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Rutin hydrate induces autophagic cell death and oxidative stress response in phytopathogenic fungus *Fusarium graminearum*

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Abstract

Fusarium graminearum infects cereal crops of high commercial and nutritional value such as barley, wheat, and maize, and causes various diseases. Due to the increasing world population and changing climatic conditions, the importance of controlling this pathogen is increasing. The aim of this study was to investigate the potential antifungal effect of the flavonoid compound rutin hydrate (RH) on the *F. graminearum* PH-1 reference strain at different levels. The EC_{50} (half effective concentration) value was determined by the agar dilution method on the PDA (potato dextrose agar) medium containing different concentrations of rutin hydrate. To examine the effect of rutin hydrate on cell viability, the WST-1 toxicity test was performed. To determine the effect of rutin hydrate on genomic stability and methylation profiles, the CRED-RA (coupled restriction enzyme digestion-random amplification) analysis was performed. To determine the expression levels of *atg5*, *cat*, *kmet1*, sporulation (*mgv1*), and apoptosis (*mst20*) related genes, the qRT-PCR (quantitative real-time polymerase chain reaction) was performed. To detect the presence of oxidative stress and autophagy, the DCF-DA (2',7'-dichlorofluorescein) and MDC (monodansylcadaverine) staining tests were used. The EC_{50} value of rutin hydrate was determined as 580 μg mL^{-1}. The WST-1 analysis revealed that rutin hydrate caused cytotoxicity (58.53%) in *F. graminearum* ($p < 0.05$). In the CRED-RA analysis, genomic template stability (GTS) decreased by 9.53%, and the methylation levels for *Hap*II and *Msp*I enzymes were 11.6% and 5.24%, respectively. There was a significant difference in the *atg5* (2.66 ± 0.48; $p < 0.01$), *cat* (3.00 ± 0.46; $p < 0.01$), and *kmet1* (6.29 ± 2.24; $p < 0.05$) expression in the rutin hydrate-treated samples. Autophagy and oxidative stress response with a significant difference ($p < 0.05$) in the rutin hydrate-treated *F. graminearum* was confirmed by DCF-DA and MDC tests.
This is the first report demonstrating the antifungal effect of rutin hydrate on *F. graminearum* at physiological, transcript, and epigenetic levels.

Keywords: cytotoxicity, epigenetics, flavonoid, fluorescence microscopy, gene expression analysis.

Introduction

From the islands to the cities, from the Oceania to the North America, *Fusarium graminearum* seems to be the predominating causal agent of Fusarium head blight (FHB) and crown rot (CR) wherever wheat is grown (Goswami, Kistler, 2004; Matny, 2015). Dean et al. (2012) reported that *F. graminearum* is a member of the top 10 fungal phytopathogenic fungi worldwide. There are several reasons to report *F. graminearum* as one of the most widespread phytopathogens round the world. In the last two decades, this species has come to be classified as a species complex with more than 10 members. Moreover, moderate to high levels of genetic variation among *F. graminearum* isolates have been reported worldwide (Wang et al., 2011; Przemieniecki et al., 2014). The *F. graminearum* species complex, which was previously known as (only) a class B-trichothecene producer, was recently announced as a class A-trichothecene producer (Varga et al., 2015). The member-specific trichothecene producing potential of *F. graminearum* species complex has made this species even more widespread.

The control of *F. graminearum* involves several management strategies. The fungicide treatment is the most used method to overcome the yield loss related to FHB and CR. However, there are several factors that reduce the efficiency of fungicides (Goswami, Kistler, 2004; Valverde-Bogantes et al., 2020). Global climatic conditions rather than the precise elimination of mycotoxins from the field, monoculture cropping practices, and (the most important issue) the development of fungicide-resistant fungi appear to be problematic for fungicide use in the near future. The resistance of *Fusarium* spp. to common fungicides such as tetraconazole and carbendazim can reduce the disease control in wheat fields (Perlin et al., 2017; Rampersad, 2020; de Chaves et al., 2022). Thus, scientists and farmers began to search for new antifungal compounds or alternative methods to eliminate the negative effects of *F. graminearum* diseases.

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In theory, the development of disease-resistant cultivars and the use of biocontrol agents such as *Trichoderma* spp. appear to be good and effective strategies for controlling *F. graminearum* diseases. However, these strategies have several limitations such as the apply to long time intervals, being labour-intensive and not cost-effective, expertise required, but post-reactions in the field are unknown. However, plant-derived secondary metabolites such as mixtures of essential oils or specific compounds in them are promising (Rampersad, 2020; Macwan et al., 2016). However, the main problem with plant-derived essential oils against FHB is the relatively higher requirement of these compounds to inhibit the growth of *Fusarium* spp. Unfortunately, the minimum inhibitory concentration (MIC) and half effective concentration (EC_{50}) values of plant-derived essential oil compounds or mixtures ranged from 33.81 μg mL^{-1} to 12.5 mg mL^{-1} of the plant-derived essential oil compounds (Kumar et al., 2016; Kong et al., 2022; Albayrak et al., 2023). Thus, perhaps experimenting with different herbal ingredients could provide more effective results against FHB.

