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Evaluation of the association of 8-hydroxyquinoline with fungicides against phytopathogenic fungi in rice seeds. Avaliação da associação de 8-hidroxiquinolina com fungicidas contra fungos fitopatogênicos em sementes de arroz.

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Abstract

Evaluation of the association of 8-hydroxyquinoline with commercial fungicides against phytopathogenic fungi where it was possible to verify the synergism between the compounds and the antifungal, which led to *in vivo* tests using rice seeds. The association of 0.5 g/L of mancozeb and 0.031 g/L of 8-hydroxyquinoline, concentration 4 and 8 times lower than the one usually used, respectively, was promising. The test of leukocyte cytotoxicity and dermal toxicity was carried out, showing, in view of all the tests performed, a potential selective agent against variations of *Fusarium graminearum* and *Fusarium meridionale* in rice seeds.
Keywords: *Fusarium graminearum*. *Fusarium meridionale*. Synergism. Mancozeb.

Resumo

Avaliação da associação da 8-hidroxiquinolina com fungicidas comerciais contra fungos fitopatogênicos onde foi possível verificar o sinergismo entre os compostos e o antifúngico, o que levou a testes *in vivo* utilizando sementes de arroz. A associação de 0,5 g/L de mancozebe e 0,031 g/L de 8-hidroxiquinolina, concentração 4 e 8 vezes inferior à usualmente utilizada, respectivamente, foi promissora. Foi realizado o teste de citotoxicidade leucocitária e toxicidade dérmica, mostrando, diante de todos os testes realizados, um potencial agente seletivo contra variações de *Fusarium graminearum* e *Fusarium meridionale* em sementes de arroz.
Palavras-chave: *Fusarium graminearum*. *Fusarium meridionale*. Sinergismo. Mancozebe.

1. Introduction

The use of fungicides is one of the most important aspects in agriculture to protect seeds during storage, as well as in the field, preventing the growth of fungi that can produce toxins (Fatma et al., 2018). The application of fungicides helps to reduce the incidence of Fusarium infection, also reducing the levels of mycotoxins in commercial grains (Shishatskaya et al., 2018).

According to the Environmental Protection Agency (EPA, 2005), mancozeb (MZ) is a broad-spectrum fungicide with multisite action, which is indicated for the control of fungi in various cultures. This fungicide belongs to the ethylene bisdithiocarbamate (EBDC) class of pesticides, a subclass of dithiocarbamate fungicides, being an antifungal agent, it inactivates amino acid sulfhydryl groups in fungal enzymes, disarms in the interruption of lipid metabolism, ATP (energy) production, it also has a EBDC backbone complexed with manganese and zinc metals. After exposure, dithiocarbamates are considered to have low acute human toxicity via the dermal, oral and respiratory routes, however, ethylenethiourea (ETU), a metabolite of EBDCs causes adverse effects after chronic exposure, where workers exposed to MZ have been shown to increase urinary excretion of manganese and ETU, hemoglobin-ETU adducts and altered thyroid function (Dhaneshwar & Hardej, 2021).

Difenoconazole being a cis, trans-3-chloro-4-[4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl-4 - chlorophenylether) phenyl-4-chlorophenylether, known commercially as Score®, belongs to the triazole group of pesticides, being a systemic inhibitor of sterol demethylation (DMI), which has the ability to interfere with mycelial growth and inhibit the germination of spores of pathogens that ultimately result in the inhibition of fungal growth (Wang et al., 2019; Li et al., 2013).

8-Hydroxyquinoline (HQ) and its derivatives are a subclass of quinolines which have many biological functions, where they have long been used for various purposes, as preservatives in the textile, paper and wood industries, as well as fungicide in agriculture, presenting importance as a fungicide, bactericide and antiproliferative agent (Oliveri et al., 2016).

Considering the importance of the economic impact caused by the contamination of grains, through phytopathogenic fungi, and the use of agricultural fungicides. Our group evaluated the effectiveness of mancozeb, difenoconazole and 8-hydroxyquinoline against *Fusarium graminearum* and *Fusarium meridionale*, both *in vitro* and *in vivo*, the latter against rice seeds, with the intention of obtaining an agricultural fungicide developed in reduced concentrations that can reduce the damage caused to agriculture. Thus, this study evaluated the antifungal susceptibility of the agricultural fungicides MZ and Difenoconazol (Score®) and of the antimicrobial 8-OH, along with an evaluation of double and three-dimensional checkboard and an *in vivo* evaluation with rice seeds, soybean against *F. graminearum* and *F. meridionale* strains.

2. Materials and methods

2.1 Antifungal susceptibility test

2.1.1 Strains

The Fusarium species (*F. meridionale* (09MI21); *F. graminearum* (09TR107); *F. asiaticum* (09AR04) were selected for a screening test, in which they are deposited in the mycological collection of the Research Laboratory in Applied Mycology - Faculty of Pharmacy, Federal University of Rio Grande do Sul.

