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Efficiency of γ-oryzanol against the complex *Fusarium graminearum* growth and mycotoxins production

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Abstract

The ability of γ-oryzanol against *Fusarium graminearum* growth and mycotoxins production were assessed and compared with the antioxidant properties in order to improve its application in food chain, beyond of the conventional. The γ-oryzanol extracted from rice bran and characterized by HPLC-UV and GG-FD. Its antifungal *in vitro*, effect on complex *F. graminearum* CQ244 (halo diameter, ergosterol and glucosamine production) and mycotoxin production (deoxinivalenol, nivalenol and zearalenone) were determined by an adapted method adapted. The antifungal activity of γ-oryzanol was compared with azoxystrobin and trifloxystrobin. MIC and specific inhibitory effects (%inhibition μg γ-oryzanol^{-1}) were also estimated. The antioxidant activity of the γ-oryzanol (DPPH, FRAP, ABTS and enzymatic inhibition) was compared with the gallic acid. Considering the MIC (1, 2 mg.g^{-1}) specific inhibitory effects on fungal growth (0.045%.μg) and mycotoxin (DON 0.001%. μg and NIV 0.002%. ug). Therefore γ-oryzanol is also a potential natural antifungal that is able to difficult the synthesis of fungal cell wall.

Keywords: deoxynivalenol; nivalenol; zearalenone; antioxidant properties.

Practical Application: Natural preservative.

1 Introduction

Protective compounds are most abundant in the outer layers of the vegetal structure, such as the in the cereal seeds, where the damage risk is more frequent. However, after harvested and benefited this parts (husk and bran) are under used and could be better employed in food chain to avoid fungal and oxidative damage (Martínez-Romero et al., 2008; Massarolo et al., 2017; Schmidt et al., 2014).

It is worth mentioning that, unlike other cereals, rice is the least contaminated one by *Fusarium* genera species in the field (Arruda et al., 2016; Dors et al., 2013). Rice bran is also a good source of γ-oryzanol, a mix of lipid and ferulic acid that has been applied for replacing synthetic antioxidant in pharmaceuticals and food chain. However, some researchers have shown its effects against fungal development (Heidtmann-Bemvenuti et al., 2016) that could be better exploited.

Regarding this issue Heidtmann-Bemvenuti et al. (2016) showed that γ-oryzanol is not only able to inhibit *Fusarium* development but also prevent DON production by toxigenic species. Therefore, estimating quantitative aspects (minimal concentration to inhibit pathogens and the mycotoxins production) before proposing new strategies to prevent damage are fundamental, so as to promote sustainability and food safety (Arruda et al., 2016). The inhibition on free radical produced by chemical or enzymatic process, carry out by peroxidase, has been related to mycotins production by pathogenic fungal species (Ferrochio et al., 2013; Heidtmann-Bemvenuti et al., 2016).

Considering the challenge related to properties of γ-oryzanol as antifungal and antioxidant activities, or both, this study assessed the effect of γ-oryzanol abiltity to inhibit the complex *F. graminearum* CQ244 development, mycotoxin production and on oxidative process. The aim was to the viability of a new strategy to replace synthetic fungicide application in food chain.

2 Materials and methods

2.1 Raw materials and analytical standards

The γ-oryzanol was obtained from rice bran donated by a company in the southern region in Rio Grande do Sul state, Brazil.

The fungus complex *F.graminearum*CQ244, was granted from collection of Federal University of Rio Grande do Sul (UFRGS). It was isolated from barley, and its potential of mycotoxins production was evaluated (Astolfi et al., 2010).

Mycotoxin (NIV, DON, and ZEA) and fungicide (azoxystrobin and trifloxystrobin) standards were purchased from Sigma-Aldrich, USA (purity> 98%). The γ-oryzanol standard was obtained from Wako Chemicals, USA (purity = 98%).

2.2 Extraction and characterization of γ-oryzanol from rice bran

Rice bran was evaluated about pH, acidez, percentual of moisture, ash, protein, lipids, fiber and carbohydrates by the Association of Official Analytical Chemists (2000) procedures. γ-oryzanol was extracted in according to the protocol described

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by Heidtmann-Bemvenuti et al. (2012a); that possess a detection limit 0.946 μg g^{-1}; repetibility 9.4% and recovery 111%. The oil containing γ-oryzanol was esterified to obtain the fatty acid methyl esters (Metcalfe et al., 1966). The separation of fatty acids was performed on a Varian 3400CX gas chromatograph equipped with a flame ionization detector (Heidtmann-Bemvenuti et al., 2012a).

