

UNIVERSIDADE FEDERAL DE VIÇOSA

**Effects of Chitosan Oligosaccharide-Amended Fungicide Treatments on the
Control of Fusarium Head Blight in Wheat**

David Aimar Sousa Silva
Magister Scientiae

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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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To my parents, Francisca e Evangelista
To my sisters, Dhully, Nataly, Francine and Elisa
To my girlfriend, Ana Paula
To professor Leonardo (In memoriam)
I dedicated.

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ABSTRACT

SILVA, David Aimar Sousa, M.Sc., Universidade Federal de Viçosa, February, 2025. **Effects of Chitosan Oligosaccharide-Amended Fungicide Treatments on the Control of Fusarium Head Blight in Wheat.** Adviser: Franklin Jackson Machado.

Fusarium Head Blight (FHB) is a destructive fungal disease of wheat caused primarily by *Fusarium graminearum*, which reduces grain yield and quality through the accumulation of mycotoxins, especially deoxynivalenol (DON). Current management strategies rely heavily on fungicide applications; however, their effectiveness varies among active ingredients, and overuse may lead to fungicide resistance and environmental concerns. Chitosan oligosaccharide (COS) is a product derived from chitin deacetylation that has been studied for its diverse biological properties and applications in various fields, including its ability to inhibit some fungi that cause plant diseases. In this study, greenhouse and field trials were conducted to evaluate the potential of COS, applied isolated and in combination with site-specific fungicides (tebuconazole, metconazole, pydiflumetofen, and thiophanate-methyl), for FHB management and reduction of DON levels in wheat grains. In greenhouse trials, FHB severity was assessed at 8-, 11-, 14-, 17- and 20-days post inoculation. Then, the area under the disease progress curve was determined. The severity at 20 days post-inoculation also was evaluated. In the field, disease incidence and severity, test weight, thousand-kernel weight, and grain yield per plot were evaluated. Additionally, DON concentration and the percentage of Fusarium-damaged kernels were determined for kernels samples harvested in all trials. Overall, COS alone had a limited effect on FHB suppression under both greenhouse and field conditions. However, when combined with pydiflumetofen, the mixture significantly improved disease control, enhanced grain quality and yield, and reduced DON accumulation compared to the fungicide applied alone. The COS–thiophanate-methyl combination also increased fungicide efficacy by reducing disease. Treatments based on tebuconazole and metconazole also showed strong performance, with efficacy rates exceeding 90% in some cases. Although COS demonstrated direct action against *F. graminearum*, its relatively low standalone efficacy suggests that it should be used as an adjuvant rather than a fungicide replacement for fungicides. These findings support the integration of COS into chemical control programs to improve FHB management.

Keywords: *Fusarium graminearum*; Chemical control; *Triticum aestivum*;

Plant protection; Deoxynivalenol

RESUMO

SILVA, David Aimar Sousa, M.Sc., Universidade Federal de Viçosa, fevereiro de 2025. **Efeitos dos tratamentos de fungicida com oligossacarídeo de quitosana no controle de giberela no trigo.** Orientador: Franklin Jackson Machado.

A Giberela é uma doença fúngica destrutiva da cultura do trigo causada principalmente por *Fusarium graminearum*, que reduz o rendimento e qualidade dos grãos por meio do acúmulo de micotoxinas, especialmente desoxinivalenol (DON). As estratégias de manejo atuais dependem fortemente da aplicação de fungicidas; no entanto, sua eficácia varia entre os ingredientes ativos, e o uso excessivo pode levar à resistência a fungicidas e a preocupações ambientais. O oligossacarídeo de quitosana (COS) é um produto derivado da desacetilação da quitina que tem sido estudado por suas propriedades biológicas diversas e aplicações em vários campos, incluindo sua capacidade de inibir alguns fungos causadores de doenças em plantas. Neste estudo, ensaios em casa de vegetação e em campo foram conduzidos para avaliar o potencial do COS, aplicado isoladamente e em combinação com fungicidas de sítio específico (tebuconazol, metconazol, pydiflumetofen e tiofanato-metílico), para o manejo da Giberela e redução dos níveis de DON em grãos de trigo. Nos ensaios em casa de vegetação, a severidade foi avaliada aos 8, 11, 14, 17 e 20 dias após a inoculação. Em seguida, foi determinada a área abaixo da curva de progresso da doença. A severidade aos 20 dias após a inoculação também foi avaliada. No campo, foram avaliadas a incidência e severidade da doença, o peso do hectolitro, o peso de mil grãos e a produtividade por parcela. Além disso, a concentração de DON e a porcentagem de grãos giberelados foram determinadas para amostras de grãos colhidos de em todos os ensaios. No geral, o COS sozinho teve um efeito limitado na supressão da Giberela em condições de campo e casa de vegetação. No entanto, quando combinado com pydiflumetofen, a mistura melhorou significativamente o controle da doença, melhorou a qualidade e a produtividade dos grãos e reduziu o acúmulo de DON em comparação com o fungicida aplicado isoladamente. Os tratamentos à base de tebuconazole e metconazol também apresentaram forte desempenho, com taxas de eficácia superiores a 90% em alguns casos. Embora o COS tenha demonstrado ação direta contra *F. graminearum*, sua eficácia isolada relativamente baixa sugere que ele deve ser usado como adjuvante, e não como substituto dos fungicidas. Essas descobertas corroboram a integração do COS em programas de controle químico para aprimorar o manejo da Giberela.

Palavras-chave: *Fusarium graminearum*; Controle químico; *Triticum aestivum*;
Proteção de plantas; Deoxinivalenol

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1. INTRODUCTION

Chitosan as well as their derivatives are abundant natural compounds with high potential for use in agriculture. Recent studies show that these molecules exhibit toxicity and inhibit fungi development, being active against viruses, bacteria, and other pests (Davydova *et al.*, 2011; Badawy *et al.*, 2014; Dodgson and Dodgson, 2017; Fan *et al.*, 2023). Moreover, chitosan has been studied as a plant resistance inducer in several crops (Jia *et al.*, 2018; Yin *et al.*, 2023). Chitosan is the deacetylated product of chitin which is naturally found in the cell walls of fungi and the exoskeleton of marine crustaceans. Chitosan oligosaccharide (COS) is a chitosan oligomer composed of glucosamine and N-acetylglucosamine units. COS has shown potential antifungal activity against a range of fungi that cause important plant disease, including Fusarium Head Blight (FHB, Xu *et al.*, 2007; Rahman *et al.*, 2014; Badawy *et al.*, 2014; Kheiri *et al.*, 2016; Wang *et al.*, 2021; Ke *et al.*, 2022 ; Paiva *et al.*, unpublished data).

FHB is a major fungal disease of wheat caused by the *Fusarium graminearum* species complex (FGSC, McMullen *et al.*, 2012). Members of FGSC infect wheat heads, reducing crop yield and depreciating grain quality through the production and accumulation of mycotoxins that are harmful to animals, such as deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZON, Malekinejad *et al.*, 2007; Del Ponte *et al.*, 2012; Huang *et al.*, 2020; Mielniczuk and Skwaryło-Bednarz, 2020). FHB is a difficult disease to control as it can be devastating under conducive weather conditions (Bolanos-Carriel *et al.*, 2020). Disease control aims to minimize yield losses and reduce mycotoxins accumulation in wheat grain. Wheat producers must comply with the maximum allowable levels of DON in small grains and food products established in Brazil and other countries (ANVISA, 2011, 2017, 2022; Van Egmond *et al.*, 2007). Therefore, integrating disease control measures such a cultural practices that reduce fungal inoculum, sowing FHB-resistant cultivars, applying fungicides or biological control agents, among others, is highly recommended (Willyerd *et al.*, 2012; McMullen *et al.*, 2012; Mielniczuk and Skwaryło-Bednarz, 2020).

Chemical control has been the main method for managing FHB in Brazil and many other producing areas around the world (Wegulo *et al.*, 2015; Duffeck *et al.*, 2020). Triazole fungicides, known as demethylation inhibitors (DMIs), which inhibits the ergosterol biosynthetic pathway leading to cellular membrane instability, are used though their efficacy varies among different active ingredients (Paul *et al.*, 2008; Machado *et al.*, 2017; Anderson *et al.*, 2020). Fungicide sprays for FHB control begin at early anthesis and can be extended several days after this period to prevent mycotoxin accumulation in the gains (Freije and Wise, 2015; Caldwell *et al.*, 2017).

Several factors can favor disease development and also affect fungicide efficacy, such as rain and mild temperatures during anthesis, increased FHB inoculum pressure, and lower coverage of wheat heads during fungicide sprays (Singh *et al.*, 2021; González-Domínguez *et al.*, 2021). Additionally, the intensive use of fungicide sprays in FHB management may increase the selection pressure on *F. graminearum* populations in wheat-producing regions of Brazil and worldwide, contributing to the emergence of resistant strains against the most used active ingredients (Chen *et al.*, 2021; Andrade *et al.*, 2022; Machado *et al.*, 2023; Nakajima *et al.*, 2024). Furthermore, the use of pesticides, in general, may also cause health problems and environmental contamination (Tleuova *et al.*, 2020).

Sustainable FHB management through chemical control, similarly to other plant diseases, can be achieved with the frequent introduction of products with new modes of action (Steinberg and Gurr, 2020). However, this scenario requires significant investment and time (Russell, 2006). Therefore, the use of alternative measures, such as products based on organic compounds, is necessary to aid in FHB control and thus reduce the number of synthetic fungicides applied.