2.2 Agricultural fungicides

The fungicides MZ and Difenoconazole (Score®) were provided by the Federal University of Pampa (UNIPAMPA), campus in Itaqui, Rio Grande do Sul, Brazil. 8-Hydroxyquinoline was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3 Determination of minimum inhibitory concentration

Minimal inhibitory concentrations (MIC) of MZ, Difenoconazole (Score®) and 8-HQ were determined by the broth microdilution method, according to the M38-A2 protocol (CLSI, 2008), where conidia inocula (1.0x103 a 3.0x103 UFC/mL-1) was prepared in cultures obtained on potato dextrose agar (PDA) at 32°C. Assays were conducted with RPMI medium, containing L-glutamine (without sodium bicarbonate), buffered to pH 7 with 0.165 mol-1 MOPS (3[N-Morpholino] propanesulfonic acid). The MIC values were defined as, the lowest concentration of antifungals, and 8-OH in which the Fusarium species did not demonstrate visible growth within 48 h.

2.4 Checkerboard test

The interaction of 8-HQ with MZ and Score® was evaluated for all strains using the chess method, where the requirements tested vary in MIC/8, MIC/4, MIC/2, MIC, MICx2, MICx4 and MICx8 for each agent. Considering that the assays, were performed in triplicate and incubated at 32 °C for 48 h, the interaction effect was defined quantitatively as an index of fractional inhibitory concentration (IFI), for knowledge, synergism was defined when FICI ≤ 0.5 , antagonism when FICI> 4 and indifference when 0.5 <FICI ≤4, defined by the following equation: (Costa et al., 2020).

$$\text{FICI} = FICa + FICb = \frac{MICa\ in\ combination}{MICa\ tested\ alone} + \frac{MICa\ in\ combination}{MICa\ tested\ alone}$$

2.5 Three-dimensional checkerboard

The three-dimensional gridded assay combining MZ, Score® and 8-HQ was performed according to Stein et al (2015), with slight modifications for each strain and agent, as selected concentration ranges depended on pre-determined in the MIC (Scheme 1) , then MIC / 8, MIC / 4, MIC / 2, MIC and MICx2 rules were used. As microplates were filled with 100 µl of a solution of the three agents at different application levels from suppliers with 100 µl of the fungal suspensions, the microplates were incubated for 48 h at 32 °C, and the experiment was carried out in duplicate and for the sake of knowledge, the synergism was defined when FICI ≤ 0.75, antagonism when FICI > 4 and indifference when 0.75 < FICI ≤ 4, and the fractional inhibitory concentration index (FICI) for triple antifungal combination was added as follows: (Costa et al., 2020).

$$\text{FICI} = FICa + FICb + FICc = \frac{MICa\ in\ combination}{MICa\ tested\ alone} + \frac{MICb\ in\ combination}{MICb\ tested\ alone} + \frac{MICc\ in\ combination}{MICc\ tested\ alone}$$

2.6 Evaluation of mycelial growth inhibition in a petri dish

Fungal colonies of isolates of *F. graminearum* (09TR107) and *F. meridionale* (09MI21) colonies, the Applied Mycology Research Laboratory - Faculty of Pharmacy, Federal University of Rio Grande do Sul, were cultivated in potato dextrose agar (BDA) for 7 days to remove discs. Mycelium with 6 mm in diameter, which were transferred to the center of new petri dishes with PDA medium containing different concentrations of the agents (25, 50, 100, 200, 200 and 800 μg/mL-1), in addition to the control, without treatment. These were kept at 25±2°C in continuous dark. Colony diameter was measured after 6 days, in two perpendicular directions. Three replicates per strain were used. (Becher et al., 2010; Spolti et al., 2012).

2.7 Quantitative analysis of the incidence of *F. graminearum* and *F. meridionale* in rice seeds

Rice seeds were obtained from IRGA (Rio Grandense Institute of Rice), in the city of Itaqui, Rio Grande do Sul, Brazil. Rice seeds of the variety BR/IRGA 409 were used. The Gerbox acrylic boxes were disinfected with 2% sodium hypochlorite, three sheets of cut filter paper (10.5x10.5 cm), previously sterilized, and were inserted inside at 160°C for 20 minutes. The paper was moistened with sterile water. Each treatment had 120 seeds collected randomly, distributed in 4 repetitions of 30 seeds each (Table 1 and Table 2) (Neves et al., 2009).

Table 1 - Concentrations tested in preventive and curative treatment in rice seeds for the fungus *Fusarium meridionale*.