Flavonoids are natural substances with a characteristic phenolic structure and can be obtained from variable plant sources. They are well known for their positive effects on health and anti-oxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties. Moreover, these natural compounds are increasingly investigated for their potential antifungal effect (Birt, Jeffery, 2013).

Rutin is a kind of flavonoid commonly found in fruits and vegetables round the world. Important sources of rutin are buckwheat, tea, and apple. Rutin and its derivatives have several health benefits including anticancer, antioxidant, and anti-inflammatory effects (Ganeshpurkar, Saluja, 2017). A potential antifungal effect of rutin against fungi has also been reported (Oliveira et al., 2016; Reddy et al., 2016). However, there is still little knowledge of the mechanisms involved in the antifungal effect of rutin.

This study aimed to demonstrate the potential antifungal activity of the flavonoid rutin hydrate, readily available from different plant species, against the worldwide phytopathogen *F. graminearum* for the first time.

Material and methods

Antifungal treatment and proliferation assay. The experiment was conducted at the Department of Molecular Biology and Genetics, Istanbul Yeni Yuzyil University, Turkey, in 2022–2023. The *Fusarium graminearum* PH-1 reference strain was a kind gift of Dr Tapani Yli-Mattila from the University of Turku, Finland. The fungal cultures were grown on the PDA (potato dextrose agar) and CMC (carboxymethyl cellulose) media for 7 days at 26 ± 2°C in a controlled growth chamber (Mikrotest, Turkey). The experimental sets were grown on the PDA medium amended with different concentrations of rutin hydrate (RH): 0, 200, 400, 600, 800, 1000, and 1200 μg mL^{-1} (Sigma-Aldrich, USA). Each experiment included seven sample sets with three biological replicates with two technical repeats. Mycelial plugs of 0.25 cm^2 obtained from the fresh PDA medium were used for cultivation. The minimum inhibitory concentration (MIC) and half effective concentration (EC_{50}) values were calculated as described by Alexander et al. (1999). EC_{50} was calculated by the formulas:

$$i_x = (100 - [(RG_{C1} \times 100) / RG_{C0}]),$$
$$i_y = (100 - [(RG_{C2} \times 100) / RG_{C0}]),$$
$$EC_{50} = C1 + ([50 - i_x] / [i_y - i_x]) \, x \, (C1 + C2) / 2,$$

where i_x is the inhibition ratio for the C1 set, i_y is the inhibition ratio for the C2 set, C0 is the concentration value for the control set, C1 is the concentration value with *in vitro* growth inhibition lower than 50%, C2 is the concentration value with *in vitro* growth inhibition higher than 50%, RGC0 is the radial growth value for the C0 set, RGC1 is the radial growth value for the C1 set, and RGC2 is the radial growth value for the C2 set.

The potential cytotoxic effect of RH was determined by the WST-1 (water soluble tetrazolium) assay. 1 cm^2 disc of control and experimental groups were dissolved in 1X PBS (phosphate-buffered saline) buffer. 1:10 (V:V) WST-1 assay (Roche, Switzerland) was added to the cells, and then samples were incubated for 3 h at 28°C in the dark. Fold changes in cell proliferation were quantified by spectrophotometric measurement at 450 and 620 nm wavelengths, as described by Albayrak et al. (2023). Each experiment set included three biological replicates with two technical repeats. The fold changes in cell proliferation were calculated by the formula:

$$\text{Fold changes in cell proliferation (\%)} = 100 \times \frac{\Delta(450 - 620) \text{ control set}}{\Delta(450 - 620) \text{ EC50 set}}.$$

Coupled restriction enzyme digestion-random amplification (CRED-RA) analysis. To detect the differences in the genomic template stability (GTS) and methylation level in the control and RH-treated cells, the CRED-RA assays were performed. For this purpose, gDNA was first extracted from 7-day-old cultures. For gDNA extraction, a spin-column based commercial kit (Anatolia Geneworks, Turkey) was used. Fresh mycelium (50 mg) was homogenised with liquid nitrogen using a sterile pestle and mortar. Then biding, washing, and elution were performed according to the protocol of the manufacturer. For the qualitative and quantitative gDNA analyses, 0.8% agarose gel and spectrophotometric measurements (Thermo Fisher Scientific Inc., USA) were used. The gDNA molecules were diluted with 50 ng μL^{-1} for use in CRED-RA analysis. The CRED-RA assay was used to detect the changes in the GTS and methylation levels of RH-treated *F. graminearum* sets. *Msp*I and *Hap*II digestion reactions were performed separately in a reaction volume of 50 μL including 20 U of digestion enzyme (Takara, Japan), 1X reaction buffer, and 500 ng of gDNA. The samples were incubated at 37°C for 1 hour and at 70°C for 10 min. Non-digested, *Msp*I-digested, and *Hap*II-digested gDNA molecules belonging to the control (RH not-treated) and experimental set (RH-treated) were used in a common RAPD (random amplified polymorphic DNA) PCR (polymerase chain reaction) protocol. In the CRED-RA assays, 20 RAPD primers (OPM3, OPM8, OPM9, OPM10, OPG1, OPC4, OPC5, and OPC7 were not included) were used (Table 1).