The major components of γ-oryzanol was performed by high performance liquid chromatography with UV detector (HPLC-UV) using γ-oryzanol standard (150 μg mL^{-1}) and rice bran extract (40 μg mL^{-1}). The elution of the blend components was performed with acetonitrile: methanol: isopropanol 50:45:5 (v/v/v) at a flow rate of 1.0 mL min^{-1}, wavelength 315 nm, loop volume 20 μL, using Hypersil ODS (5 μm) 250 × 4.6 mm column as the stationary phase. The majority four peaks corresponded respectively to the cycle artenilferulate; 2,4-methylene cicloartenilferulate; campesterilferulate; and β-sitosterilferulate(Paucar-Menacho et al., 2007). The linearity was evaluated by external standardization with a calibration curve using γ-oryzanol standard solution at concentrations from 30 to 150 μg mL^{-1}.

2.3 Adaptation of the procedure to extract Fusarium toxins from fungal biomass

Mycotoxins nivalenol (NIV), deoxynivalenol (DON) and zearalenone (ZEA) were extracted from the medium whose recovery had been previously evaluated by 6 procedures: Heidtmann-Bemvenuti et al. (2012b), Monaci et al. (2011), Nielsen & Smedsgaard (2003), Sospedra et al. (2010), Vaclavik et al. (2010) and Zachariasova et al. (2010).

The Petri dish with 15 mL dextrose potato agar was fortified with each mycotoxins at three levels, three-fold their limits of detection (0.219 μg for DON; 0.150 μg for NIV and 0.229 μg for ZEA). After 24 hr, the mycotoxins were extracted according to the previously mentioned protocols. Detection and quantification were carried out by thin layer chromatography eluting toluene: ethil acetate: formic acid (30:20:5); the chromatogram was sprinkled with a solution of aluminum chloride and methanol (15% w/v) dried at 130 °C for 10 min. The chromatogram was kept in a black chamber under UV light (364 nm), aligned to get the optimal resolution of the photographic image and analyzed by the software *ImageJ* (Hoeltz et al., 2010; Rocha et al., 2017). The screening showed that the best mycotoxin recovery was obtained by the Vaclavik et al. (2010) method, that was adjusted by a factorial planning 2^2 by a vortex (experiments 1 to 7) and orbital shaker (experiments 8 to 14). The variables were solventeproportion acetonitrile: water 1 (12.5:7.5), 0 (10:10) and -1 (7.5:12.5) and the level of salts MgSO4:NaCl (g): 1 (4:1), 0 (2.5:2.5) e -1 (1:4) applied to both group of experiments.

The experiments were carried out by weighing 2 g of the sample (culture medium), adding distillated water and acetonitrile, homogenizing it in a vortex for 4 min or orbital shaker for 20 min, adding salts ($MgSO_4$ and NaCl), vortexing for 3 min or shaking for 15 min and centrifuging (5 min, 3220 x g, 20 °C). After that, 4 mL of organic layer was dried. The procedure which had the best simultaneous recovery of the mycotoxins and resulted in the minimal wastes was chosen to extract them from the culture media of the experiment. The quantification of the *Fusarium* toxins (DON and NIV) was carried out by HPLC-DAD equipped with a C18 Supelco column (250 × 4.6 mm, 10 μm). ZEA was quantified by HPLC-FLD (fluorescence detector). The chromatographic conditions were: mobile phase water: methanol (88:12, v/v) at 0.8 mL min^{-1} for 8 min, methanol 1 mL min^{-1} for 10 min, and water: methanol (88:12, v/v) at 0.8 mL min^{-1} for 9 min, totaling 27 min.