Samples	**Concentration**
***Fusarium meridionale* (Positive Control)**	2,5 X 105
DMSO	2%
MZ	2 g/L
MZ	0,5 g/L
8-HQ	0,25 g/L
8-HQ	0,031 g/L
Score®	2 g/L
Score®	0,5g/L
MHQ	2 g/L + 0,25 g/L
MHQ	0,5 g/L + 0,031 g/L
MHQ	0,25 g/L + 0,031 g/L
MHQ	0,125 g/L + 0,031 g/L
SHQ	2 g/L + 0,25 g/L
SHQ	1 g/L + 0,25 g/L
SHQ	0,5 g/L + 0,25 g/L
SHQ	0,25 g/L + 0,25 g/L

DMSO: Dimethylsulfoxide; MZ: Mancozeb 8-HQ: 8-hydroxyquinoline; MHQ: Mancozeb + 8-hydroxyquinoline; SHQ: Score® + 8-hydroxyquinoline.

Table 2 - Concentrations tested in preventive and curative treatment in rice seeds, for the fungus *Fusarium graminearum*.

Samples	Concentration
***Fusarium graminearum* (Positive Control)**	2,5 X 105
DMSO	2%
MZ	2 g/L
MZ	0,5 g/L
8-HQ	0,25 g/L
8-HQ	0,031 g/L
MHQ	2 g/L + 0,25 g/L
MHQ	0,5 g/L + 0,031 g/L
MHQ	0,25 g/L + 0,031 g/L
MHQ	0,125 g/L + 0,031 g/L

DMSO: Dimethylsulfoxide; MZ: Mancozeb; 8-HQ: 8-hydroxyquinoline; MHQ: Mancozeb + 8-hydroxyquinoline.

The preventive treatment was evaluated, where first the fungicide was applied after 1 hour the seeds are infected, and after 48 hours, 14 days are counted for the final reading. As for the curative treatment, the seeds are first infected, wait 48 hours, then apply the fungicide and count 14 days for the final reading.

2.8 Cytotoxicity Test

To assess the cytotoxicity of the agents used, the cell viability assay was performed.

Cell culture

1 mL of venous blood collected by venipuncture, from a young adult volunteer over 18 years of age, without the use of medication was used lymphocytes (protocol approved by the Ethics Committee of the Federal University of Pampa, under number 27045614.0.0000.5323). Were obtained, by centrifugation gradient, immediately transferred to culture medium containing 9 ml of RPMI 1640, medium supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin. The cell culture flasks were collected for 72 hours in an incubator at 37°C, the positive control with 3 μg mL-1 of hydrogen peroxide and the negative control was prepared with 500 μL of PBS buffer 7.4 (Santos Montagner et al., 2010).

Cell Viability

Assessed by the loss of leukocyte membrane integrity, cell viability was performed using tripamide blue dye (BUROW et al., 1998), the technique uses leukocytes which, if unknown in Tripam blue reagent, after three minutes, an aliquot is interrupted in the Neubauer chamber for visualization under a microscope (400X magnification), it is known that dead cells acquire a blue color, therefore, being visually differentiated from living cells, 300 cells were counted.
Data will be directed to statistical analysis ANOVA, followed by Tukey test, with data expressed as mean ± SD, where differences will be considered statistically significant when $p < 0.05$. Data will be analyzed using GraphPad Prism 5.0 software.

2.9 Permeation determination and histopathological evaluation

Using tissue from adult male pigs, slaughtered at the Federal Institute of Santa Catarina - Concordia Campus, the formation of tissue damage caused by the action of the MZ and 8-HQ was evaluated, where these animals were slaughtered following the rules of the Ministry of Agriculture of Brazil. Respecting animal welfare (Brazil, 2013), pig ear skin was used, which was removed 5 minutes after slaughter; hairs were removed by an electric trimmer and transported to the laboratory in an ice-cold Krebs-Hepes buffer, and the original skin was mounted in Franz diffusion cells (Logan Instrument Corp., NJ) with diffusion area of approximately 1.75 cm2.

The epidermal side of the skin, was exposed to a solution of 0.1 M NaOH (positive control) and PBS pH 7.0 (negative control), for 6 hours, in this test, MZ (at a concentration of 0.5 and 2.0 g/l), 8-HQ (at a concentration of 0.031 and 0.25 g/l) and "T Fungicide" (solution MZ and 8-HQ in procedures of - 0.5 + 0.031 and 2.0 + 0.25 g/l, respectively). The epidermal surface of the skin was subjected to contact of the extract with this solution for a period of 6 hours, fragments of these tissues were collected, fixed in 10% neutral buffered formalin, routinely processed and stained with hematoxylin and eosin (HE) and examined in light microscopy and experiments were performed in triplicate.

3. Results and discussion

3.1 Susceptibility antifungal test

All agents tested were able to inhibit the growth of *F. graminearum*, *F. meridionale* and *F. asiaticum*. MIC values ranged from 0.25 μg/mL to 8 μg/mL (Table 3).

Table 3 - Values of minimum inhibitory concentrations (MICs) (μg/mL) for fungicides.