The initial denaturation and final elongation steps were 94°C for 5 min and 72°C for 10 min. Two amplification loops were carried out as 5 cycles (94°C for 1 min, 37°C for 1 min, and 72°C for 3 min) and 35 cycles (1 min at 94°C, 1 min at 40°C, and 2 min at

Table 1. Genomic stability and percentage of methylation values obtained from CRED-RA

Primer	5'-3' sequence	Resolution power (RP)	Polymorphism information content (PIC)	Total number of control set		Total number of band		Total number of polymorphic band		Polymorphism %	
						EC_{50}		EC_{50}		EC_{50}	
				*Hap*II	*Msp*I	*Hap*II	*Msp*I	*Hap*II	*Msp*I	*Hap*II	*Msp*I
OPM1	GTTGGTGGCT	2.01	0.18	12.0	12.0	13.0	13.0	1.0	1.0	0.08	0.08
OPM2	ACAACGCCTC	3.98	0.46	4.0	5.0	5.0	5.0	1.0	0.0	0.20	0.00
OPM4	GGCGGTTGTC	2.64	0.49	2.0	2.0	2.0	2.0	0.0	0.0	0.00	0.00
OPM5	GGGAACGTGT	2.66	0.35	6.0	7.0	6.0	7.0	0.0	0.0	0.00	0.00
OPM6	CTGGGCAACT	4.30	0.49	2.0	2.0	2.0	2.0	0.0	0.0	0.00	0.00
OPM7	CCGTGACTCA	4.30	0.49	3.0	3.0	2.0	3.0	1.0	0.0	0.50	0.00
OPB10	CTGCTGGGAC	3.34	0.44	5.0	5.0	5.0	5.0	0.0	0.0	0.00	0.00
OPB13	TTCCCCCGCT	2.66	0.47	1.0	1.0	1.0	1.0	0.0	0.0	0.00	0.00
OPB14	TCCGCTCTGG	3.66	0.49	1.0	2.0	1.0	2.0	0.0	0.0	0.00	0.00
OPG13	CTCTCCGCCA	3.62	0.48	1.0	2.0	2.0	4.0	1.0	2.0	0.50	0.50
OPG16	AGCGTCCTCC	3.98	0.49	1.0	2.0	1.0	2.0	0.0	0.0	0.00	0.00
Mean of RP and PIC		3.38	0.44	Mean of methylation values						11.6%	5.24%

72°C). Amplicons were separated on 1.7% agarose gel and photographed with a UV transilluminator. As a reference DNA size marker, 1 kb DNA ladder (Thermo Fisher Scientific Inc.) was used. After scoring a band as 0 or 1 for its presence or absence, GTS, resolution power (RP), polymorphism information content (PIC), and methylation values were calculated according to Prevost and Wilkinson (1999), Roldan-Ruiz et al. (2000), and Nardemir et al. (2015). Six sample sets included three biological replicates. PIC was calculated by the formula:

$$PIC = 2 \times (\text{amplified band number / total band number}) \times \left(1 - \frac{\text{null band number}}{\text{total band number}}\right).$$

RP was calculated by the formula:

$$ib = 1 \times 2 \times (0.5 - p),$$

$$RP = \Sigma\ ib,$$

where ib is the informative band, and p is the percentage of the number for individual with any polymorphic band and the number for the total individual number.

Quantitative real-time polymerase chain reaction (qRT-PCR) assays. To detect the changes in genes related to different physiological processes as indicated *kmet1* for methylation (methyl transferase; FGSG_02778), *cat* for oxidative stress (catalase; XM_011328072.1), *atg5* for autophagy, *mgv1* for sporulation (MAP kinase; AF492766.1), and *mst20* for apoptosis (serine/threonine-protein kinase; XM_011329927.1), qRT-PCR was used. *β-tubulin* was used as a housekeeping gene (beta-tubulin; AY303689.1). The primers designed by Gazdağlı et al (2018) and the primers developed in this experiment were used (Table 2).