2.4 Antifungical activity on F. graminearum

The fungus was incubated in SpezielleNahrstoffarmer Agar (SNA) at 25 °C for sporulation and kept at 4 °C in SNA medium. For the experiment, it as propagated in Potato Dextrose Agar (PDA) and incubated for 7 days at 25 °C in an environmental chamber (12 h light/12 h dark). γ-oryzanol was added to the medium so as to make up the concentrations of 0.1; 0.3; 0.6; 0.8, 1.0 and 1.2 g kg^{-1}. Synthetic fungicides were used at concentrations of 0.2 mg kg^{-1} for azoxystrobin and 0.4 mg kg^{-1} for trifloxystrobin, (the maximum limit allowed by the Brazilian law in rice crops (Heidtmann-Bemvenuti et al., 2012b). Mycelial disks (0.5 cm diameter) of fungal biomass were placed in the center of the Petri dish (8 cm diameter) containing 20 mL 3.9% PDA and fungal inhibitors (natural or synthetic). In the control medium it was not added inhibitor (Pagnussatt et al., 2014). Cultures were incubated in an environmental chamber (12 h light/12 h dark) for 7 days.

The inhibitory effects of the extracts were determined by using the agar dilution method measuring of the mycelium diameter daily, and the structural compounds of the fungal cell: glucosamine (wall) (Scotti et al., 2001) and ergosterol (membrane) (Pagnussatt et al., 2014) at the on the 7th day. The percentage of fungal inhibition by comparing the responses of these determinations in control plates. The minimum inhibitory concentration (MIC) was defined as the minimum concentration at μg kg^{-1} in which mycelium growth was not observed. The adapted and validated procedure was carried out to determine the mycotoxins production.

2.5 Antioxidant activity of γ-oryzanol extract

For purposes of comparison, the antioxidant activity of γ-oryzanol and gallic acid standards was also evaluated by different methods.

The consumption of free radical DPPH• were performed in UV-Vis spectrophotometer (Varian Cary-100) at 515 nm. For that, 0.5 mL methanol (control) and 0.5 mL antioxidant substance: γ-oryzanol extract (5, 10, and 25 μg mL^{-1}), γ-oryzanol standard (25 mg mL^{-1}) and gallic acid (3 μg mL^{-1}) were added to tubes containing 3.0 mL DPPH methanolic solution (5.2 x 10^{-5} mol l^{-1}). The reactive mixture was kept at room temperature in the dark, and the change from purple color to yellow was measured after 15 min. The ability to scavenge free radicals was expressed as percent inhibition (Schmidt et al., 2014).

The radical $ABTS^{\bullet+}$was generated by the oxidation of 7 mM ABTS with 2.45 mM potassium persulphate, allowing the mixture to stand in the dark for 16 h at room temperature. The solution containing the radical was diluted in ethanol to give an absorbance of 0.700 ± 0.020 at 734 nm. For that, 30 μL

antioxidant substance (γ-oryzanol extract (35 μg mL^{-1}), γ-oryzanol standard (35 μg mL^{-1}) and gallic acid (10 μg mL^{-1}) were added to 3 mL dilute $ABTS^{\bullet+}$. The absorbance was measured at 734 nm after 6 min of reaction. The results were expressed as trolox equivalent antioxidant activity (TEAC), as μmol $_{Trolox}$. $g_{extract}^{-1}$, using a Trolox calibration curve (0.1 to 2 μmol mL^{-1}) (Schmidt et al., 2014).

The ferric reducing antioxidant power (FRAP method) was assessed in each extract was prepared at different dilutions. The concentrations were 300, 500, and 600 μg mL^{-1} of γ-oryzanol extract; 100 μg mL^{-1} for the γ-oryzanol standard; and 1, 3, and 10 μg mL^{-1} for gallic acid. In the dark, an aliquot of 90 mL of each dilution was transferred to tubes containing 270 μL distilled water and 2.7 mL FRAP solution was added. The mixture was homogenized and placed in a water bath at 37 °C for 30 min, followed by absorbance measurement at 595 nm. The FRAP solution was prepared daily by mixing 25 mL of 0.3 M acetate buffer, 2.5 mL of 10 mM TPTZ solution, and 2.5 mL of 20 mM ferric chloride. The standard curve of ferrous sulfate was built between 500 and 2000 μM.