Some studies on the antifungal activity of natural compounds against FGSC members, extracted from living organisms show potential to inhibit mycelial growth *in vitro* and suppress mycotoxins accumulation in wheat grains, including γ -oryzanol extracted from rice plants (Bemvenuti *et al.*, 2019), *Curcuma longa* alcohol extract (Chen *et al.*, 2018), mustard-based botanicals (Drakopoulos *et al.*, 2020) and timol (Gao *et al.*, 2016). However, there are few studies with tests in field trials.

Given the limitations of traditional fungicide-based approaches, combining COS with fungicides may offer a more effective strategy for FHB chemical control and mycotoxin reduction in wheat grains. Application of COS in wheat plants has shown potential to reduce the severity of FHB in greenhouse and field trials, in addition to reducing the amount of DON concentration in wheat kernels inoculated under greenhouse conditions (Paiva *et al.*, unpublished data). Poznanski *et al.* (2023) demonstrated that the combination of chitosan and azoxystrobin resulted in increased fungicide's efficacy against *F. graminearum*. Another recent study observed that spraying COS mixed with tebuconazole was more effective in controlling FHB compared to the application of tebuconazole alone in greenhouse trials (Dopp *et al.*, unpublished data). By synergistically enhancing antifungal activity this integrated approach could contribute to improved disease management. COS is a water-soluble biodegradable compound with antioxidant activity, potentially reducing oxidative stress that triggers mycotoxins biosynthesis in *F. graminearum* (Sun *et al.*, 2008; Audenaert *et al.*, 2010). In this

study, we propose to quantify the efficacy of different fungicides and chitosan oligosaccharide, both individually and in combination, in FHB control and DON accumulation. The main objective of this study was to investigate whether the addition of COS enhances control efficacy and whether COS can reduce the required amount of fungicide to FHB control.

2. MATERIALS AND METHODS

2.1 Greenhouse trials

The greenhouse trials were carried out at the Universidade Federal de Viçosa, Viçosa, Minas Gerais state, Brazil (20° 45 '29.12 " S, 42° 52' 11.92" W, 660 m altitude) from late April to August 2024. The *F. graminearum* isolate CML 3066 was used in all trials (Del Ponte *et al.*, 2015; Machado Wood *et al.*, 2020). The COS product used in this work was provided by America Latina Agricultura Sustentável (ALAS). The fungicide treatments evaluated in our study used, applied either individually or in combination with COS, along with their respective rates, are listed in **Table 1**.

Ten seeds of wheat of the BRS 264 cultivar (Albrecht, 2006), susceptible to FHB, were sown in 1-liter pots filled with a 1:1 mixture of red latosol soil and commercial substrate prepared. A 50 mL/pot nutrient solution was applied weekly to adjust fertility conditions. The nutrient solution applied was 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). Two weeks after sowing, pots were thinned out to leave five plants/pot. The experiment was designed in a completely randomized design, including 16 treatments and 4 replicates. Each experimental unit consisted of a pot containing five wheat plants.

Plants were sprayed at the early anthesis stage (62 of Zadoks scale; Zadoks *et al.*, 1974). Early anthesis was observed as the first day that 50% of the plants were extruding 50% of their anthers. A second spray was performed seven days later (**Table 1**). At the time of treatment application, COS was dissolved in 500 mL of sterile distilled water at a concentration of 2000 mg/L. Fungicides were diluted in the same volume of water to the doses specified in Table 1. For preparing the fungicide-COS mixtures, COS was first diluted in 250mL of water and then mixed with the fungicide solution, with all components adjusted to a final volume of 500 mL. The final volume of each fungicide-COS mixture was 500 mL. The concentration for each fungicide tested in mixture treatments was the same used for each single product applied. Plants were sprayed using a 1.5 m boom sprayer equipped with four fan-type nozzles and a CO₂ cylinder for pressurization, adjusted to a flow rate of 200 L/ha. Control treatments included

nontreated plants (CHECK, Table 1) and non-inoculated plants (blank). For the treatments used in field trials, the volume of solution prepared for each treatment was 2L.

Table 1. Summary of the treatments applied in the greenhouse and field experiments.

Treatments	Code	Product	Rate (mL or g/ha)	Number of sprays
Nontreated check	CHECK	-	-	-
Tebuconazole ^a	TEBU	Tebufort®	750	2
Metconazole ^a	METC	Caramba® 90	1000	2
Pydiflumetofen ^a	PYDI	Miravis®	800	2
Thiophanate-methyl ^a	TMET	Cercobin® 875 WG	900	2
COS ^a	COS 1×	Chitosan oligosaccharide	420	1
COS ^a	COS 2×	Chitosan oligosaccharide	420	2
Tebuconazole + COS	TEBU+COS 1×	Mixtures	750 + 420	1
Metconazole + COS	METC+COS 1×	Mixtures	1000 + 420	1
Pydiflumetofen + COS	PYDI+COS 1×	Mixtures	800 + 420	1
Thiophanate-methyl + COS	TMET+COS 1×	Mixtures	900 + 420	1
Tebuconazole + COS ^a	TEBU+COS 2×	Mixtures	750 + 420	2
Metconazole + COS ^a	METC+COS 2×	Mixtures	1000 + 420	2
Pydiflumetofen + COS ^a	PYDI+COS 2×	Mixtures	800 + 420	2
Thiophanate-methyl + COS ^a	TMET+COS 2×	Mixtures	900 + 420	2

^a Treatments also used in field trials.

COS = Chitosan oligosaccharide (Concentration = 95% and N = 5%; pH = 4-6; MO = 80%, molecular weight ≤ 1.500 kDa and water-soluble) provided by América Latina Agricultura Sustentável (ALAS). Nontreated check = no product application. Mixtures = COS pre-diluted in water and then added to the indicated commercial fungicide solution. Number of sprays = “1” indicated one application once after inoculation during the early anthesis stage (62 of Zadoks scale; Zadoks *et al.*, 1974), and = “2” indicated that a second application was performed seven days later. In field trials no inoculation was performed, and the first application was performed during the early anthesis stage. Application flow rate = 200 L/ha. Source: prepared by the author.

For the inoculation procedure, the CML 3066 isolate was cultivated on potato dextrose agar (PDA) for 7 days at $25 \pm 1^\circ$ C with a 12-h dark/light cycle. Mycelial plugs were transferred to plates containing *Spezieller Nährstoffarmer Ágar* (SNA) and incubated under the same conditions for 7 days. Macroconidia were harvested from SNA plates by scraping them with sterile distilled water and subcultured onto fresh SNA plates for sporulation. On the day of inoculation, macroconidia were harvested from 7-days-old SNA cultures by washing and scraping the mycelium with sterile water. The macroconidial suspension was filtered through sterile gauze and adjusted to 1×10^4 macroconidia/mL. Inoculation was performed one day after treatment application, by spraying 1 mL of the macroconidial suspension onto the wheat heads until runoff. Inoculated plants were covered with moistened transparent plastic bags. Plants were incubated in a growth chamber at 25° C with a 12-h photoperiod. After 24 hours, plants

were transferred to the greenhouse and kept until grain harvest. The experiment was conducted twice.

In both experiments, the severity of FHB in wheat plants was evaluated at 8-, 11-, 14-, 17- and 20-days post inoculation (d.p.i.) by counting symptomatic spikelets in each head. The FHB severity (%) was calculated by proportion of symptomatic spikelets by the total number of spikelets per head multiplied by 100. Disease progress was monitored over time. After 20 d.p.i., plants were grown until senescence. Heads were harvested, stored in paper bags, and threshed manually. The percentage of Fusarium damaged kernels (FDK) was calculated for each pot by dividing the number of symptomatic kernels by the total number of grains and multiplying by 100.

2.2 Field trials

Field experiments were conducted during the growing season of 2024, from May to August, in the experimental station of the Department of Plant Pathology, Universidade Federal de Viçosa, Viçosa, MG, Brazil (20°44'48.11" S, 42°50'57.51" W, 679 m altitude). The experimental site has a history of natural occurrence of FHB. The first sowing was established on May 17, 2024, using BRS 264 wheat cultivar in experimental plots of 6 m² using a row spacing of 0.2m and a seeding rate of 150 kg.ha⁻¹. A second sowing was carried out 10 days later under the same conditions. Both plantings were conducted in adjacent fields that had been prepared with conventional tillage after a corn crop.

A basal application of 435 kg.ha⁻¹ NPK 8-28-16 fertilizer was applied at sowing, followed by a topdress of 375 kg.ha⁻¹ NPK 20-0-20 at 21 days after sowing. Weed control was achieved with a single foliar application of 0.65 L.ha⁻¹ 2,4D (Aminol®) at 24 days after sowing.

The experiment was arranged in a randomized block design with 11 treatments and 4 blocks, resulting in 44 experimental plots for each experiment. The same treatments used in greenhouse assay, excluding the single-application mixtures and the blank (Nontreated and mock inoculated), were applied in the field (**Table 1**).

FHB incidence and severity were assessed on plants reaching the soft dough stage (85 of Zadoks scale; Zadoks *et al.*, 1974), on August 7 and 20 August 2024. Assessments were made by randomly selecting fifty wheat heads from the central five rows of each plot over a 1-m length. FHB incidence was calculated by counting the number of symptomatic heads among the total number of heads observed in that plot. Severity was rated using Stack and McMullen scale (1995).

