm) for the effect of different timing of chitosan oligosaccharide and tebuconazole sprays in relation to the inoculation on inoculated wheat heads under greenhouse conditions.

Treatments	Timing of sprays	Trial 1		Trial 2	
		Inoculated	Non inoculated	Inoculated	Non inoculated
Chitosan oligosaccharide	-5	70.93[ns]	69.35[ns]	80.65[ns]	81.70[ns]
	- 2	74.73	69.35	83.90	82.00
	0	71.10	71.70	79.80	83.15
	+2	73.00	68.30	83.25	79.01
Tebuconazole	-5	69.80	70.65	80.75	78.75
	-2	72.68	74.95	83.90	80.40
	0	73.13	71.80	84.10	80.20
	+2	68.15	73.25	81.40	80.35
Nontreated Check		73.24	72.25	82.45	84

[ns] Means not differ from each other at 5% of significance by Tukey test.

Table S2. Mean values of *Fusarium*-damaged kernels (FDK, %) for the effect of different timing of chitosan oligosaccharide and tebuconazole sprays in relation to the inoculation on inoculated wheat heads under greenhouse conditions.

Treatments	Timing of sprays	Trial 1		Trial 2	
		Inoculated	Non inoculated	Inoculated	Non inoculated
Chitosan oligosaccharide	-5	25 abc	0 aA	36.50 c	0 aA
	- 2	26 abc	0 aA	36 c	0 aA
	0	29.50 bc	0 aA	21 bc	0 aA

	+2	38.50 c	0 aA	25 bc	0 aA
Tebuconazole	**-5**	10 ab	0 a	23 bc	0 aA
	-2	9.5 ab	0 a	17 ab	0 aA
	0	7 a	0 a	1 a	0 a
	+2	12 ab	0 a	10 ab	0 aA
Nontreated Check		45.50 c	0 aA	33.50 c	0 aA

The means followed by the lowercase letters in the columns and not followed by uppercase letter in the columns did not differ from each other at 5% of significance by Tukey test.

Table S3. Mean values of thousand-kernels weight (TKW, g) for the effect of different timing of chitosan oligosaccharide and tebuconazole sprays in relation to the inoculation on inoculated wheat heads under greenhouse conditions.

Treatments	**Timing of sprays**	**Trial 1**		**Trial 2**	
		Inoculated	Non inoculated	Inoculated	Non inoculated
Chitosan oligosaccharide	**-5**	37.90 abc	50.05 aA	25.80 d	46.50 a
	- 2	35.50 bc	48.80 aA	28.20 c	43.60 aA
	0	33.50 bc	48.40 aA	34.20 ab	47.50 aA
	+2	32.75 c	49.80 aA	31.90 abc	47.10 aA
Tebuconazole	**-5**	47.90 ab	44.75 a	33.80 abc	46.10 aA
	-2	46.40 abc	48.75 a	37.70 ab	48.25 aA
	0	51.80 a	46.60 a	53.20 a	48.10 a
	+2	45.50 abc	51.65 a	41.80 a	51.80 aA
Nontreated Check		33.90 bc	49.70 aA	29.80 bc	44.40 aA

The means followed by the lowercase letters in the columns and not followed by uppercase letter in the columns did not differ from each other at 5% of significance by Tukey test.

Table S4. Mean values of plant height (cm) for the effect of different chitosan oligosaccharide concentrations sprayed on inoculated wheat heads under greenhouse conditions.

Treatments	Concentrations (mg/L)	Trial 1	Trial 2
Chitosan oligosaccharide	500	75.80[ns]	77.00[ns]
	1000	77.30	80.30
	2000	76.50	78.70
	3000	79.50	77.60
Tebuconazole	2.5	78.20	78.80
Mock		76.80	76.10
Nontreated check		81.10	85.50

[ns] Means not differ from each other at 5% of significance by Tukey test.

Table S5. Mean values of *Fusarium*-damaged kernels (FDK, %) for the effect of different chitosan oligosaccharide concentrations sprayed on inoculated wheat heads under greenhouse conditions.

Treatments	Concentrations (mg/L)	Trial 1	Trial 2
Chitosan oligosaccharide	500	49.50 b	35.50 bc
	1000	45.50 b	34.00 b
	2000	44.50 b	35.50 bc
	3000	47.50 b	49.50 bc
Tebuconazole	2.5	0 a	8.00 a
Mock		0 a	0 a
Nontreated check		53.50 b	57.00 c

The means followed by the lowercase letters in the columns and not followed by an asterisk in the columns did not differ from each other at 5% of significance by Tukey test.

Table S6. Mean values of thousand-kernels weight (TKW, g) for the effect of different chitosan oligosaccharide concentrations sprayed on inoculated wheat heads under greenhouse conditions.

Treatments	Concentrations (mg/L)	Trial 1	Trial 2
Chitosan oligosaccharide	500	17.90 b	28.50 b
	1000	23.60 b	27.10 b
	2000	24.20 b	27.70 b

	3000	26.10 b	25.10 b
Tebuconazole	**2.5**	45.10 a	42.40 a
Mock		40.50 a	50.90 a
Nontreated check		21.90 b	25.40 b

The means followed by the lowercase letters in the columns and not followed by an asterisk in the columns did not differ from each other at 5% of significance by Tukey test.

Table S7. Mean values of *Fusarium*-damaged kernels (FDK, %) for the effect of different timing of chitosan oligosaccharide and tebuconazole sprays in relation to the inoculation on inoculated wheat heads under field conditions.

Treatments	Timing of sprays	Inoculated	Non inoculated
Chitosan oligosaccharide	**-5**	4 ab	0.50 a
	- 2	8 ab	0 aA
	0	6 ab	1.5 a
	+2	10.5 ab	0 aA
Tebuconazole	**-5**	1 a	0 a
	-2	2 a	0 a
	0	0 a	0 a
	+2	1.5 a	0 a
Nontreated Check		6 ab	1 a

The means followed by the lowercase letters in the columns and not followed by uppercase letter in the columns did not differ from each other at 5% of significance by Tukey test.

Table S8. Mean values of thousand-kernels weight (TKW, g) for the effect of different timing of chitosan oligosaccharide and tebuconazole sprays in relation to the inoculation on inoculated wheat heads under field conditions.

Treatments	Timing of sprays	Inoculated	Non inoculated
Chitosan oligosaccharide	**-5**	34.80 a	38.50 ab
	- 2	32.10 a	33.60 ab
	0	32.90 a	28.90 c
	+2	38 aA	30.90 bc

Tebuconazole	**-5**	38.90 a	43.90 a
	-2	35 aA	29 bc
	0	34.50 a	38.50 a
	+2	30.20 a	38 abA
Nontreated Check		33.40 a	30.60 bc

The means followed by the lowercase letters in the columns and not followed by uppercase letter in the columns did not differ from each other at 5% of significance by Tukey test.