Isolados	MZ	Score®	8-HQ
	MD - SD	MD - SD	MD - SD
09MI21	4,0 - 0	4,0 - 0,94	0,5 - 0
09TR107	8,0 - 1,88	4,0- 1,88	0,5 - 0,12
9AR04	0,25 - 0,12	8,0 - 1,88	2,0 - 0,47

09MI21: *F. meridionale*; 09TR107: *F. graminearum*; 09AR04: *F. asiaticum*. MZ: Mancozeb; 8-HQ: 8-hydroxyquinoline.

In our study MZ and 8-HQ combination present synergism against *F. graminearum* and *F. meridionale* strains and in an unprecedented way, in an *in vivo* experiment, using rice seeds infected with these two strains. When MZ was tested at a concentration 4 times, lower than that usually used and 8-HQ, 8 times lower than usual, it showed inhibition of the growth of *F. graminearum* and *F. meridionale*, in preventive treatment and similarly these concentrations "cured" the seeds, in their curative treatment, making their potential visible.

The concentration reduction of, MZ and 8-OH was taken from the *in vitro* test performed where comparing the individual MIC and the MIC of these association, a reduction 4 to 8 times was

obtained, there is no comparison in the literature between MZ, Score® and 8-HQ fungicides directly related to rice seeds, *F. graminearum* and *F. meridionale*. This can be emphasized in a study carried out by Silva et al. (2008), where the effect of plant extracts on the control of *F. oxysporum* f. sp. tracheiphilum from cowpea seeds, compared with the effect of MZ in association with plant extracts. Still, coming to corroborate our findings, we can analyze the package insert of the agricultural fungicide Unizeb gold®, a product that prescribes MZ, which does not talk about fighting as much *F. graminearum* and *F. meridionale*, in curative and preventive treatments.

3.2 Checkerboard Test

In a double association test, MZ + 8-HQ combination presented synergism against *F. meridionale* and *F. graminearum*. However, the Score® + 8-HQ combination showed synergism only against *F. meridionale*. In addition, both associations did not show synergism against *F. asiaticum* (Table 4).

Table 4 - MIC values (μg/mL) for fungicides.

Strains	MZ + 8-HQ	ICIF	Score® + 8-HQ	ICIF
	MD - SD		MD - SD	
09MI21	1,0 + 0,06	0,31*	2 + 0,5	0,5*
09TR107	0,5 + 0,06	0,28*	1 + 0,06	1,0
09AR04	0,5 + 0,12	1,12	2 + 0,06	1,25

*: Synergism; 09MI21: *F. meridionale*; 09TR107: *F. graminearum*; 09AR04: *F. asiaticum*; MZ: Mancozeb; 8-HQ: 8-hydroxyquinoline.

3.3 Three-dimensional Checkerboard

In triple association, both fungicides, MZ Score® and 8-HQ presented synergism against *F. meridionale* (09MI21) and *F. graminearum* (09TR107) (Table 5).

Table 5 - Checkerboard values (μg/mL) in triple association of Mancozeb, Score® and 8-Hydroxyquinoline fungicides against *Fusarium meridionale* (09MI21) and *Fusarium graminearum* (09TR107).

Strains	**MZ**	**Score®**	**8-HQ**	**FICI**
	MD - SD	MD - SD	MD - SD	
09MI21	0,25 + 0,11	0,25 + 0,11	0,5 + 0,17	0,5*
09TR107	0,25 + 0	0,25 + 0,05	0,5 +0,11	0,5*

*: Synergism; 09MI21: *F. meridionale*; 09TR107: *F. graminearum*; MZ: Mancozeb; 8-HQ: 8-hydroxyquinoline.

Thus, once again, our study brings exceptionally these results, coming to collaborate with the agronomic area, coming up with a probable new option to avoid the contamination of seeds during their storage, thus bringing several benefits. The generation of reactive oxygen species (ROS) is the main component of EBDC pesticide-induced toxicity, metals present in MZ can generate ROS,

particularly through Fenton-like reactions, resulting in mitochondrial dysfunction and ultimately leading to apoptotic cell death (Srivastava et al., 2012; Dhaneshwar & Hardej, 2021).

Difenoconazole is the typical triazole fungicides, which inhibits ergosterol biosynthesis by inhibiting the activity of the cytochrome P450 enzyme (CYP450) and thus inhibits fungal growth; therefore, it is widely used to control fungal diseases of fruits and vegetables. With the increase in its use in agricultural production, its residue gradually accumulated in surface water, and it was reported that the residue in rice field water was still as high as 1.98 mg/L after spraying it for 7 days, thus, it can be seen that Difenoconazole has a high residue, so its risk to the health of the human body cannot be ignored (Wang et al., 2021).

3.4 Evaluation of mycelial growth inhibition in a petri dish

In the mycelial growth assay, the fungicide MHQ was tested. Then, where for *F. meridionale* (09MI21) there was a small growth observed up to the 50 µg/mL-1 (0.3 X 0.4 X 0 0.3 X 0.2 mm), after this concentration, no growth was observed. As for *F. graminearum*, there was only growth in the control, with no growth being observed in any of the concentrations tested (Figure 1).

