

Naturally Occurring Phenols Modulate Vegetative Growth and Deoxynivalenol Biosynthesis in *Fusarium graminearum*

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Cite This: *ACS Omega* 2020, 5, 29407–29415



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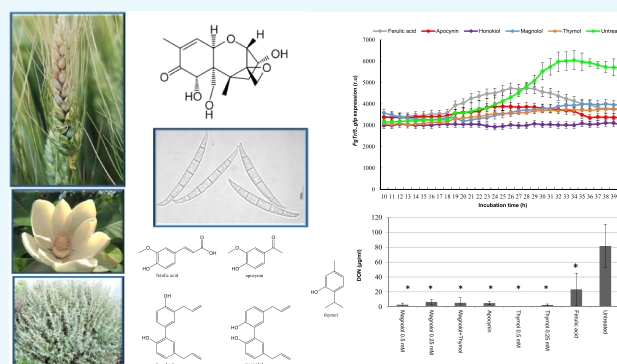


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ABSTRACT: To assess the in vitro activity of five naturally occurring phenolic compounds (ferulic acid, apocynin, magnolol, honokiol, and thymol) on mycelial growth and type B trichothecene mycotoxin accumulation by *Fusarium graminearum*, three complementary approaches were adopted. First, a high-throughput photometric continuous reading array allowed a parallel quantification of *F. graminearum* hyphal growth and reporter *TRIS* gene expression directly on solid medium. Second, RT-qPCR confirmed the regulation of *TRIS* expression by the tested compounds. Third, liquid chromatography–tandem mass spectrometry analysis allowed quantification of deoxynivalenol (DON) and its acetylated forms released upon treatment with the phenolic compounds. Altogether, the results confirmed the activity of thymol and an equimolar mixture of thymol–magnolol at 0.5 mM, respectively, in inhibiting DON production without affecting vegetative growth. The medium pH buffering capacity after 72–96 h of incubation is proposed as a further element to highlight compounds displaying trichothecene inhibitory capacity with no significant fungicidal effect.



1. INTRODUCTION

Fusarium species are ranked among the most diverse and widespread plant-infecting fungi. *Fusarium graminearum* Schwabe is a prevalent pathogen on cereals, and it is known to be the most relevant species occurring worldwide on wheat as a causal agent of *Fusarium* head blight.^{1–4} Its infection generates decreased yield and poor grain quality, leading to significant economic losses.^{5–7} *F. graminearum* produces type B trichothecene mycotoxins such as deoxynivalenol (DON) and its acetylated forms (3-ADON and 15-ADON), which are dangerous to human health.^{8–10}

Type B trichothecenes are characterized by a tetracyclic form of sesquiterpenes including a 9,10-double bond and a 12,13-epoxide ring that is crucial for their toxicity and are distinguished by a keto group at C-8 in the trichothecene A-ring.^{11,12} The presence of the epoxide group at positions 12–13 confers the ability to bind to ribosomes, leading to the activation of various protein kinases, the modulation of gene expression, and the inhibition of protein synthesis and cell toxicity.^{13,14}

The function of nearly all genes implicated in the trichothecene biosynthesis pathway, commonly named *TRI* genes, has been elucidated.^{15–18} Among the clustered genes, only *TRI5*, *TRI4*, *TRI6*, and *TRI10* are involved in trichothecene skeleton synthesis.^{14,16,19} *TRI5* plays a crucial role because it encodes a trichodiene synthase and provides the

first trichothecene isotrichodermol from farnesyl pyrophosphate.^{16,20,21}

Although the appropriate use of conventional fungicides such as tubulin polymerization inhibitors (e.g., carbendazim and thiophanate-methyl) and sterol demethylation inhibitors (e.g., tebuconazole and metconazole) results in a significant reduction of *Fusarium* diseases, these fungicides cause severe environmental pollution and may result in the selection of resistant populations of the pathogen, hence leading to a progressive reduction in the available options for control.^{22,23} Therefore, it appears urgent and appropriate to identify new-generation inhibitory compounds exerting a low selection pressure that do not act on the saprophytic phase of *Fusaria* but rather on their pathogenic and mycotoxigenic potential or that are able to stimulate defense reactions by the host plant.^{24–28}