First and second trials were harvested 13 and 16 days after the disease assessment. In both cases, the plants were nearing physiological maturity. After harvest, the material from each

plot was placed on a cover screen to allow them to dry. Wheat heads were threshed, and the samples were stored for later analysis. Grain yield ($\text{kg}\cdot\text{ha}^{-1}$) was based on weight kernels of the central 4 m^2 of each plot. Thousand-kernels weight (g) was estimated by the hundred-kernels weight of each sample multiplied by 1000 and divided by 100. The percentage of Fusarium-damaged kernels—grains with a shrunken and wizened appearance—was determined by dividing the number of symptomatic grains by the total number of grains in 100g samples and multiplying by 100. The test weight was determined by weighing a 100mL volume of kernels.

2.3 Mycotoxins analysis

After harvest and drying, 10 g subsamples grains were identified and stored at -20°C until mycotoxins analysis. DON levels were determined in grains from both greenhouse and field experiments. For greenhouse samples, DON was determined by bulking the kernels from each treatment, while for field samples, DON was determined by a sub-sample of the kernels harvested from each plot. The amount of DON was quantified in the samples using an enzyme-linked immunosorbent assay (ELISA) following the AgraQuant® Deoxynivalenol PLUS protocol kit (Romer Labs®).

2.4 Data analysis

The maximum FHB severity in greenhouse trials was determined at 20 d.p.i. (hereafter FHB severity). Severity data were used to estimate the mean severity and the area under the disease progress (AUDPC) for each treatment using function ‘AUDPC()’ from the ‘epifitter’ package (Alves and Del Ponte, 2021). Control efficacy was calculated as the percentage reduction in FHB severity compared to the CHECK treatment in both greenhouse and field trials. Data from each individual trial of the greenhouse and field were analyzed separately.

To ensure the validity of subsequent analyses, all collected data were tested for normality and homoscedasticity. The values of FHB severity, AUDPC and FDK were square-root transformed prior to analysis. Greenhouse trials data were subjected to the analysis of variance (ANOVA) with ‘aov()’ function in R. Post-hoc analyses were performed with the ‘emmeans’ package to obtain the estimates of the marginal means and the respective confidence intervals (Lenth *et al.*, 2025). The ‘SK’ function from ‘ScottKnott’ (Jelihovschi and Allaman, 2023) was used for multiple comparisons of treatment means using the Scott-Knott clustering testing (Scott and M. Knott, 1974) at 5% significance.

The incidence values from the first field trial were arcsin square root-transformed prior to analysis. The FDK data from the first field trial, and FHB severity, from the second field trial, and yield data, from both field trials, were square root-transformed prior to analysis. When differences by the F test were significant ($P < 0.05$), the means were compared by the Scott-

Knott test at 5% significance with the add-on package ‘easyanova’ (Arnhold, 2013). All analyses were performed in software R (R Core Team, 2025).

3. RESULTS

3.1 Disease progress in greenhouse trials

Fusarium Head Blight severity on wheat heads varied over time among the treatments in both greenhouse trials (**Figure 1**). Overall, disease severity was greater in the first trial (**Figure 1A**) compared to the second (**Figure 1B**), except for the plants from the CHECK, which exhibited similar severity progress in both trials.

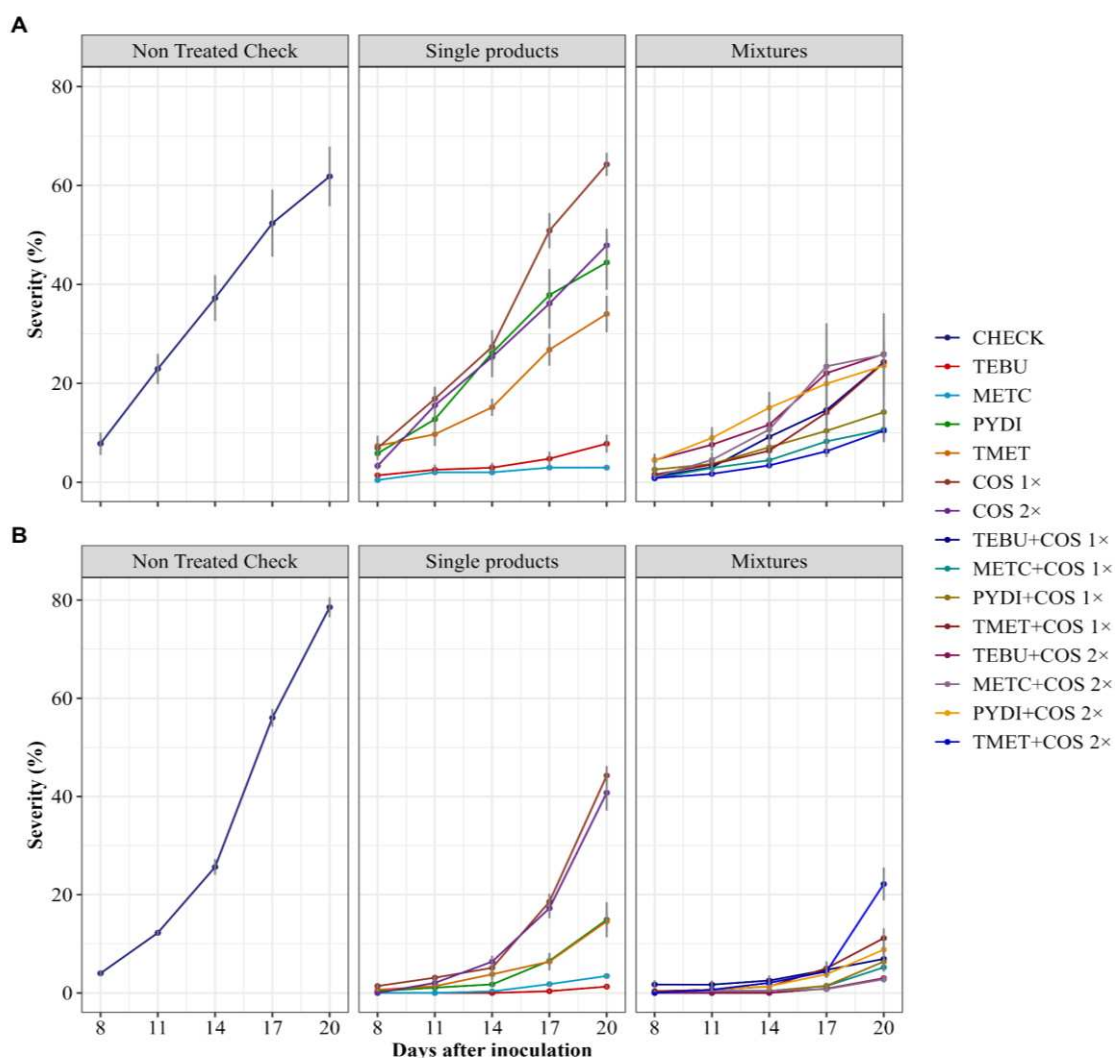


Figure 1. Progress of Fusarium Head Blight severity and respective standard error in wheat heads in the first (A) and second greenhouse trial (B). Treatments included synthetic fungicides (TEBU = tebuconazole; METC = metconazole; PYDI= pydiflumetofen; TMET= thiophanate-methyl), chitosan oligosaccharide (COS), mixtures (synthetic fungicides and chitosan oligosaccharide) and non treated control (CHECK). Treatments followed by “1×” were applied once after inoculation in the early anthesis stage (62 of Zadoks scale; Zadoks *et al.*, 1974), while treatments followed by “2×” indicate that a second application was performed seven days later. Single sprayed fungicides also were applied twice. Source: prepared by the author.

Disease progress was higher in plants treated with COS 1× in the first trial (Figure 1A), with disease progress like that observed in CHECK. The application of COS 2× in the first trial and COS 1× and COS 2× in the second trial resulted in a reduction in disease severity over time compared to the CHECK. However, the effect of COS application was not superior to that of other treatments. Fungicides singly applied suppressed disease severity over time compared to the treatments with COS alone, with the exception of PYDI in the first trial (Figure 1A). Efficacy, however, varied among the tested fungicides. Plants treated with TEBU and METC exhibited the lowest severity over time.

Plants sprayed with fungicide-COS mixtures exhibited lower severity over time compared to those treated with PYDI and TME*t* alone, starting in 11 and 14 d.p.i. in the first and second trial, respectively, regardless of the number of sprays (**Figure 1**). However, at 20 d.p.i., plants sprayed with TMET+COS 2× showed greater severity compared to plants treated with the fungicides alone in the second trial (**Figure 1B**). PYDI+COS regardless of the number of applications, showed lower disease severity in both trials compared to PYDI. In the first trial, TMET+COS also performed better compared to the TMET alone, regardless of the number of sprays (**Figure 1A**). However, it was observed only for TMET+COS 1× in the second trial (**Figure 1B**).

3.2 Severity, control efficacy, AUDPC and FDK in greenhouse trials

Data from the two greenhouse trials were analyzed separately due to the significant interaction between trials for all variables. There was a significant effect of the treatments in all analyzed variables for each trial ($P < 0.05$; **Table 2**).