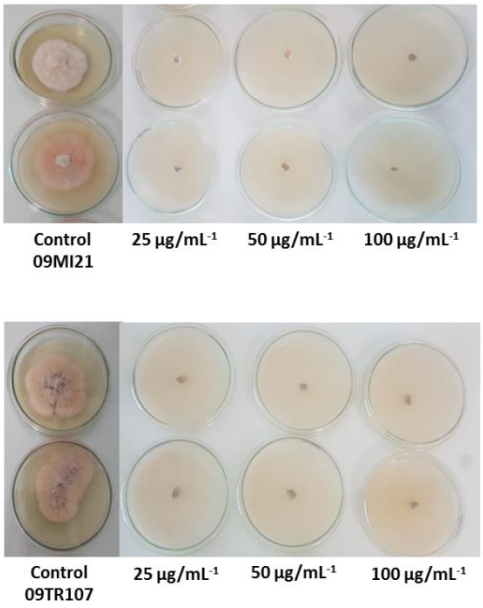

Figure 1 - Mycelial growth assay. 09MI21: *Fusarium meridionale*; 09TR107: *Fusarium graminearum*. Concentrations of 25, 50 and 100 µg/mL-1.

When the mycelial growth was evaluated, it was possible to verify the effectiveness of our association, since the mycelial growth was only observed in low drops and evaluated in terms of growth measures. We were able to demonstrate reduced growth compared to our control, when it was *F. meridionale,* and for *F. graminearum* there was no growth, which corroborates our *in vivo* assay.

3.5 Quantitative analysis of the incidence of *F. graminearum* and *F. meridionale* in rice seeds

For curative treatments, the inoculum showed a total reduction in seed viability, providing minimum counts in all tests performed. The 2% DMSO solution was not able to have a great increase in antifungal activity. However, it was significant, but different from all proposed treatments, not being the agent responsible for the decrease in infection (Table 6 and 7) (Figure 2).

Table 6 - Quantitative analysis evaluated in the curative treatment of rice seeds contaminated with *F. meridionale*.

Samples	**Concentration**	**Number of healthy seeds**	**Mean (120 seeds)**
***Fusarium meridionale* (Positive Control)**	2,5 X 105	0	0
DMSO	2%	12	3
MZ	2 g/L	70	17,5
MZ	0,5 g/L	61	15,25
8-HQ	0,25 g/L	65	16,25
8-HQ	0,031 g/L	58	14,5
Score®	2 g/L	90	22,5
Score®	0,5g/L	73	18,25
MHQ	2 g/L + 0,25 g/L	115	28,75
MHQ	0,5 g/L + 0,031 g/L	112	28
MHQ	0,25 g/L + 0,031 g/L	79	19,75
MHQ	0,125 g/L + 0,031 g/L	40	10
SHQ	2 g/L + 0,25 g/L	102	25,5
SHQ	1 g/L + 0,25 g/L	95	23,75
SHQ	0,5 g/L + 0,25 g/L	71	17,75
SHQ	0,25 g/L + 0,25 g/L	49	12,25

DMSO: Dimethylsulfoxide; MZ: Mancozeb; 8-HQ: 8-hydroxyquinoline; MHQ: Mancozeb + 8-hydroxyquinoline; SHQ: Score® + 8-hydroxyquinoline.

Table 7 - Quantitative analysis evaluated in the curative treatment of rice seeds contaminated with *F. graminearum*.

Samples	**Concentration**	**Number of healthy seeds**	**Mean (120 seeds)**
***Fusarium graminearum* (Positive Control)**	2,5 X 105	0	0
DMSO	2%	13	3,25
MZ	2 g/L	67	16,75
MZ	0,5 g/L	58	14,5
8-HQ	0,25 g/L	86	21,5
8-HQ	0,031 g/L	58	14,5
MHQ	2 g/L + 0,25 g/L	117	29,25
MHQ	0,5 g/L + 0,031 g/L	113	28,25
MHQ	0,25 g/L + 0,031 g/L	95	23,75
MHQ	0,125 g/L + 0,031 g/L	51	12,75

DMSO: Dimethylsulfoxide; MZ: Mancozeb; 8-HQ: 8-hydroxyquinoline; MHQ: Mancozeb + 8-hydroxyquinoline.