The total RNA was extracted from the 7-day-old control and experimental sets using 50 mg of fresh mycelia. After the homogenisation via liquid nitrogen as described in the gDNA isolation protocol, the homogenisation was completed with 0.8 mL of monophasic NucleoZOL compound (Macherey-Nagel, Germany). To obtain the total RNA, the protocol provided by the manufacturer was followed. After the DNaseI treatment (Zymo, USA), qualitative (0.8% agarose gel)

Table 2. qRT-PCR primers used in this study

Primer set	5'-3' sequences	Band size in bp	Designer
betaF/betaR	agggtcattacaccgagggt / gtaccaccaccaagagagtgg	121	
MgvRTF/MgvRTR	aggttcaacgattccgacag / gaccattaccctgaggcaga	100	Gazdağlı et al., 2018
Mst20rtF/Mst20rtR	cctgaaaaggaacgacgaga / gccagcatgatggaatttct	117	
CatrtF/CatrtR	aattccacgttcgtttcgtc / ccatactaggctcgctttgc	130	
Atg5F/Atg5R	atgtcttctcccatcccgc / gctgaagcgtggaatactgg	108	this study
Kmet1F/Kmet1R	agactttgaggttctccccg / ggcgtcgttatcacaggaac	115	this study

and quantitative (spectrophotometer, Thermo Fisher Scientific Inc.) analyses of RNA and cDNA synthesis were performed using a commercial first strand cDNA synthesis kit following the two-step protocol of the manufacturer (Nepenthe, Turkey). Gene expression analysis was performed based on the SYBR Green I fluorophore using the QuantStudio 5.0 platform (Thermo Fisher Scientific Inc.). The reactions were conducted in a 12 µL volume containing 50 ng cDNA, 5 pmol forward primer, and 5 pmol reverse primer with 1X SYBR Green Mix (Episozyme, Turkey). After the pre-denaturation at 95°C for 3 min, the amplification was performed with 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s. After the melting curve temperature scan step, Cp values were recorded for each sample. The fold changes in gene expression levels were evaluated according to the $2^{-\Delta\Delta CT}$ formula (Livak, Schmittgen, 2001). The control and experimental sets included three biological replicates with two technical repeats. The fold changes in gene expression were calculated by the formula:

$$\text{Fold changes in gene expression} = 2 - [^{(\Delta Cp\text{-treated (target-reference)})} - (\Delta Cp\text{-untreated (target-reference)})].$$

Fluorescence microscopy assays. To detect the oxidative stress and autophagic cell death responses in *F. graminearum* treated with RH DCF-DA (2',7'-dichlorofluorescein) and MDC (monodansylcadaverine), fluorescence microscopy staining tests (Carl-Zeiss,

Germany) were used. The protocols provided by Hu et al. (2011) and Teker et al. (2021) were followed. Five to six mycelium plugs of 7-day-old PDA cultures were transferred to the CMC medium (with or without the addition of 580 µg mL^{-1} RH). The samples were incubated on a rotary shaker at 150 rpm at 26 ± 2°C. The 7-day-old cultures were filtered twice through sterile gauze to remove mycelia. For the fluorescence microscopy analysis, 1 × 10^6 macroconidia cultures (dissolved in 1X PBS) were used. The cells were first collected by centrifugation at 14.000 rpm for 5 min and then fixed with 4% paraformaldehyde and 0.1% Triton X-100 treatment for 1 hour in the dark (at room temperature). After the fixation, in the DCF-DA staining assay, the control and RH-treated cells were suspended in PDB including 5 µg mL^{-1} DCF-DA for 30 min. The cells were washed with 1X PBS and after the centrifugation were suspended in 100 µL of 1X PBS, and 100 µL of 10 mM glucose was added to the mixture. After 5 min incubation in the dark, the cells were analysed via an FITC filter (494 nm excitation / 518 nm emission). The fixed cells were exposed to 3 µL of MDC (100 mg mL^{-1}) and incubated for 30 min in the dark. After the centrifugation, the cells were washed three times with 1X PBS. The cells were visualised via a DAPI filter (350 nm excitation/465 nm emission). Quantitative assays were performed using 100 cells per biological replicate (n = 3). The fold changes in oxidative stress and autophagic responses related to the fluorescence of the cells were calculated by the formula:

$$\text{Fold changes in fluorescence (\%)} = 100 \times \frac{\text{Cell number for control set}}{\text{Cell number for EC50 set}}$$

in planta tests.

The potential plant protective effect of RH against *F. graminearum* infection was determined by a pathogenicity test. As a host plant, the common wheat (*Triticum aestivum* L.) cultivar 'Midas' was used. For *in planta* analysis, three biological replicates including three technical repeats were used. The inoculum source was obtained from the *F. graminearum* PH-1 strain grown on the CMC medium for 7 days. 1 × 10^6 macroconidium filtered through 2X gauze was added to the autoclaved soil with a ratio of 5:95/ W:V/ macroconidium: soil (Moya-Elizondo, Jacobsen, 2016; Moradi et al., 2017). After three days of inoculation, surface-sterilised wheat seeds (Gargouri-Kammoun et al., 2009) were planted in 13-cm-diameter plastic pots and allowed to growth at 26°C in 16/8 light/dark with 1500 lux for three weeks. Plantlets were watered every other day with distilled water (control set) or RH solution (EC_{50} dose for the experimental set). Disease severity was calculated based on the crown rot (CR) status of the seedlings with a scale ranging from 0 (no disease symptoms) to 4 (no root development). The effect of RH on CR disease severity was determined by the Abbott formula (Beccari et al., 2011):

$$\text{Disease severity (\%)} = 100 \times (\Sigma\ (n \times V)) / (Z \times N),$$

where n is the number of samples with different scores, V is the value for the scale, Z is the highest value of the sample scale, and N is the sample number.