In general, control efficacy was higher in the second trial, although the severity of CHECK treatment at 20 d.p.i. was greater. In the first trial, COS either sprayed once or twice resulted in similar FHB severity as the CHECK. However, in the second trial, the same treatments significantly reduced disease severity compared to the CHECK, although their efficacy was not superior to the other treatments evaluated (**Table 2**).

Table 2. Estimated mean severity values at 20 days after inoculation, area under the disease progress curve (AUDPC), and percent Fusarium-damaged kernels (FDK) in wheat plants from two greenhouse trials and Fusarium head blight control efficacy for each treatment.

Treatment codes	Trial 1				Trial 2			
	Severity ^a (%)	Efficacy (%)	AUDPC ^a	FDK ^a (%)	Severity ^a (%)	Efficacy (%)	AUDPC ^a	FDK ^a (%)
CHECK	59.9a	-	419.9a	38.4a	78.4a	-	404.2a	55.8a
TEBU	5.5c	87.4	29.0b	8.9b	0.6d	98.8	1.4d	7.5c
METC	2.2c	95.1	19.3b	5.6b	3.2d	96.2	9.9d	0.6d
PYDI	42.7a	28.2	294.9a	47.6a	11.5c	88.9	39.6c	8.0c
TMET	33.0a	45.0	207.5a	33.3a	13.5c	84.0	48.1c	7.1c
COS 1×	64.0a	-4.0	387.6a	51.8a	44.0b	44.1	148.1b	39.9a
COS 2×	47.2a	22.5	290.1a	52.8a	39.8b	50.3	132.9b	40.2a
TEBU+COS 1×	22.4b	60.7	107.7b	23.4b	4.6c	94.9	23.4c	11.7c
METC+COS 1×	8.7c	82.7	50.4b	16.7b	4.8d	94.2	12.4d	2.5d
PYDI+COS 1×	9.9c	77.0	55.3b	14.4b	6.3d	92.1	14.4d	6.1c
TMET+COS 1×	22.8b	61.0	101.3b	17.5b	9.9d	88.5	30.4c	21.4b
TEBU+COS 2×	25.2b	58.1	167.6a	27.9a	2.7d	96.7	5.8d	3.5d
METC+COS 2×	19.5b	58.3	113.1b	13.9b	1.9d	97.4	6.7d	0.8d
PYDI+COS 2×	20.0b	61.8	149.2b	16.1b	8.2c	90.2	28.2c	7.4c
TMET+COS 2×	9.6c	83.2	48.7b	9.8b	19.5c	78.8	46.6c	16.8c

Means followed by equal letters in the columns belong to the same grouping, by Scott-Knott's test, at 5% probability

^a Data were square root-transformed prior to analysis. Back-transformed data are presented. Treatments included synthetic fungicides (TEBU = tebuconazole; METC = metconazole; PYDI = pydiflumetofen; TMET= thiophanate-methyl), chitosan oligosaccharide (COS), mixtures (synthetic fungicides and chitosan oligosaccharide) and non treated control (CHECK). Treatments followed by "1×" were applied once after inoculation during the early anthesis stage (62 of Zadoks scale; Zadoks *et al.*, 1974), while treatments followed by "2×" indicate that a second application was performed seven days later. Single sprayed fungicides also were applied twice. Source: prepared by the author.

AUDPC values were similar for PYDI, TMET, COS 1×, COS 2× and the CHECK. In the second trial, AUDPC was higher for the CHECK compared to all treatments. In general, all applied mixtures reduced AUDPC values compared to the control. However, in the first trial, only the mixtures of PYDI+COS (1× and 2×) and TMET+COS 2× were more effective than the single-sprayed fungicides. In the second trial, COS improved PYDI control efficacy when the mixture was sprayed twice (Table 2). TEBU and METC and all applied fungicide-COS mixtures were able to reduce the percentage of FDK compared to the CHECK. Triazoles and its mixtures with COS performed best to suppress FDK in the harvested sample. COS alone was not able to

reduce the percentage of FDK compared to CHECK. The combined treatments of PYDI+COS (1× and 2×) and TMET+COS (1× and 2×) significantly reduced FDK values compared to the isolated applications of PYDI and TMET (**Table 2**).

3.3. Field trial results

Since significant interaction existed between the two field trials, the data were analyzed separately. Isolated fungicides, fungicide-COS mixtures, and COS (1× and 2×) did not significantly affect FHB incidence in either field trial ($P > 0.05$), nor did significantly affect FHB severity in the second field trial ($P = 0.948$). However, these treatments did significantly affect FHB severity in the first trial ($P = 0.025$) and FDK in both trials ($P < 0.05$) (**Table 3**).

Table 3. Estimated mean values for FHB incidence, severity, and percentage of Fusarium-damaged kernels (FDK) in wheat plants from two field trials and Fusarium head blight control efficacy for each treatment.

Treatment codes	Trial 1				Trial 2			
	Incidence ^a (%)	Severity (%)	Efficacy (%)	FDK ^b (%)	Incidence (%)	Severity ^b (%)	Efficacy (%)	FDK (%)
CHECK	99.9 ^{ns}	72.0a	-	50.0*	71.5 ^{ns}	30.2 ^{ns}	-	51.0a
TEBU	99.7	65.6b	8.8	41.9	63.5	26.8	11.2	13.2b
METC	98.9	60.5b	16	45.4	60.5	26.6	12.2	23.2b
PYDI	98.2	72.7a	-0.9	39.6	69.0	25.4	16.0	28.5b
TMET	99.6	68.2b	5.3	35.6	74.5	31.0	-2.5	20.0b
COS 1×	99.7	72.0a	0.0	65.4	69.5	31.3	-3.4	58.8a
COS 2×	98.9	78.2a	-8.7	75.2	75.0	31.9	-5.4	34.5b
TEBU+COS	99.6	60.8b	15.5	27.0	59.5	28.4	6.2	17.5b
METC+COS	99.9	75.1a	-4.3	45.7	77.0	29.4	2.7	27.2b
PYDI+COS	97.2	65.5b	9.0	48.8	68.5	25.6	15.4	35.5b
TMET+COS	98.0	61.2b	15.0	62.6	77.5	39.4	-30.4	24.0b

Means followed by equal letters in the columns belong to the same grouping, by Scott-Knott's test, at 5% probability.

^{ns} Not significant by *F* test ($P > 0.05$).

* The *F*-test was significant, but the Scott-Knott test did not separate the groups.

^a Data were arcsin square root-transformed prior to analysis. Back-transformed are presented.

^b Data were square-root transformed prior to analysis. Back-transformed are presented.

FDK (%) = Fusarium-damaged kernels. Treatments included synthetic fungicides (TEBU = tebuconazole; METC = metconazole; PYDI= pydiflumetofen; TMET= thiophanate-methyl), chitosan oligosaccharide (COS), mixtures (synthetic fungicides and chitosan oligosaccharide) and non treated control (CHECK). Treatments followed by "1×" were applied once after inoculation during the early anthesis stage (62 of Zadoks scale; Zadoks *et al.*, 1974), while treatments followed by "2×" indicate that a second application was performed seven days later. Single fungicides and fungicides-COS mixtures also were applied twice. Source: prepared by the author.

Although FHB incidence and severity were considerably high in both trials, the conditions for the first trial were more conducive for FHB development. FHB incidence and severity ranged from 97.2% to 99.9% and from 59.5% to 77.5%, respectively. In the first trial, wheat plants sprayed with the TEBU, METC, and TMET, as well as those sprayed with mixtures TEBU+COS, PYDI+COS, and TMET+COS, showed significantly reduced FHB severity when compared to CHECK (**Table 3**).

In general, all treatments poorly suppressed FHB severity compared to the CHECK. In the first field trial, PYDI sprays did not effectively reduce FHB severity. However, when the mixture PYDI+COS 2× was sprayed, wheat plants showed a 9% reduction in disease severity. The efficacy was also greater when mixtures of TEBU+COS 2× and TMET+COS 2× were applied, compared to the single sprayed fungicides. The results of the first trial contrasted with the results of the second trial, where the highest FHB control efficacy was observed in plants sprayed with PYDI (23.2%). The lowest control efficacy in the second trial occurred in plants sprayed with TMET+COS 2× (**Table 3**).

In the first trial, despite the significant difference detected by the F-test, the Scott-Knott test did not allocate the treatments into statistical distinct groups according to the mean FDK values of the wheat heads. In the second trial, the application of the all isolated fungicides (TEBU, METC, PYDI and TMET), COS 2× and all fungicide-COS mixtures significantly reduced FDK values, grouped together due to their lower mean values, compared to the CHECK treatment. However, the percentage of FDK did not differ significantly between the mixtures and the isolated fungicides (**Table 3**).

The application of treatments did not significantly affect test weight ($P = 0.0959$) and TKW ($P = 0.9571$) in the first trial. In the second trial, there was a significant difference for TKW ($P < 0.001$) and test weight ($P = 0.0439$). However, the Scott-Knott test did not allocate the treatments into statistical distinct groups according to mean test weight values. Test weight values of wheat plants range from 36.4 to 46.2 kg in the first trial and from 61.3 to 76.7 kg in the second trial (Table 4). Four statistical groups were formed for mean values of TKW. Plants treated with the TEBU and with the mixture PYDI+COS showed the highest mean TKW values, differing significantly from the other treatments. PYDI and TMET provide a return in TKW, compared to CHECK. The application of METC, COS 1×, COS 2× and the mixtures TEBU+COS, METC+COS and TMET+COS did not cause a significant increase in the TKW of harvested wheat heads compared to the CHECK treatment (**Table 4**).