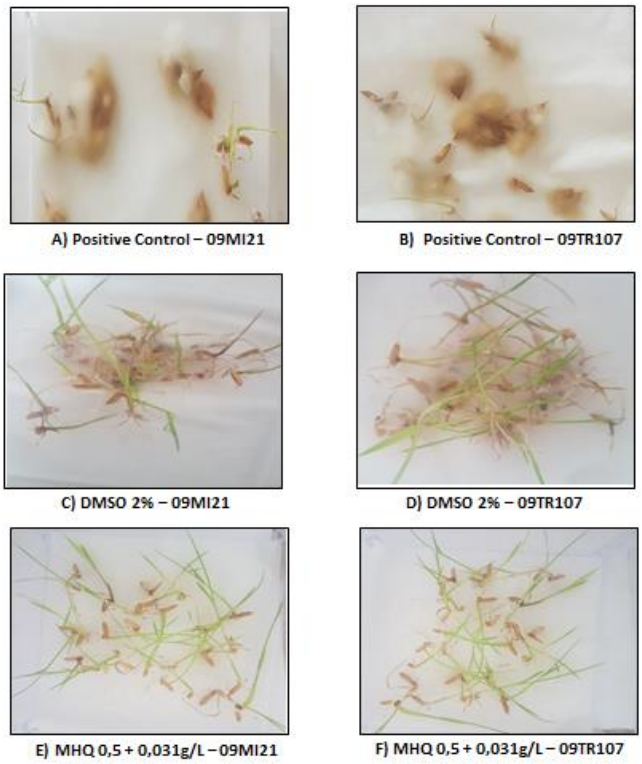

Figure 2 - Curative treatment of rice seeds against fungal control and fungicide treatment. A) Control performed with the fungus *F. meridionale* (09Mi21); B) Control performed with the fungus *F. graminearum* (09TR107); C) Control performed with DMSO 2% with the fungus *F. meridionale* (09Mi21); D) Control performed with DMSO 2% with the fungus *F. graminearum* (09TR107); E) Test performed with the fungicides MZ + 8-HQ (MHQ) at a concentration of 0.5 + 0.031g/L, with the fungus *F. meridionale* (09Mi21); F) Test performed with the fungicides MZ + 8-HQ (MHQ) at a concentration of 0.5 + 0.031g/L, with the fungus *F. graminearum* (09TR107).

Among the samples tested, two treatments, and for both strains tested there was only no statistical difference between MZ (0.5 g/L) and 8-HQ (0.031 g/L) ($P>0.9999$). The MHQ (2 + 0.25 g/L) and MHQ (0.5 + 0.031 g/L) combinations no showed statistical difference ($P>0.1721$). All other comparisons were highly significant ($p<0.0001$).

Similar results for *F. meridionale* related to the DMSO an inoculum were observed. However, the treatments tested show a very modified profile and responsive to the concentrations tested. The MZ at different concentrations showed statistical differences. For the Score®, low concentrations are sufficient to maintain an effective treatment.

The MZ and 8-HQ at 2, 0.250, 5 and 0.031 g/L combinations were equally effective, probably due to a synergistic effect between these agents. At lower concentrations, however, it was not possible to maintain this similar activity. Effect not observed in associations with Score® and 8-HQ, as only the treatment with 2 and 0.25 g/L obtained good results.

For a preventive treatments of seeds (Figure 3), a similar effect is observed against *F. meridionale* on the combinations (Table 8). The same effect is present MZ and 8-HQ combinations at the same concentrations. However, this effect was not observed at lower concentrations of Score® and 8-HQ.

The preventive treatment performed for *F. graminearum* showed that the associations were more efficient than the isolated treatments (Table 9). The two associations already important in the other examples with MZ and 8-HQ (2 + 0.25 and 0.5 and 0.031 g/L).

Table 8 - Quantitative analysis evaluated in the preventive treatment of rice seeds contaminated with *F. meridionale*.

Samples	**Concentration**	**Number of healthy seeds**	**Mean (120 seeds)**
***Fusarium meridionale* (Positive Control)**	2,5 X 105	0	0
DMSO	2%	15	3,75
MZ	2 g/L	115	28,75
MZ	0,5 g/L	109	27,25
8-HQ	0,25 g/L	99	24,75
8-HQ	0,031 g/L	80	20
Score®	2 g/L	116	29
Score®	0,5g/L	115	28,75
MHQ	2 g/L + 0,25 g/L	120	30
MHQ	0,5 g/L + 0,031 g/L	120	30
MHQ	0,25 g/L + 0,031 g/L	94	23,5
MHQ	0,125 g/L + 0,031 g/L	33	8,25
SHQ	2 g/L + 0,25 g/L	120	30
SHQ	1 g/L + 0,25 g/L	110	27,5
SHQ	0,5 g/L + 0,25 g/L	108	27
SHQ	0,25 g/L + 0,25 g/L	107	26,75

DMSO: Dimethylsulfoxide; MZ: Mancozeb; 8-HQ: 8-hydroxyquinoline; MHQ: Mancozeb + 8-hydroxyquinoline; SHQ: Score® + 8-hydroxyquinoline.

Table 9 - Quantitative analysis evaluated in preventive treatment of rice seeds contaminated with *F. graminearum*.