Statistical analysis. The column statistics including the mean and standard error values, normality tests, T-test, and correlation matrix were obtained using the software GraphPad Prism, version 9.0 (Dotmatics, USA). The principal component analysis (PCA) of the numerical data, obtained by the WST-1 analysis, gene expression findings, and fluorescence microscopy values, was performed using the software R/RStudio with 'readxl', 'devtools', and 'ggbiplot' packages. The confidence interval was 0.05.

Results and discussion

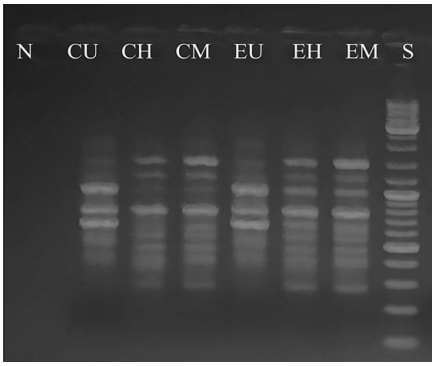
Antimicrobial activity analysis. The agar dilution technique was used to determine the MIC and EC_{50} values of the *F. graminearum* cultures grown on PDA medium amended with rutin hydrate (RH). Rutin hydrate inhibited the *in vitro* growth of *F. graminearum*. EC_{50} and MIC values were calculated as 580 and 1160 µg mL^{-1}, respectively. As an experimental set for further analysis, PDA + 580 µg mL^{-1} RH was used. Similarly, inhibition for growth *in vitro* was detected by the WST-1 analysis. Relative absorbance values ($\Delta_{450-620}$) for the control and experimental sets were calculated as 0.23 ± 0.01 and 0.09 ± 0.01, respectively. The fold changes in cell proliferation were 58.92%; the change in cell proliferation was significant ($p < 0.001$).

Both the agar dilution technique and the cytotoxicity test showed that rutin hydrate strongly inhibited the *in vitro* growth of *F. graminearum*, even at low concentrations. Previous investigations have shown that the amount of plant-derived secondary metabolites (required to determine the MIC value) was usually higher than that of the synthetic fungicides. MIC values (for sensitive isolates) from 0.1 to 10 µg mL^{-1} for synthetic fungicides can reach more than 10 mg mL^{-1} values for plant-derived essential oil compounds such as myrcene or α-thujone (Macwan et al., 2016; Perlin et al., 2017; Yörük, 2018; Teker et al., 2021; de Chaves et al., 2022; Albayrak et al., 2023). In this study, it was found that a relative low amount of rutin hydrate was effective to determine the MIC value of *F. graminearum*. Similarly, a relatively low level of the RH treatment produced a similar cytotoxic effect on *F. graminearum* compared to α-thujone (75%) and myrcene (65.1%) (Teker et al., 2021; Albayrak et al., 2023). Compared to plant-derived essential oil compounds or mixtures, rutin hydrate (with a low level of need) resulted in a particularly good inhibitory effect on *F. graminearum*. Oliveira et al. (2016) found that the combination of rutin (125 µg mL^{-1}) with amphotericin B (common fungicidal compound) provided a synergistic effect in inhibiting the growth of *Candida* sp. and *Cryptococcus neoformans* strains *in vitro*. The potential antifungal effect of rutin derivatives on *Candida albicans*, *Candida glabrata*, *Aspergillus flavus*, and *Fusarium verticillioides* has been previously reported (Lupaşcu et al., 2017). In this study, using comprehensive methods, the potential antifungal effect of rutin hydrate compound on *F. graminearum* was determined for the first time.

Genomic and epigenetics analysis. To detect potential instability in the genome and epigenome of RH-treated *F. graminearum*, type-II (*Hap*II) and type-III (*Msp*I) methylation-specific enzymes and common CRED-RA PCR were used. The first GTS was calculated for the control-undigested and undigested experimental sets. The GTS was calculated as 90.47% meaning the genomic mobility of 9.53% due to the RH treatment was present in the *F. graminearum* genome. The mean RP and PIC values were calculated as 3.38 and 0.44,

respectively (Table 1). The OPM6 primer had the highest level of polymorphism with 4.30 RP and 0.49 PIC values among the 6 sets (control-undigested, control-*Hap*II-digested, control-*Msp*I-digested, EC_{50}-undigested, EC_{50}-*Hap*II-digested, and EC_{50}-*Msp*I-digested) among the primers used in this study. The mean values of type-II and type-III methylation changes were 11.6% and 5.24%, respectively.