Table 4. Estimated mean values for test weight, thousand-kernels weight (TKW), and yield of wheat plants from two field trials and yield return for each treatment.

Treatment codes	Trial 1				Trial 2			
	Test weight	TKW	Yield ^a	Return	Test weight	TKW	Yield ^a	Return
	(kg)	(g)	(kg.ha ⁻¹)	(%)	(kg)	(g)	(kg.ha ⁻¹)	(%)
CHECK	36.4 ^{ns}	18.4 ^{ns}	1958.5c	-	66.8*	25.6c	2806.9 ^{ns}	-
TEBU	45.9	19.8	2763.3a	41.1	66.9	35.9a	3454.5	23.1
METC	44.5	19.4	2349.7b	20.0	65.8	25.6c	2693.7	-4.0
PYDI	40.0	20.2	2198.1c	12.2	76.7	31.0b	4060.0	44.6
TMET	43.2	18.9	2529.1b	29.1	71.3	29.8b	3429.0	22.2
COS 1×	39.7	19.8	2188.4c	11.7	61.3	19.9d	2808.0	0.0
COS 2×	42.3	17.8	2200.5c	12.4	72.4	26.9c	3255.5	16.0
TEBU+COS	45.5	20.1	2443.2b	24.7	69.2	26.5c	2918.3	4.0
METC+COS	43.9	19.0	2251.2c	14.9	67.6	25.2c	3134.9	11.7
PYDI+COS	44.1	19.4	2410.1b	23.1	75.1	33.5a	3652.3	30.1
TMET+COS	46.2	19.4	2382.6b	21.7	65.7	22.8d	2655.7	-5.4

Means followed by equal letters in the columns belong to the same grouping, by Scott-Knott's test, at 5% probability

^{ns} Not significant by F test ($P > 0.05$).

* The F-test was significant ($P < 0.05$), but the Scott-Knott test did not separate the groups.

^a Data were square root-transformed prior to analysis. Back-transformed are presented.

TKW (g) = Thousand-kernels weight. FDK (%) = Fusarium-damaged kernels. The values of AUDPC and FDK were square-root transformed prior to analysis. Treatments included synthetic fungicides (TEBU = tebuconazole; METC = metconazole; PYDI = pydiflumetofen; TMET= thiophanate-methyl), chitosan oligosaccharide (COS), mixtures (synthetic fungicides and chitosan oligosaccharide) and non treated control (CHECK). Treatments followed by "1×" were applied once after inoculation during the early anthesis stage (62 of Zadoks scale; Zadoks *et al.*, 1974), while treatments followed by "2×" indicate that a second application was performed seven days later. Single fungicides and fungicides-COS mixtures also were applied twice. Source: prepared by the author.

Overall, all treatments substantially provided a return in yield in the first trial. However, TEBU performed best in preventing yield losses compared to the CHECK and the other treatments. On the other hand, PYDI, COS a and METC+COS sprayed plots produced a similar amount of grains to the CHECK. In the second trial, although there was no significant difference between treatments ($P = 0.245$), the applied treatments resulted in an increase in yield, except for METC, COS 1× and TMET+COS (**Table 4**).

3.4 Mycotoxin analysis

In the greenhouse trials varying levels of DON were detected in samples of bulked kernels, except in the MOCK treatment in the second trial. DON levels in the CHECK treatment were higher in the second trial (**Figure 2**).

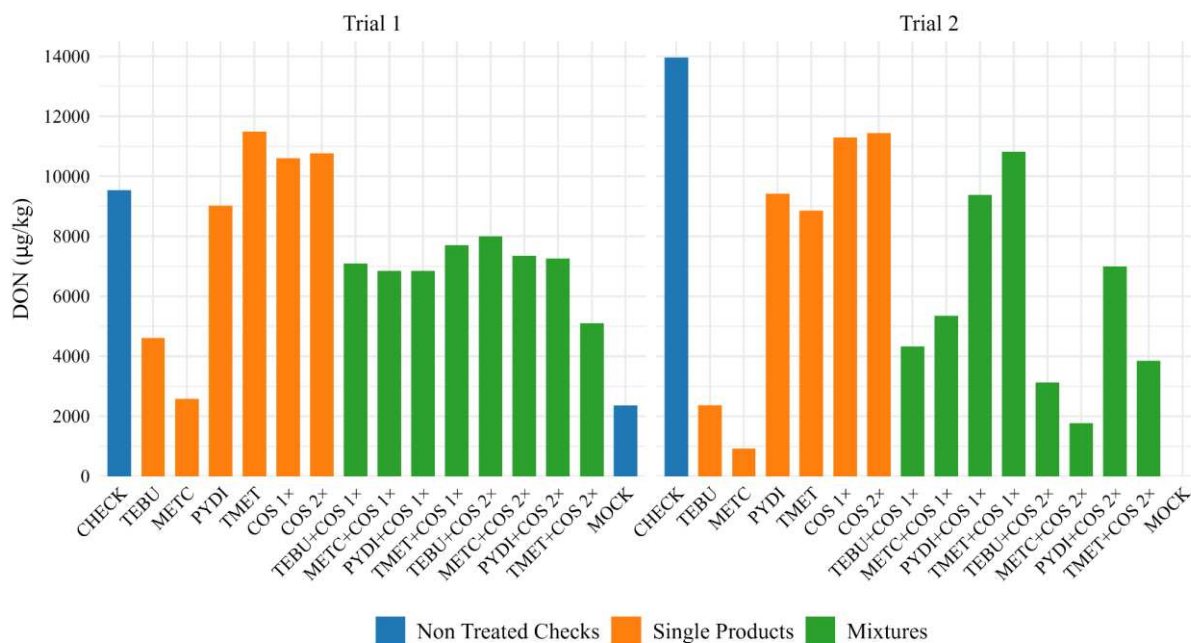


Figure 2. Deoxynivalenol levels accumulated in wheat 10g kernel samples (n = 20 bulked wheat heads from each treatment) in the first (left) and second (right) greenhouse trial. Treatments included synthetic fungicides (TEBU = tebuconazole; METC = metconazole; PYDI = pydiflumetofen; TMET = thiophanate-methyl), chitosan oligosaccharide (COS), mixtures (synthetic fungicides and chitosan oligosaccharide) and non treated control (CHECK). Treatments followed by “1×” were applied once after inoculation during the early anthesis stage (62 of Zadoks scale; Zadoks *et al.*, 1974). Treatments followed by “2×” indicate that a second application was performed seven days later. Single fungicides also were applied twice. Source: prepared by the author

DON levels on bulked kernels from the COS sprayed plants were high and similar to the CHECK in the first trial. Conversely, grains from COS sprayed plants (1× e 2×) showed 19% less DON than CHECK. In general, some treatments of fungicide+COS mixture showed an effect to reduce DON accumulation. Mixtures of COS with PYDI and TMET were more effective in suppressing DON accumulation (ranging from 19 to 46%) compared to single sprayed fungicides in the first trial, regardless of the number of sprays. In the second trial, the same mixtures applied twice performed better than the same fungicides, with reduction ranging from 50 to 72%. This pattern was not observed when comparing TEBU and METC with the fungicides+COS mixtures in both trials (**Figure 2**).

DON was also detected in all samples of harvested kernels from field trial plots. Because there was significant interaction between the data from field trials, DON data were analyzed separately. In the first trial, there was no significant effect in DON accumulation for treatments applied ($P = 0.0646$). However, in the second trial there was a significant effect of treatments sprayed for DON accumulation in wheat kernels ($P < 0.001$). Fungicides+COS mixtures showed higher suppression in DON accumulation compared with CHECK, ranging from 77.8 to 86.2% (for PYDI+COS and TEBU+COS). This effect was not different compared with fungicide synthetic applications (**Figure 3**).