Samples	**Concentration**	**Number of healthy seeds**	**Mean (120 seeds)**
***Fusarium graminearum* (Positive Control)**	2,5 X 105	0	0
DMSO	2%	10	2,5
MZ	2 g/L	110	27,5
MZ	0,5 g/L	104	26
8-HQ	0,25 g/L	114	28,5
8-HQ	0,031 g/L	74	18,5
MHQ	2 g/L + 0,25 g/L	120	30
MHQ	0,5 g/L + 0,031 g/L	120	30
MHQ	0,25 g/L + 0,031 g/L	75	18,75
MHQ	0,125 g/L + 0,031 g/L	36	9

DMSO: Dimethylsulfoxide; MZ: Mancozeb; 8-HQ: 8-hydroxyquinoline; MHQ: Mancozeb + 8-hydroxyquinoline.

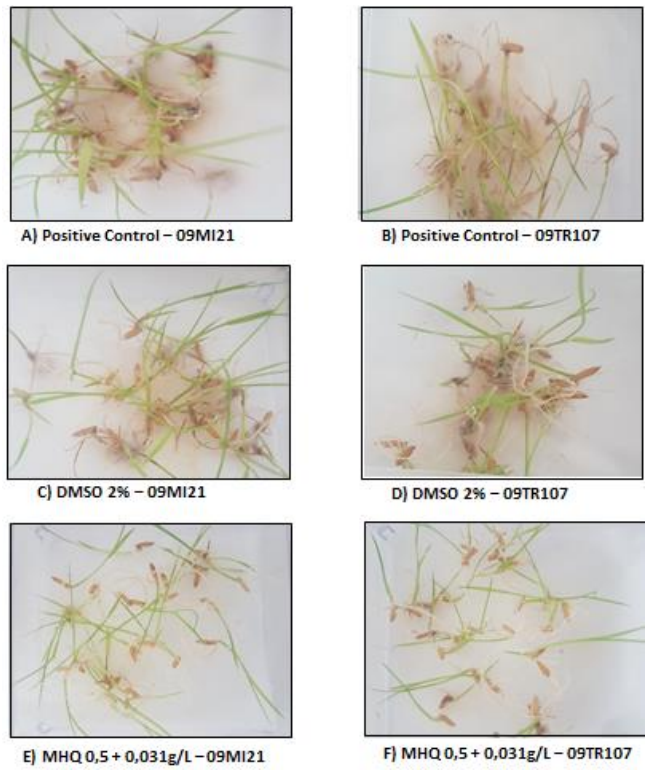

Figure 3 - Preventive treatment of rice seeds against fungal control and fungicide treatment. A) Control performed with the fungus *F. meridionale* (09Mi21); B) Control performed with the fungus *F. graminearum* (09TR107); C) Control performed with DMSO 2% with the fungus *F. meridionale* (09Mi21); D) Control performed with DMSO 2% with the fungus *F. graminearum* (09TR107); E) Test performed with the fungicides MZ + 8-HQ (MHQ) at a concentration of 0.5 + 0.031g/L, with the fungus *F. meridionale* (09Mi21); F) Test performed with the fungicides MZ + 8-HQ (MHQ) at a concentration of 0.5 + 0.031g/L, with the fungus *F. graminearum* (09TR107).

3.6 Cytotoxicity Assay

The one-way ANOVA test, showed statistical difference between the different samples: positive control, negative control and agents tested ($p<0.0001$). All MZ, Score® and 8-OH concentrations were different of the positive control (Figure 4).

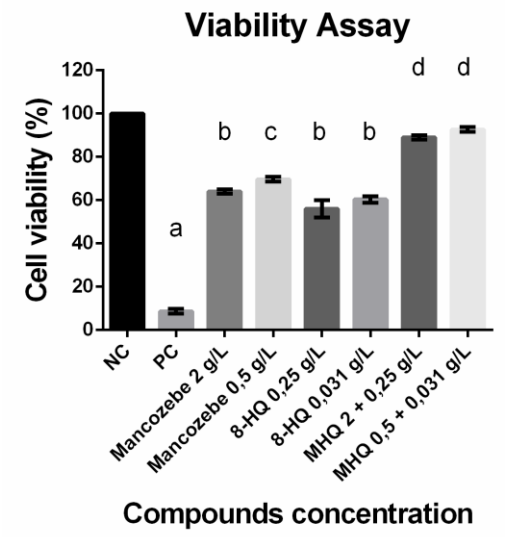

Figure 4 - Cell viability of fungicides performed on human leukocytes. NC: negative control; PC: positive control; 8-HQ: 8-hydroxyquinoline; MHQ: Mancozeb + 8-hydroxyquinoline.

MZ 2g/L is different (significantly) (P<0.05) from MZ 0.5 g/L, different (P<0.01) 8-HQ 0.25 g/L and different (P<0.01) from MHQ 0.5 + 0.031 g/L. However, it is not different (P>0.05) from treatment with 8-HQ 0.031 g/L. Treatment with MZ 0.5 g/L is different (P<0.01) from all other treatments.