Figure 1 shows the fingerprinting profile of the 6 sets obtained from the OPM1 primer.

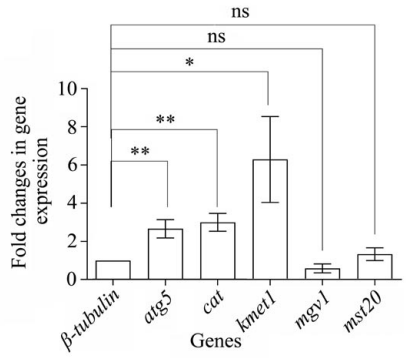


N – no template control; control set: CU – non-digested, CH – *Hap*II-digested, CM – *Msp*I-digested, EC_{50} set: EU – non-digested, EH – *Hap*II-digested, EM – *Msp*I-digested, S – 1 kb DNA ladder

Figure 1. DNA fingerprinting profile of six sets obtained from the OPM1 primer

Previous studies related to the determination of GTS values in regard with the specific treatment of chemical compounds on *Fusarium* spp. revealed GTS% of 66.27% to 90.9% (Gazdağlı et al., 2018; Yörük, 2018; Harshitha, Nair, 2020; Teker et al., 2021; Albayrak et al., 2023). The GTS value of 90.47% for the RH treatment appears to be a relatively lower level of genomic instability in the *F. graminearum* genome. However, the RP and PIC values were relatively similar or higher (Silva et al., 2013; Consolo et al., 2015; Alkan et al., 2019). It can be concluded that the RH treatment can induce a relatively moderate level of genomic instability. In addition to the genomic instability test, changes in methylation level were also evaluated by CRED-RA analysis. Both *Hap*II- and *Msp*I-specific methylation differences were detected in the RH-treated *F. graminearum* PH-1 strain. However, the values of type-II and type-III methylation differences can be denoted as a relatively lower level of epigenetic changes. The relatively lower or moderate methylation changes in *F. graminearum* may be due to the relatively low amount of rutin hydrate or no effective stimulation between the rutin hydrate flavonoid compound and the epigenome of fungus. Further studies could include the relationship between different types of flavonoid use and the 5-mC DNA methylation level of fungal genome.

Gene expression analysis. A total of 5 target genes related to different physiological processes were used in the qRT-PCR analysis. The fold changes of *atg5*, *cat*, *kmet1*, *mgv1*, and *mst20* genes were recorded as 2.66 ± 0.48, 3.00 ± 0.46, 6.29 ± 2.24, 0.59 ± 0.23, and 1.33 ± 0.33, respectively. Although there were no significant differences between the control and EC_{50} sets for the *mgv1* ($p > 0.05$) and *mst20* ($p > 0.05$) genes, there were significant alterations in the fold changes of *atg5* ($p < 0.01$), *cat* ($p < 0.01$), and *kmet1* ($p < 0.05$) gene expression (Figure 2).



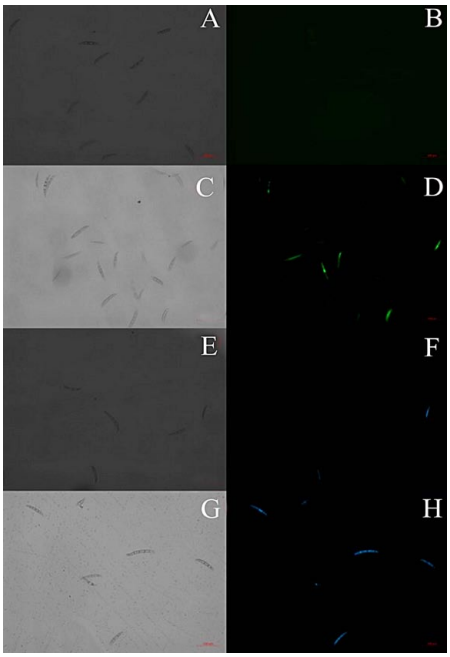
*, ** – significant at $p < 0.05$ and $p < 0.01$; ns – not significant ($p > 0.05$)

Figure 2. Fold changes in the gene expression values related to the T-test in the *Fusarium graminearum* PH-1 strain

To our knowledge, this is the first report showing the autophagic cell death effect of any plant-derived specific compound on a phytopathogenic fungus. Previous gene expression studies related to the response of FHB and CR pathogens to biotic and/or abiotic stressors have mainly focused on fold changes in the expression of gene(s) located in a trichothecene biosynthetic cluster (Ponts et al., 2007; Merhej et al., 2010; Yörük et al., 2018). Only a limited number of studies have investigated the apoptotic cell death and oxidative stress response of *Fusarium* spp. against stressors. These studies revealed that synthetic fungicide or plant-derived essential oil compounds induced apoptotic cell death and oxidative stress in FHB and CR pathogens. Similar to previous studies, the RH treatment altered the oxidative stress-related gene expression (Gazdağlı et al., 2018; Yörük, 2018; Teker et al., 2021; Albayrak et al., 2023). However, no significant differences in the apoptosis related gene expression were recorded in the RH treatment. The results of this study showed that the RH treatment induced the autophagic cell death mechanism rather than apoptotic cell death.