The enzymatic browning inhibion reaction was carried out at 30 °C at pH 6.5 using 1% guaiacol as substrate, 0.08% H_2O_2 and peroxidase extracted from potato (enzyme extract was obtained by mixing potato peel and buffer at a ratio of 1:25), besides the enzyme inhibitor (γ-oryzanol 25 μg mL^{-1}, γ-oryzanol standard 25 μg mL^{-1}, and gallic acid standard 5 μg mL^{-1}). An aliquot of the exctract (1 mL) was added as inhibitor of the reaction, which was replaced by distilled water in the control group. The reaction consisted of adding 1.5 mL of pH 6.5 phosphate buffer, 1 mL of distilled water, 1 mL of 0.08% hydrogen peroxide; 0.5 mL of 1% guaiacol, 1 mL of inhibitor (extract), and 1 mL of enzyme extract. Absorbance was measured at 470 nm on a UV-Vis spectrophotometer (Cary 100, Varian model) at 5, 10, 15, 20, 30, and 40 min. The antioxidant power was expressed as the percent inhibition of browning reaction in relation to the control (100%) (Oliveira & Badiale-Furlong, 2008).

3 Results and discussion

3.1 Composition of the rice bran and γ-oryzanol

Rice bran acidity and pH were 3.4% ± 1.2 and 6.0 ± 0.1; moisture 8.4 ± 0.1%; lipids 18.7 ± 0.6%; ash 8.9 ± 0.6%; protein 20.3 ± 1.7%; fiber 8.9 ± 1.4% carbohydrates 34.8 ± 0.9% (estimated by difference). This profile content in rice bran is similar to routinely related by others researches (Massarolo et al., 2017; Schmidt et al., 2014). The lipid fraction of rice bran present the main content of γ-oryzanol, so it is important to use crude bran (Pestana-Bauer et al., 2012; Massarolo et al., 2017).

γ-oryzanol extracted from rice bran was dark yellow oil with a characteristic odor and the extraction yield of rice bran oil was 1.4%. Among the 37 fatty acids found most of them are polyunsaturated (51.6%), followed by monounsaturated (32.3%) and saturated (16.1%) fatty acids. The alpha-linolenic acid (18:3 n3) was present in greater amount (34.6%), followed by oleic acid (18:1 n9c) (31.5%), palmitic acid (16:0) (14.6%), linoleic acid (18:2 n6c) (9.0%), and gamma linolenic acid (18:3 n6) (6.5%). Other fatty acids were found in amounts below 1%. Despite the health benefits of unsaturated fatty acids, they are also more susceptible to oxidation, which may require more intense γ-oryzanol antioxidant activity.

The correlation coefficient (r) of standard curves of γ-oryzanol applied to evaluate the components of the extracts are bigger than 0.9. The major components of γ-oryzanol are present in the sample with the same retention time of the standard, confirming the presence of γ-oryzanol in the rice bran extract.

3.2 Effects on the complex F.graminearum CQ244 growth and mycotoxins production

The complex *F. graminearum*CQ244 was previously studied by Pagnussatt et al. (2014) regarding its response under the effect of enzymatic inhibitor extracted from wheat and rice. Its toxigenic potential was also tested by the authors (data not shown). Although many studies have shown the antioxidant property of γ-oryzanol, its antifungal activity has not been studied deeply (Heidtmann-Bemvenuti et al., 2016). Likewyseγ-oryzanol extract was studied as an antifungal by applying to the culture media. The effects were compared to synthetic fungicides azoxystrobin and trifloxystrobin.

Increase in the γ-oryzanol concentration (1 g kg^{-1}) led to decrease in the mycelium growth, reaching 53% inhibition by comparison with the control on the 3th day and 37% on the 7th day. It suggests that the inhibition is reversible at this level. Regarding the synthetic fungicides, the highest halo inhibition was observed with trifloxystrobin (14%) by comparison with azoxystrobin (0%) on the 7th day. Mycellium growth was not observed when γ-oryzanol concentration was 1.2 g kg^{-1} was used. Therefore, it was considered the minimum inhibitory concentration (MIC) for 100% of inhibition of the microorganism.