Treatment with 8-HQ 0.25 g/L is not different from 8-HQ 0.031 g/L, but it is different (P<0.01) from mixtures with MHQ. Treatment with 8-HQ 0.031 g/L is different from treatment with MHQ blends. And there is no difference between the treatments with the mixtures carried out.

Regarding the cytotoxicity assay on leukocytes, the MHQ association presented higher values of cell viability compared to isolated fungicides. This fact reinforces the potential and safety of this association to be used in agriculture for the treatment of plant infections caused by Fusarium. This result, along with the *in vivo* assay, has been demonstrated the efficacy and safety of MHQ, presenting a significant number of viable seeds.

3.7 Permeation determination and histopathological evaluation

In the dermal toxicity test (Figure 5), it is possible to observe that all the treatments (MZ, 8-HQ and our combinations) have a histopathological profile different from the positive control of tissue damage (NaOH) and similar to the negative control (PBS).

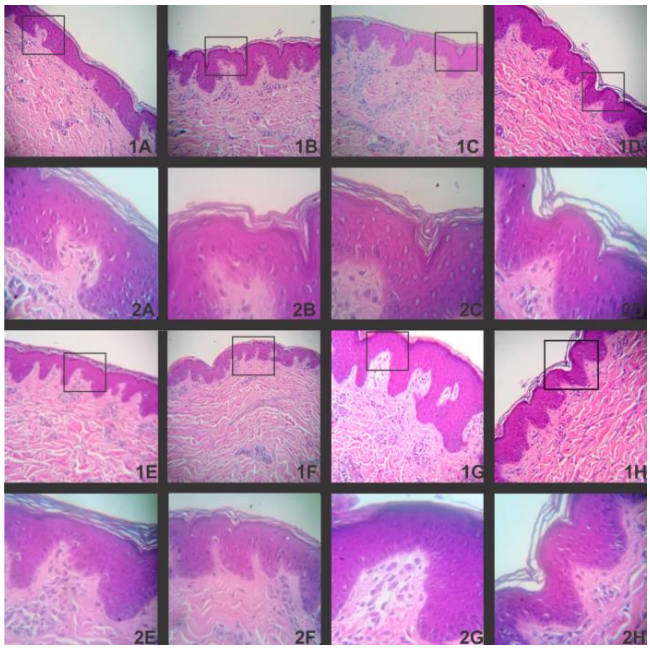

Figure 5 - Histopathological evaluation through the dermal toxicity test. 1A. MZ (0.25 g/l); 1B. MZ (2.0 g/l); 1C. 8-HQ (0.031 g/l); 1D. 8-HQ (0.25 g/l); 1 AND. MZ + 8-HQ 0.5 and 0.031 g/l, respectively); 1F. MZ + 8-HQ (at concentrations of 2.0 and 0.25 g/l, respectively); 1G. PBS (negative control); 1H. NaOH (positive damage control).

Finally, the dermal toxicity test at different concentrations and associations revealed the absence of apparent microscopic lesions, even at higher concentrations. Thus, no difference was compared to the positive control, corroborating all the results obtained. As noted, there were no apparent microscopic lesions in any of the dermal toxicity treatments, even considering the highest concentrations analyzed, contrary to what is seen for the positive control.

These findings support, an excellent perspective of the application of these antifungals in the agronomic area, alone or in association, because when used in crops they would not cause harmful effects to consumers and professionals involved, who are somehow exposed to the products at the

time of application or consumption. Other studies have used histopathological evaluation to investigate the harmful potential of some substances to the skin (Lana et al., 2020).

We have the visibility of a prosperous panorama of the application of these fungicides with a perspective that, when used, they will not cause harmful effects both for the professionals involved, who are exposed to the product at the time of application, as well as for the final consumers.

4. Conclusions

It was possible to satisfactorily observe the effectiveness of the associations, and especially of MZ with 8-HQ (0.25 g/L + 0.031 g/L), allowing us to approach an effective and safe product. Which will be available in the market in the future, to its use as a fungicide, being able to either treat infection by *F. graminearum* and *F. meridionale* or prevent it from occurring.

Interest conflicts

There was no conflict of interest of the authors.

Author contributions

Taís Fernanda Andrzejewski Kaminski: Conceptualization, Methodology, Software, Formal analysis, Writing - Review & Editing, Visualization, Project administration; Anderson Ramos Carvalho: Methodology; Bruna Batista: Methodology; Marcela Silva Lopes: Methodology; Gabriella da Rosa Monte Machado: Review & Editing; Methodology; Guilherme Ribeiro: Methodology; Saulo Fernandes Andrade: Supervision; Alexandre Meneghello Fuentefria: Conceptualization, Supervision, Writing - Review & Editing, Project administration.

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