Fluorescence microscopy analysis. In the DCF-DA staining analysis, the control sets revealed a limited amount of green-coloured (oxidative-stress related) cells at 14.5 ± 2.41% (Figure 3A, B). However, the cells of the EC_{50} set showed a relatively higher level of the green-coloured cell profile at 31.50 ± 2.91% (Figure 3C, D). A significant difference ($p < 0.001$) was found between the control and EC_{50} sets in the level of oxidative stress. Similar results were obtained in the MDC-staining analysis. The frequency of a blue-coloured (autophagic) cell was 20.17 ± 2.31% and 75.33 ± 4.45% in the control and EC_{50} sets, respectively (Figure 3E, F, G, H). The RH treatment resulted in a significant level of autophagic cell death ($p < 0.0001$) in the *F. graminearum* PH-1 strain.

Using the gene expression analysis, the presence of the oxidative stress response was determined in the RH-treated *F. graminearum* cells by fluorescence microscopy analysis. Yörük (2018) found that the common fungicide tetraconazole increased oxidative stress by 20.75% in the *F. graminearum* H-11 reference strain. A similar level of oxidative stress induction up to +1.5-fold increase in the IC_{50} (half maximal inhibitory concentration) set was found in the *Schizosaccharomyces pombe* treated with the plant-derived essential oil compound



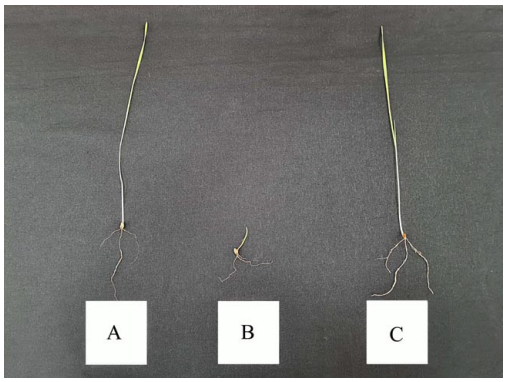
Note. Day-light filter (A and C) and FITCH filter (B and D) profile for DCF-DA staining of the control (A and B) and EC_{50} set (C and D) of *F. graminearum* PH-1; day-light filter (E and G) and a DAPI filter (F and H) profile for MDC staining of the control (E and F) and EC_{50} set (G and H) of *F. graminearum* PH-1; day-light filter photographs were presented for the internal control of fluorescence micsropcopic analysis; FITCH and DAPI filters were used in order to show the presence of oxidative stress and autophagic response, respectively.

Figure 3. Imaging of the oxidative stress and autophagy responses in rutin hydrate-treated *Fusarium graminearum* with fluorescence microscopy

terpinolene (Agus et al., 2018). These results indicated that rutin hydrate can induce relatively high levels of oxidative stress in fungi. Similar results were obtained by the MDC staining analysis, where the autophagic cell death response increased up to +3.17-fold in the RH-treated *F. graminearum*. To our knowledge, this is also the first report showing the quantitative data of MDC-positive macroconidium in response to the plant-derived secondary metabolite treatment. The results showed that rutin hydrate can strongly affect the autophagic cell death mechanisms in phytopathogenic fungi in response to abiotic stress.

In planta disease severity analysis. The effect of RH treatment on CR disease severity was tested using the Abbott formula. *F. graminearum* PH-1 infection resulted in the 2.33 ± 0.23 infection degree on a disease scale of 0–4. The plantlets infected with *F. graminearum* PH-1 and treated with RH showed 0.44 ± 0.24 disease severity values. The changes in disease severity values between the PH-1-infected and PH-1 infected/RH-treated plantlets were significantly different ($p < 0.0001$). The RH treatment resulted in healthy plantlets with no infection, as in the control sets (Figure 4).

There are several reports including the beneficial effects of common fungicides on FHB and/or CR hosts (Ponts et al., 2007; Merhej et al., 2010; Spolti et al., 2014; Perlin et al., 2017; Ye et al., 2017; de Chaves et al., 2022). Most of these previous studies focused on disease severity changes in response to different fungicides (or conditions) and *TRI* gene expression. However, there



A – non-infected plant, B – *F. graminearum* PH-1 infected plant, C – *F. graminearum* PH-1 infected and RH-treated plant

Figure 4. Crown rot (CR) disease severity analysis of wheat plants

is still a lack of research on the beneficial effects of plant-derived secondary metabolites on *F. graminearum* diseases. In this study, rutin hydrate was shown to reduce up to 80% the disease severity of common wheat infected with *F. graminearum*. Oufensou et al. (2021) revealed the potential antifungal activity and trichothecene biosynthesis inhibitory effects of specific plant-derived essential oil compounds by molecular docking and *in planta* assays. Further studies could include the inhibitory effect of rutin hydrate or another flavonoid compounds on trichothecene production.