Mycelium growth in the control culture on the last day of the experiment was equal the media with synthetic fungicides (Figure 1). It is noteworthy that the chemical fungicide concentration was lower than that of the natural fungicide, because in the experiment Maximum Residual Limits (MRL) for processed rice, in according to Braziliam law, was observed, i. e., 0.1 mg kg^{-1} for azoxystrobin and 0.2 mg kg^{-1} for trifloxystrobin.

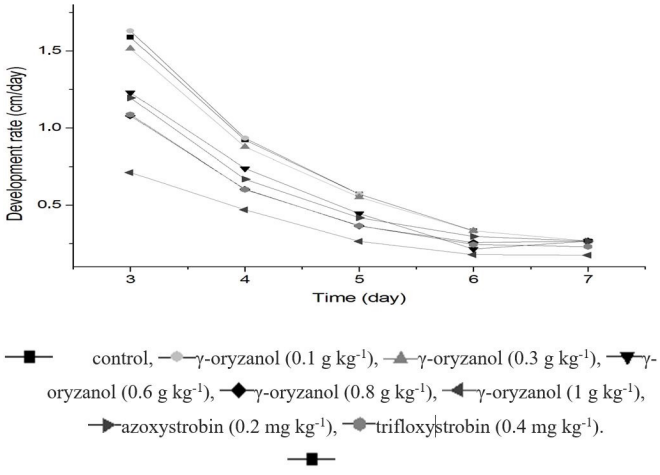

Figure 1. *F. graminearum* CQ244 complex development rate (cm day^{-1}).

Above these limits, fungicides are dangerous to human health, specially in the case of rice that is daily consumed in many countries (Hýsek et al., 2005).

The determination of fungal structural components, such as glucosamine and ergosterol, provides better insight to understand the natural extract mechanism to prevent fungal contamination at different points in the food chain. γ-oryzanol, in addition to its antioxidant capacity reported in literature, also has a promising effect against *Fusarium* growth. Table 1 shows the mean values and the percentage of fungal inhibition (%) in agreement with halo diameter, glucosamine and ergosterol measurements under different active-principles.

Again, γ-oryzanol exhibited fungal inhibition by comparison with chemical fungicides. It is worth mentioning that γ-oryzanol, at the lowest concentration (which is about one hundred times lower than the one naturally found in rice bran), did not inhibit fungal halo development. Besides, the chemical fungicide present a low inhibition of the ergosterol production, either. In the plants, γ-oryzanol acts synergistically with other chemical families that inhibit fungal growth. In fact, this corroborates the assumption that γ-oryzanol is associated to the natural defense mechanism of rice grain against fungal pathogens.

γ-oryzanol inhibited the fungal glucosamine in a range from 38% (for γ-oryzanol, 0.1 g kg^{-1}) to 77% (for γ-oryzanol, 0.8 g kg^{-1}), while the ergosterol inhibition varied from 0.6% (for γ-oryzanol, 0.1 g kg^{-1}) to 36% (for γ-oryzanol, 1.0 g kg^{-1}). The fungicide azoxystrobin inhibited the glucosamine production by 48% and ergosterol by 15%, while 48% glucosamine and 10% ergosterol inhibition was observed in the experiments with trifloxystrobin. This is another evidence that γ-oryzanol can be more effective to inhibit the fungus than synthetic fungicides.

The glucosamine (wall) was more inhibited by the antifungal agent than ergosterol (membrane), confirming that γ-oryzanol plays an important role in the decrease fungal cell protection efficiency. Data suggest that γ-oryzanol may adhere to the surface of the fungal cell, thus preventing the exchange with the environment. Decrease in the production of components of the fungal cell wall by phenolic extracts from *Spirulina* was previously demonstrated by other authors (Scotti et al., 2001; Pagnussatt et al., 2014).

Mycotoxins determined in culture media containing antifungals and control are shown in Table 2. The adapted procedure to determine them showed a media recovery around 90% and a variability 8%, therefore suitable to apply in this study. For effective inhibition of the three mycotoxins in this study, γ-oryzanol concentrations above 0.6 g kg^{-1} were required. All treatment decreased DON production, however synthetic fungicides at low concentration stimulated NIV and ZEA production.