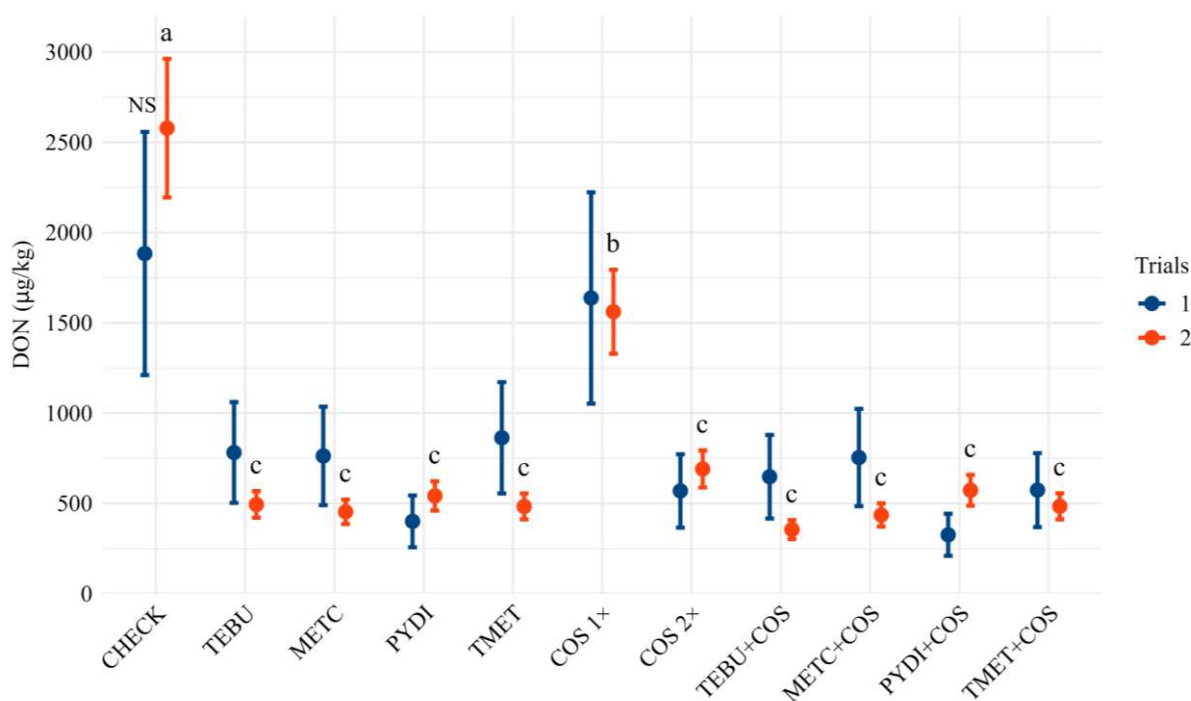


Figure 3. Estimated deoxynivalenol (DON) levels ($\mu\text{g}/\text{kg}$) in 20g kernel samples from wheat heads harvested in each plot of the two field trials (harvested). Error bars represent the standard error of the mean. Different letters above error bars indicate significant differences among treatments in the second trial by Skott-Knott test ($\alpha = 0.05$), while “NS” denotes non-significant differences in the first trial. Treatments included synthetic fungicides (TEBU = tebuconazole; METC = metconazole; PYDI= pydiflumetofen; TMET= thiophanate-methyl), chitosan oligosaccharide (COS), mixtures (synthetic fungicides and chitosan oligosaccharide) and non treated control (CHECK). Treatments followed by “1x” were applied once after inoculation during the early anthesis stage (62 of Zadoks scale; Zadoks *et al.*, 1974). Treatments followed by “2x” indicate that a second application was performed seven days later. Single fungicides and mixtures also were applied twice.

4. DISCUSSION

In this study, we evaluated the synergistic effect of COS with different site-specific fungicides in controlling FHB on wheat, as well as its impact on DON accumulation in grains. A recent study also investigated the efficacy of this compound singly sprayed against FHB

under in vivo conditions. Although the authors described that COS sprays suppressed FHB, its overall efficacy was somewhat low (Paiva *et al.*, unpublished data). Another study evaluated the application of COS mixed with tebuconazole at the beginning of anthesis stage and compared this strategy with the isolated application of COS, tebuconazole, and the sequential single application of each product. The authors described that the tebuconazole+COS mixture led to a slower disease increase over time and it was more effective in reducing AUDPC, suggesting a synergistic effect between the compounds (Dopp *et al.*, unpublished data). Thus, we aimed to evaluate the effect of COS both individually and in combination with different fungicides, including tebuconazole.

In the greenhouse trials, we evaluated whether the combination of fungicides with COS, in addition to enhancing the efficacy of synthetic fungicides, could reduce the number of applications without compromising disease control. Through severity assessments at 8, 11, 14, 18, and 20 d.p.i., we observed that the isolated application of COS suppressed disease development compared to the CHECK but was not as effective as the fungicide-COS mixtures or the fungicides alone. Similar results were reported by Dashies *et al.* (2022), who also observed a reduction in FHB progress in infected spikes assessed at 7, 14, and 21 d.p.i., compared to the water-treated control. Additionally, we found that disease progress over time decreased with fungicide application, although variations were observed among the tested products. TEBU and METC resulted in the lowest disease increase over time, whereas PYDI and TMET were less effective. It may be related to the intermediate rate used for PYDI, which was lower than the highest recommended dose in the Miravis® label, the timing of application, and the reduced sensitivity of the CML3066 isolate to TMET (data not shown). The controlled conditions in the greenhouse, combined with the exposure of wheat heads to inoculum at the optimal infection stage, promote a sharp increase in disease severity over time. This may indicate the need for higher recommended doses of each fungicide to ensure greater efficacy in FHB control.

We also observed that applying the mixtures at the early anthesis stage (62 of Zadoks scale; Zadoks *et al.*, 1974) resulted in a greater reduction in disease severity over time compared to the fungicides PYDI and TMET applied alone. These findings suggest that the addition of COS enhances the efficacy of these fungicides and indicate that a single application of a COS-fungicide mixture at early anthesis stage can reduce FHB severity as effectively as or even more than two applications of PYDI or TMET. To the best of our knowledge, no studies have evaluated the combined application of chitosan or its derivatives with pydiflumetofen or thiophanate-methyl. As previously mentioned, the reduction in FHB severity following the

application of a COS-tebuconazole mixture at early anthesis, compared to a fungicide applied twice at a seven-day interval, has already been observed (Dopp *et al.*, unpublished data).

The comparison of FHB severity values at 20 d.p.i. in greenhouse trials allowed us to assess the efficacy of the treatments when the disease reached its peak within the time frame of the evaluations. The overall efficacy of the treatments was high, which can be attributed to the use of a single highly aggressive *F. graminearum* isolate, as well as the standardized inoculation timing for all spikes, the uniform phenological stage of the plants at inoculation, and the synchronization of the spraying with fungal inoculation at the wheat heads. These conditions ensured greater and more homogeneous exposure of the pathogen to the tested products. In both greenhouse experiments, environmental conditions were similar (with comparable FHB severity in the CHECK); however, the overall treatment efficacy was higher in the second trial. Error effects are generally minimal in greenhouse experiments. However, even when ensuring similar conditions across multiple experiments, variability may still occur (Brien *et al.*, 2013).

Our findings demonstrated a partial efficacy of COS treatment in controlling FHB, similar to the results obtained by Paiva *et al.* for the same pathosystem, in which a reduction in FHB severity was observed, although without a statistically significant difference compared to the control (*unpublished data*). In this study, only in the second greenhouse experiment COS significantly reduced disease severity at its peak (20 d.p.i.) compared to CHECK, with an efficacy of approximately 40–50%, regardless of the number of applications. However, treatments with COS alone were still inferior to fungicides and tested mixtures.

Previous studies have demonstrated an inhibitory effect of chitosan and COS on *F. graminearum* growth in vitro (Francesconi *et al.*, 2020; Loron *et al.*, 2023; Paiva *et al.*, unpublished data). In the study by Francesconi *et al.*, (2020), FHB severity was significantly reduced in two wheat cultivars inoculated with an isolate of *F. graminearum* and sprayed with chitosan at 21 d.p.i., compared to inoculated plants sprayed only with water. These studies, along with our findings, indicate a direct effect of COS on *F. graminearum*, although with limited intensity.

We confirmed the efficacy of the tested triazoles in controlling FHB in our greenhouse trial, although efficacy varied among them. We also observed that a single application of the metconazole-chitosan mixture performed as well as the fungicide applied twice, suggesting that two applications of the fungicide could be replaced by a single application of the mixture. The high efficacy of the two tested triazoles was expected, as some studies indicate that the triazole chemical group is more effective in controlling FHB compared to other groups (Paul *et al.*, 2008; Machado *et al.*, 2017; Bolanos-Carriel *et al.*, 2020). In southern Brazil, multiple

fungicides, either single-active or multi-active ingredients, were applied three times from wheat flowering onward and compared, with metconazole proving to be the most effective in reducing FHB severity and DON levels (Barro *et al.*, 2023). The study by Paul *et al.* (2008) demonstrated that tebuconazole is generally more effective in reducing FHB under moderate disease conditions. Additionally, we observed a trend of improved performance for pydiflumetofen and thiophanate-methyl when mixed with COS, except for the TMET+COS 2× treatment.

The AUDPC is used to consolidate multiple observations of disease progress into a single value. COS reduced the AUDPC compared to CHECK, although the difference was not statistically significant in the first trial. Khan and Doohan (2009) evaluated the efficacy of chitosan and other natural compounds in controlling FHB in wheat and barley, both in greenhouse trials and small-scale field experiments. The authors observed a reduction of over 70% in AUDPC values for FHB in wheat plants sprayed with chitosan. Another study demonstrated that chitosan, applied either before or after inoculation in wheat plants, was effective in significantly reducing the AUDPC of FHB (Deshaies *et al.*, 2022). The PYD+COS and TMET+COS mixtures tended to reduce the AUDPC compared to PYD and TMET, supporting the results obtained for other variables that help us understand disease behavior.

Regarding the suppression of FDK percentage, we observed that COS does not exhibit effective performance, whereas triazoles and their mixtures with COS are more effective compared to CHECK. These findings align with the strong performance of triazole-based fungicides in both trials. Another finding consistent with other results from greenhouse trials was that the combined treatments of PYDI+COS (1× and 2×) and TMET+COS (1× and 2×) reduced FDK values compared to PYDI and TMET. We believe that the combined action of PYDI+COS and TMET+COS involves different mechanisms that, when combined, may affect the fungus through distinct metabolic pathways. In the literature, some studies report that fungal cell membranes are the primary target of the antifungal activity of chitosan and its various formulations (Singh *et al.*, 2008; García-Rincón *et al.*, 2010; Ing *et al.*, 2012; Xing *et al.*, 2018). However, recent studies indicate that the antifungal efficacy of chitosan may vary depending on the target organism (Ke *et al.*, 2022).