Statistical analysis. For each experimental procedure, mean and standard error values were calculated. Based on these data, each set was tested for normality by the Shapiro-Wilk test, and each data set from each experimental procedure (from WST-1 assay to fluorescence microscopy analysis) was determined as normal (parametric) distribution with $p > 0.05$. The Pearson R correlation test showed that there was only a positive correlation between the DCF-DA fluorescence microscopy data and cat expression ($p = 0.04$, $R = 0.82$). This correlation can be taken as a confirmation of oxidative stress presence due to the RH treatment in *F. graminearum*. However, the remaining data from different experimental procedures showed a great level of variation. To confirm the high or moderate level of variation of the data obtained from the experimental procedures, PCA by R/RStudio was performed. According to the PCA tests, the percentages of PCA1 and PCA2 axis were 59.4% and 21.3%, respectively. While the *mgv1* gene expression and cell proliferation were found to have different distributions compared to other test types, the *atg5* gene expression and autophagy level were nearly distributed. In the PCA graphic, the oxidative stress data and methylation levels co-clustered similarly (Figure 5).

Autophagy and oxidative stress can be strong indicators of the external stressors to living cells and disruption of homeostasis. The detection of parallel or opposing effects of these phenomena is important to have information about the damage that can occur in the cell and the future of cell homeostasis (Shintani, Klionsky, 2004). In the present study, rutin hydrate, a plant secondary metabolite, was found to induce oxidative stress and autophagic response in the plant pathogen *F. graminearum*. Contrary to the literature data, where autophagy and apoptosis are evaluated together (Shintani, Klionsky, 2004; Thorburn, 2014), in the present study, oxidative stress and

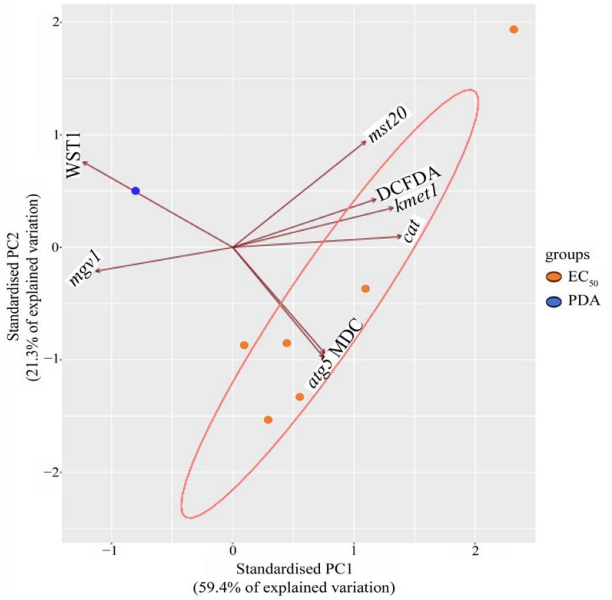


Figure 5. Principal component analysis (PCA) plot from different experimental sets in the *Fusarium graminearum* PH-1 strain

autophagy were evaluated together. It has been shown that rutin hydrate can damage the phytopathogenic fungus through multiple pathways. Furthermore, this research is the first study demonstrating the autophagic effect of rutin hydrate on phytopathogenic fungi.

The results showed that different gene expression analysis and fluorescence microscopy data were closely related to each other. Those two methods were used in subsequent studies to confirm the presence of autophagic cell death and oxidative stress in response to abiotic stressors. Further studies could include the effects of different plant-derived secondary metabolites on phytopathogenic fungus in terms of different types of autophagic, apoptotic, or necrotic cell death.

Conclusions

The results of this experiment suggest that plant-derived secondary metabolites such as flavonoids may be a potential new strategy against *Fusarium graminearum* diseases. It was shown that a relatively low amount of rutin hydrate (RH) showed the antifungal effect on the global phytopathogen *F. graminearum* by inducing methylation changes, oxidative stress, and autophagic cell death.

The main results of the study are the following:

1. Even small amounts of the rutin hydrate compound are sufficient to inhibit the growth and cell proliferation of the global plant pathogen *Fusarium graminearum*.

2. Rutin hydrate compound reduced the genomic template stability and altered the methylation levels in the genome and epigenome of *F. graminearum*.

3. Rutin hydrate flavonoid induced the responses related to oxidative stress and autophagic cell death in *F. graminearum* due to homeostasis disruption.

4. The RH treatment can be a promising, effective, and protective strategy against the rot, blight, or related fungal diseases.

5. The combined evaluation of gene expression analysis, fluorescence microscopy studies, and principal component analysis provided an approach to confirm the mechanism of RH-induced oxidative stress and autophagic cell death in *F. graminearum*.

6. The RH treatment has been shown to have the antifungal effect by inducing multiple different physiological processes in *F. graminearum*.

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