Some studies have shown that the use of synthetic fungicides can lead to higher mycotoxin contamination of grains by comparison with crops which are not subjected to chemical treatment (Dors et al., 2013; Heidtmann-Bemvenuti et al., 2012b). It can cause more stress to the fungus, triggering its

Table 1. *F. graminearum* CQ244 complex growth in the presence and absence of antifungal agents (7^{th} day).

Antifungal Agent	antifungal	Halo		Glucosamine		Ergosterol	
		Mean ± SD (cm)	Inhibition (%)	Mean ± SD (mg/g)	Inhibition (%)	Mean ± SD (mg/g)	Inhibition (%)
Control	-	8.0 ± 0	-	31.0 ± 2.9	-	1.4 ± 0.3	-
Azoxystrobin	0.2 mg kg^{-1}	8.0 ± 0	0	16.0 ± 2.1	48	1.2 ± 0.1	15
Trifloxystrobin	0.4 mg kg^{-1}	6.9 ± 0	14	16.1 ± 3.4	48	1.3 ± 0.3	10
Oryzanol	0.1 g kg^{-1}	8.0 ± 0	0	19.2 ± 2.6	38	1.4 ± 0.2	1
Oryzanol	0.3 g kg^{-1}	8.0 ± 0	0	15.9 ± 3.1	49	0.9 ± 0.1	32
Oryzanol	0.6 g kg^{-1}	8.0 ± 0	0	13.2 ± 3.5	57	0.9 ± 0.1	35
Oryzanol	0.8 g kg^{-1}	8.0 ± 0	0	7.1 ± 0.0	77	0.9 ± 0.1	32
Oryzanol	1.0 g kg^{-1}	5.0 ± 0.9	37	20.7 ± 0.5	33	0.9 ± 0.1	36

Values are shown as mean ± standard deviation (SD) (n=3).

Table 2. Mycotoxins NIV, DON, and ZEA under treatments with natural and synthetic fungicides.

Main active	Antifungal	Mycotoxins (μg kg^{-1})		
		NIV	DON	ZEA
Control	-	84.59	115.11	70.89
Azoxystrobin	0.2 mg kg^{-1}	87.25	78.58	78.59
Trifloxystrobin	0.4 mg kg^{-1}	89.75	67.51	72.88
Oryzanol	0.1 g kg^{-1}	81.29	87.14	70.60
Oryzanol	0.3 g kg^{-1}	91.24	81.47	67.39
Oryzanol	0.6 g kg^{-1}	97.53	113.22	65.46
Oryzanol	0.8 g kg^{-1}	61.03	105.66	69.14
Oryzanol	1.0 g kg^{-1}	54.88	88.43	70.66

NIV = nivalenol; DON = deoxynivalenol; ZEA = zearalenone.

toxigenic potential, and thus leading to mycotoxin production. In this experiment, NIV and ZEA production was favored when chemical fungicides were used. Accordingly, the use of a natural compound was more effective to inhibit mycotoxin production.

The antioxidants may act as antifungal agents and aflatoxin inhibitors (Oliveira & Badiale-Furlong, 2008; Souza et al., 2011). According to the authors, the inhibition of aflatoxin synthesis is due to the decrease lipid peroxidation and consequent oxidative stress that is related to the toxin biosynthesis. This effect may be the same as the one found in this experiment. Table 3 shows the effects of the treatments with antifungal compounds as specific halo diameter inhibition, ergosterol and glucosamine in the fungal biomass under estimate, considering the conditions in which each one is more efficient.

3.3 Antioxidant activity of the γ-oryzanol

The DPPH method has already been adopted by researches of antioxidant properties, because reaction conditions is well established. It was carried out in the experiments with different levels of γ-oryzanol and reaction time, based on the literature, to infer about conditions to conducte other experiments with γ-oryzanol extracts (Table 1). The extract containing 5μg mL^{-1} was sufficient to promote the major specific inhibition (0.2%/μg/min). After 15 min there was no improvement on the inhibition. γ-oryzanol standard solutionwas less efficient than the crude natural extract.(specific activity 0.02%/μg/min)whereas gallic acid solution showed thehighest specific inhibition: (0.9%/μg/min).