Pydiflumetofen belongs to the carboxamide group and acts as a succinate dehydrogenase inhibitor (SDHI), impairing cellular respiration and reducing ATP synthesis. Meanwhile, thiophanate-methyl, part of the benzimidazole group (MBC), inhibits fungal cell division (FRAC, 2024). The overall strong performance of both combinations suggests that COS may influence each fungicide differently, as pydiflumetofen has a predominantly protective effect, while thiophanate-methyl is more effective in reducing disease symptoms.

The field trials were conducted to validate the performance of treatments under real cultivation conditions. The high disease severity in the CHECK treatment and the overall low efficacy of the treatments ($\leq 16\%$), suggest that the disease may have been influenced by highly favorable climatic conditions, particularly in the first experiment. In general, efficacy in our field trials has been low and variable, including other products ($<35\%$, unpublished data and Figure S1).

We observed that among the tested fungicides, TEBU, METC, and TMET reduced disease severity, whereas PYDI did not. This finding, combined with the high incidence of FHB, suggests that infection was not effectively reduced. Thus, chemical control appears to have had a greater impact on symptom progression rather than protecting wheat spikes from *F. graminearum* in the first trial. Under field conditions, the diversity of the present inoculum and variations in plant phenological stages result in different infection timings, which may not always coincide. Some authors report that FHB can exert high pressure on crops, usually due to favorable climatic conditions, leading to disease incidences exceeding 60% even when fungicides are applied (Singh *et al.*, 2021; González-Domínguez *et al.*, 2021). Additionally, we found that the PYDI+COS mixture improved the performance of PYDI in the first trial, enhancing the curative effect of the fungicide and making it as effective as other growth-inhibiting fungicides.

The efficacy of treatments in suppressing FHB severity was generally low compared to the untreated control (CHECK). Specifically, the application of PYDI did not significantly reduce disease severity in the first field trial, but the PYDI+COS 2 \times mixture resulted in a 9% reduction, reinforcing the hypothesis that combining COS with PYDI may enhance its efficacy. However, the effectiveness of the mixtures was inconsistent between trials. In the second trial, the highest disease control efficacy was observed with the standalone application of PYDI (23.2%), while the TMET+COS 2 \times mixture showed the lowest efficacy. These results suggest that COS does not enhance fungicide efficacy under lower FHB pressure, as observed in the second trial, where even the CHECK treatment showed relatively low severity (30.2%). This also indicates that using mixtures may be justified under specific conditions.

The use of only two applications may have contributed to the overall low efficacy of the fungicides in the field trials, which ranged from -8.7% to 16% in the first trial and from -30.4% to 16% in the second trial. A study conducted in Brazil evaluating the efficacy of different fungicides for FHB control applied three sprays, starting at the wheat heading stage (60 of Zadoks scale; Zadoks *et al.*, 1974) with 7- to 12-day intervals between applications, achieving

higher control efficacy (41.5% to 62.8%) compared to the values obtained in this study (Barro *et al.*, 2023).

The assessment of FDK percentage provided insights into the effect of treatments on one of the main quality parameters of wheat grains. Despite variation among treatments, the dispersion of FDK values in the first trial may have limited the formation of statistically distinct treatment groups. Spraying COS 2× on wheat spikes reduced FDK levels compared to the control and COS 1×, suggesting that the product may have potential for protecting grains against late infections. In the literature, the percentage of FDK has been reported to have moderate to high phenotypic and genetic correlations with DON accumulation (Gaire *et al.*, 2021). Our results also indicate that while fungicides and their mixtures with COS reduced FDK values compared to the control, there is no significant additional benefit of combining COS with the tested fungicides in this regard.

We observed that the application of treatments did not significantly affect test weight or thousand-kernel weight (TKW) in the first field trial. In the second trial, the higher test weight values remained above 80 kg, which is the average value for the wheat cultivar used in the experiments (Albrecht, 2006). The results from the second field trial indicate that the treatments TEBU, PYDI, TMET, and PYDI+COS had a positive effect on TKW. Additionally, the PYDI+COS mixture proved to be more effective than PYDI alone in increasing TKW. It has long been established that high FHB severity levels result in poor grain quality (McMullen *et al.*, 1997). In our first field trial, the high disease severity observed was reflected in low test weight and TKW values, high FDK levels, and the absence of statistically significant differences among these variables. Both FHB and wheat blast contributed to the reduction in test weight, TKW, and grain yield observed in this experiment. Among the treatments in the first trial, the fungicide TEBU resulted in the highest grain yield.

A meta-analysis on chemical control of FHB in Brazil, which evaluated data from 2000 to 2015 on the triazole fungicides tebuconazole and propiconazole, as well as the benzimidazole fungicide carbendazim, concluded that tebuconazole provided the highest yield response (Machado *et al.*, 2017). In the first trial, COS-based treatments showed a positive return in yield (an average increase of 18.8%), although they did not differ statistically from the untreated control. The PYDI+COS treatment resulted in a significantly higher yield than PYDI alone. All treatments applied in the first trial led to an increase in yield compared to CHECK, ranging from 229.9 kg.ha⁻¹ to 804.8 kg.ha⁻¹. This yield gain was likely due to the effective control of foliar diseases, such as leaf spots and rust, as the FHB control was low in this trial.

In the second trial, the treatments PYDI, PYDI+COS, TEBU, and TMET provided the highest overall yield increases. In general, most applied treatments contributed to grain yield improvement. However, in the second trial, METC, COS 1×, and TMET+COS resulted in yield reductions. Nevertheless, these treatments were still among the highest-yielding when compared to those in the first trial, suggesting that, in this case, the CHECK treatment yield and disease severity were similar (Figure S2).

Our results reveal important trends regarding wheat grain contamination by DON under different treatments. In the greenhouse, the isolated application of COS resulted in little or no reduction in DON levels. The TEBU+COS and METC+COS mixtures did not reduce the accumulated DON levels in grains in either trial compared to fungicides applied alone. This result may be related to the higher efficacy of individual fungicides compared to the mixtures. Additionally, a possible negative synergy between COS and triazoles may have reduced the inhibition of DON. The PYDI+COS and TMET+COS mixtures reduced DON levels in the first trial, regardless of the number of applications, suggesting a synergistic effect between the fungicides and COS. In the second trial, DON reduction was observed when the same mixtures were applied twice. In this case, mixtures that showed greater efficacy in controlling FHB than individual fungicides also had lower accumulated DON levels.

We observed reductions in DON levels for all field-applied treatments in both trials, ranging from 13.1% (COS 1×, first trial) to 86.2% (TEBU+COS, second trial), although this reduction was not statistically significant in the first trial. All COS mixtures reduced DON levels compared to triazole fungicides applied alone in both trials. The reductions were 1.2% and 17% in the first trial and 3.9% and 28.2% in the second trial for METC+COS and TEBU+COS, respectively. The PYDI+COS and TMET+COS mixtures reduced DON levels in the first trial by 18.6% and 33.5%, respectively, compared to PYDI and TMET. However, this pattern was not observed in the second trial, when disease pressure was lower. Although reductions in mean DON values were observed, these differences were not statistically significant.

Our results suggest that COS may have an additional effect on grain quality when combined with tebuconazole, even if it does not significantly contribute to FHB control. Some authors have already demonstrated the effect of chitosan derivatives in reducing DON and other mycotoxins produced by *F. graminearum* in vitro (Loron *et al.*, 2023) and in wheat and durum wheat grains (Zachetti *et al.*, 2019; Palazzini *et al.*, 2022). Although these studies observed a significant reduction in mycotoxins, the authors emphasize that the exact mechanism is still not fully elucidated, and that further research is needed to confirm these hypotheses. The use of

different chitosan formulations and various mycotoxin detection methods may pose a challenge to elucidating this mechanism. Additionally, the regulation of secondary metabolism in mycotoxigenic fungi can be influenced by multiple regulatory systems in response to external environmental factors (Chen *et al.*, 2019). Some authors suggest that oxidative stress triggers mycotoxin biosynthesis (Ponts *et al.*, 2006; Audenaert *et al.*, 2010). Chitosan derivatives have demonstrated strong antioxidant activity (Xie *et al.*, 2001; Sun *et al.*, 2006, 2008), which may be related to the results observed in this study. Furthermore, Liu *et al.*, (2023) investigated the mechanisms of action of metconazole against *Fusarium pseudograminearum* strains, which cause Fusarium crown rot in wheat in China, using transcriptomic and biochemical analyses. The authors concluded that the fungicide reduced the activity of fungal antioxidant enzymes and inhibited toxin accumulation.

5. CONCLUSIONS

Among the four fungicides tested, PYDI exhibited the highest synergy when combined with COS. The PYD+COS mixture resulted in greater efficacy in controlling FHB, improved grain quality and yield, and reduced mycotoxin accumulation in grains, outperforming standalone fungicide in most cases. The TMET+COS combination also stood out, as it enhanced the fungicide's performance for certain evaluated variables, including the suppression of DON production. Therefore, incorporating COS with azole fungicides may be a promising strategy to reduce DON levels.

Our findings also reinforce the direct action of COS against *F. graminearum*. However, its overall low efficacy compared to the tested fungicides suggest that it should be used as an adjuvant in chemical control to the tested rather than a replacement.

These results are encouraging and may contribute to the development of new strategies for FHB management. Nevertheless, further studies are needed to evaluate the effectiveness of the mixtures under different spray schedules in wheat crops and across multiple growing seasons. Additionally, as previously mentioned, the inclusion of a third spray should also be considered.

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SUPPLEMENTARY MATERIAL

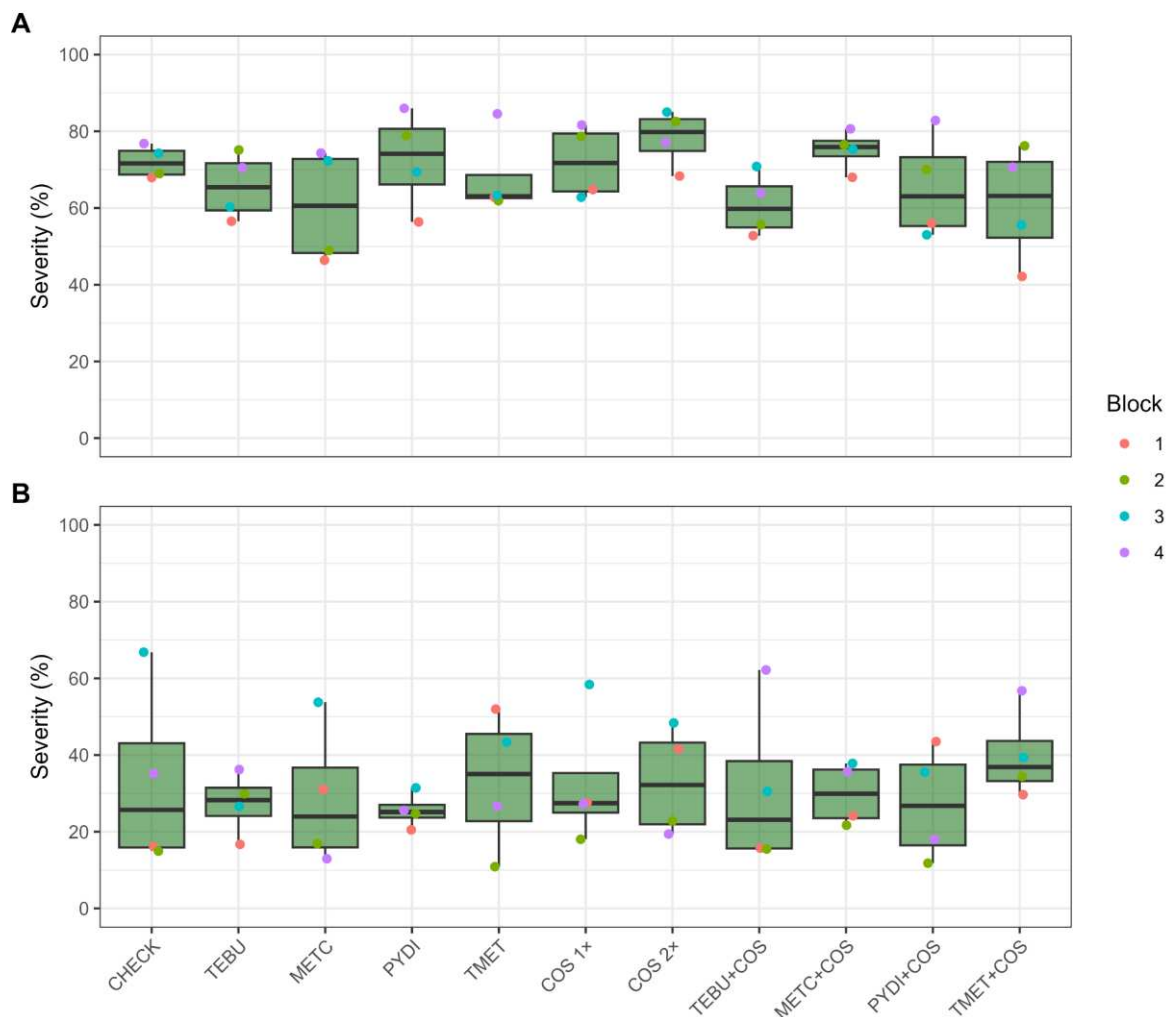


Figure S1. Fusarium head blight (%) severity means rated using Stack and McMullen scale (1995) in the first (A) and second field trial (B). Treatments included synthetic fungicides (TEBU = tebuconazole; METC = metconazole; PYDI= pydiflumetofen; TMET= thiophanate-methyl), chitosan oligosaccharide (COS), mixtures (synthetic fungicides and chitosan oligosaccharide) and non treated control (CHECK). Treatments followed by “1×” were applied once after inoculation in the early anthesis stage (62 of Zadoks scale; Zadoks *et al.*, 1974), while treatments followed by “2×” indicate that a second application was performed seven days later. Single sprayed fungicides and mixtures also were applied twice. Source: prepared by the author.

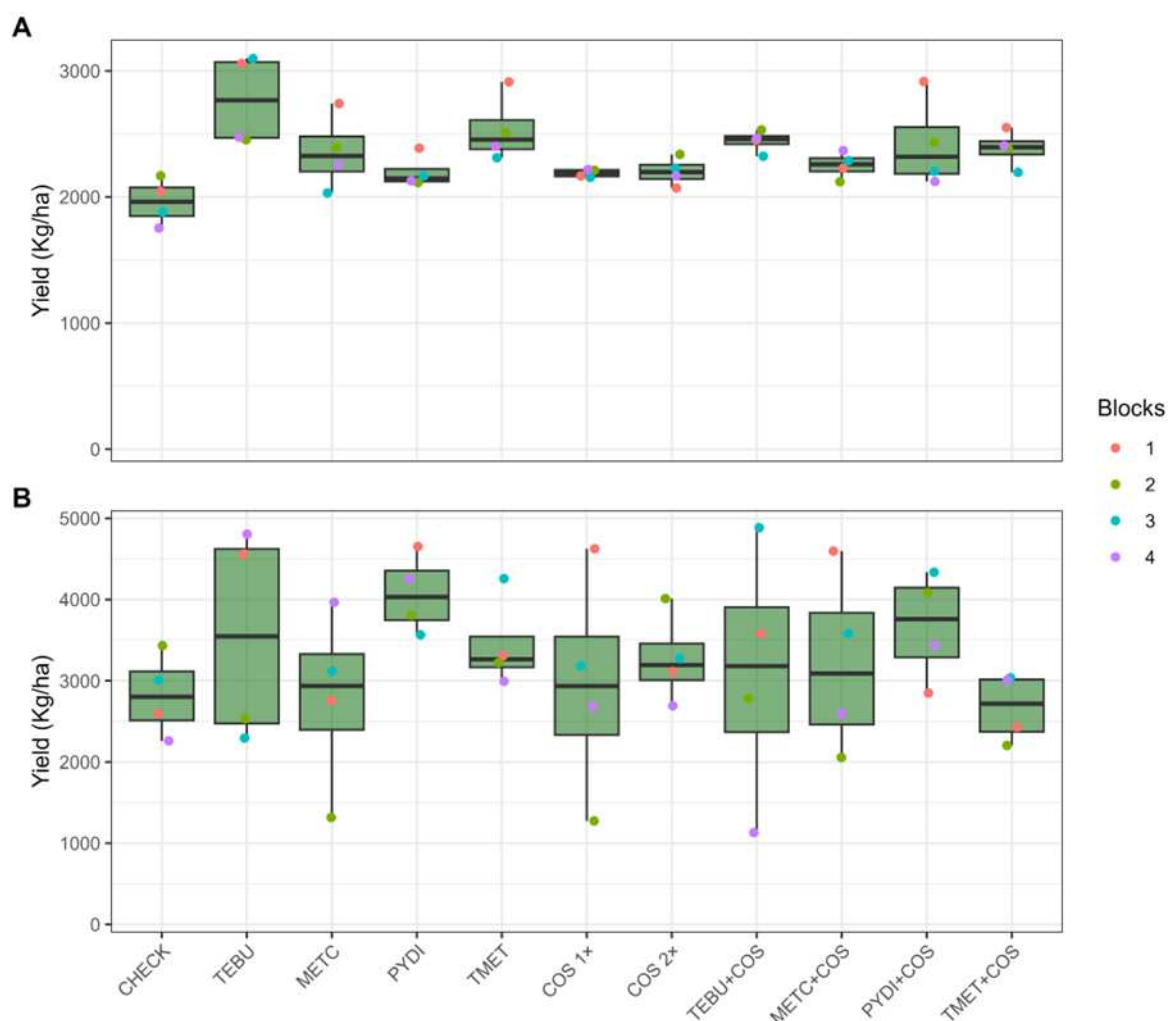


Figure S2. Yield of grain (kg/ha) based on weight kernels of the central 4 m² of each plot in the first (A) and second field trial (B). Treatments included synthetic fungicides (TEBU = tebuconazole; METC = metconazole; PYDI= pydiflumetofen; TMET= thiophanate-methyl), chitosan oligosaccharide (COS), mixtures (synthetic fungicides and chitosan oligosaccharide) and non treated control (CHECK). Treatments followed by “1^x” were applied once after inoculation in the early anthesis stage (62 of Zadoks scale; Zadoks et al., 1974), while treatments followed by “2^x” indicate that a second application was performed seven days later. Single sprayed fungicides and mixtures also were applied twice. Source: prepared by the author.