γ-oryzanol crude extract is interesting because its ability to scavenge the $DPPH^{\bullet}$ radical is higher than the one of the standard, because the unsatured fatty acids can acts synergistically to capture free radicals. It was possible to estimate that the amount of extract required to decrease the initial DPPH concentration by 50% (MIC_{50}) was 29 μg mL^{-1} for γ-oryzanol extract; 129 μg mL^{-1} for γ-oryzanol standard solution and 3.5 μg mL^{-1} for gallic acid solution.

The $ABTS^{\bullet+}$ method is fast and allow the analysis of compounds of both lipophilic and hydrophilic (Kuskoski et al., 2005). Considering that fatty acids are components of γ-oryzanol extract an experiment was conducted with crude extract and standard solution concentration containing 35 μg γ-oryzanol mL^{-1} to estimate the specific inhibition of ABTS radical. The FRAP power was also estimated the specific inhibition for crude, standard solutions of γ-oryzanol and gallic acid. The gallic acid standard provided greater ability to reduce iron (200 fold the natural extract).

The antioxidant activity can also inhibit the effects of enzymatic browning by oxidoreductases, since they have the function of oxidizing electron donor compounds under their action. Specific inhibition of peroxidase was also estimated in this study too. Again gallic acid solution showed the highest antioxidant activity in a similar way to other experiments. The peroxidase inhibition by γ-oryzanol is lower compared to other procedure evaluated. This may be attributed to its low polarity and high content of unsaturated fatty acids, that do not allow the interaction between the γ-oryzanol and the peroxidase (Oliveira & Badiale-Furlong, 2008). Therefore these structures are less effective in aquos media where the enzyme acts. To improve the comparation between the antioxidant effect of γ-oryzanol were estimated as their specific inhibition (Table 4).

Regarding fungal property inhibition, γ-oryzanol extract was the most efficient because it promotes the strongest effect on the inhibition in relation to synthetic fungicides. It is worth mentioning that nivalenol and zearalenone were not inhibited by any synthetic fungicide. Comparing both specific and antifungal activity antioxidant of γ-oryzanol, it was more efficient as an antioxidant than an antifungal but it was the best inhibitor of manifestation of the complex *F. graminearum* toxigenic potential. It stands out as the only one that was able to inhibit nivalenol production that would be attributed to the antioxidant activity on the fungal biomass. Estimating the specific activity of the extracts for each method made it possible to confirm that apply the γ-oryzanol crude extract is the best for avoid oxidative process promote by any mechanism.

Table 3. Specific inhibition of fungal biomass by antifungal compounds.

Specific inhibition	Azoxistrobine	Trifloxistrobine	γ-oryzanol
% Halo growth/μg antifungal	NI	0.014	0.04
% ergosterol/ μg antifungal	0.048	0.05	0.05
% glucosamine/ μg antifungal	0.015	0.010	0.04
% deoxynivalenol/ μg antifungal	0.001	0.0005	0.001
% nivalenol/ μg antifungal	NI	NI	0.002
% zearalenone/ μg antifungal	NI	NI	NI

NI = no inhibition.

Table 4. Specific antioxidants properties of the crude and standard solutions.

antioxidants	γ-oryzanol standard	Gallic acid standard	γ-oryzanol Crude
DPPH	0.07 ± 0.01	14.2 ± 0.18	2.1 ± 0.22
mM rolox/ μg	0.14 ± 0.01	2.3 ± 0.07	0.07 ± 0.02
FRAP/ μg	0.39 ± 0.06	2591 ± 1.2	0.03 ± 0.00
Inibição %PO/ μg	0.96 ± 0.01	7.5 ± 0.12	0.5 ± 0.00

4 Conclusions

The ability of γ-oryzanol as an antifungal and its action mechanism, using complex *F. graminearum*CQ244 as a model, was demonstrated for the first time. Glucosamine (fungal wall) production during cultivation was inhibited by the antifungal agents (natural and chemical ones), rather than by ergosterol (fungal membrane), suggesting that antifungals play an important role in reducing the fungal cellular defense system. It was possible the effective inhibition of the three mycotoxins under study (NIV, DON and ZEA) using γ-oryzanol.

This study shows that γ-oryzanol extract is, beyond and antioxidant compound, a natural antifungal, since it inhibits growth and prevents the manifestation of the toxigenic potential of the chosen fungus model.

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