Plant resistance to *Fusarium* and mycotoxin accumulation is a highly complex mechanism, and only recently it has been partially elucidated.^{29–34} Fungal cell wall degrading enzymes

Received: September 1, 2020

Accepted: October 15, 2020

Published: November 9, 2020



are likely involved in the penetration and infection of plants.^{35,36} As a result, reactive oxygen species (ROS) generated in plants during the fungal attack are a key factor as their presence may affect both the host plant and the pathogen.^{37,38}

Comprehensive studies on *Fusarium* spp., performed by spectroscopic techniques, shed light on the mechanism of fungal attack evidencing the role of an external environment in influencing the fungal metabolome that adapts as a function of external inputs.^{39–41}

Plants are inexhaustible reservoirs of secondary metabolites, mainly terpenoids and phenylpropanoids. The latter often possesses antioxidant properties and antimicrobial activity.³² The majority of these compounds are bound to the cell wall, participating in its reinforcement and indicating their potential involvement in the general plant defense system against fungal pathogens.^{42–44} Some of them, like ferulic acid, may also interfere with mycotoxin biosynthesis.^{32,37,45}

In previous *in vitro* and *in silico* studies devoted to the search of effective sustainable fungicides in agriculture, we observed that ferulic acid, apocynin, and magnolol are able to inhibit trichothecene production and/or fungal vegetative growth in both *Fusarium culmorum* (W.G. Smith) Saccardo and *F. graminearum*.^{27,46,47,59} These compounds are considered as attractive candidates for natural approaches to synthetic fungicide considering first their structure similarity to ferulic acid, the most potent phenolic acid with antifungal activity against *Fusarium* species,^{37,56} their commercial availability at relatively low cost, and especially the fact that they share a different mode of action compared to conventional fungicides. The exact mechanism of their antifungal and mycotoxin production modulation activities is still poorly understood, yet new approaches to plant pathogen control would be highly desirable.

The research into traditional medicine often focuses on a single compound or a single target, which has led to the identification of many effective components. However, in many cases, the synergistic result of multiple components, targets, and pathways appears more efficient.⁴⁸ A possible strategy is to use a combination of natural compounds with differing modes of action: two compounds may interact to produce synergistic interactions, leading to more effective inhibition of mycotoxins or fungal growth.⁴⁹

Phenotyping fungal growth is routinely explored by harvesting mycelium accumulated in liquid media and by quantifying its dry weight; however, this approach may not accurately reflect the fungal growth in its natural habitat because liquid media are not natural substrates for filamentous plant pathogens. Many filamentous fungi cannot grow in submerged culture, and several studies confirmed that the physiology and developmental biology of fungi greatly depend on whether they grow on a solid substrate or submerged in liquid media.^{50–52}

As reported by three independent groups,^{53–55} high-throughput phenotyping of fungal growth by means of absorbance (optical density, OD) measurement can be used to monitor growth of *Fusarium* colonies. Cánovas et al.⁵⁴ developed a successful platform to quantitatively detect fungi growing on top of solid media and, in parallel, to achieve monitoring of their gene expression using promoter fusions with fluorescent reporter proteins.

Using this technology, the objective of this study was to evaluate the efficacy of a set of naturally occurring phenolic

compounds, including their synergistic combination on vegetative growth and DON production by *F. graminearum* through the use of three complementary *in vitro* assays, with the aim to achieve a significant inhibition of DON production without affecting fungal growth.

2. RESULTS AND DISCUSSION

With an aim to develop naturally occurring inhibitors of trichothecene production, four compounds sharing the ferulic acid structure with some differences in the functional groups and mostly known for their antimicrobial activity were screened by complementary methods.

To provide a better understanding of the mode of action of the selected naturally occurring phenols, we focused on *TRIS*, a gene coding for the key enzyme in the trichothecene biosynthesis pathway. We used a multiplexing platform equipped with high-throughput photometric continuous reading that allows parallel quantification of *F. graminearum* hyphal growth and reporter gene expression directly on solid medium.⁵⁴ This allowed monitoring the inhibitory effect of selected compounds on trichothecene biosynthesis as well as on growth inhibition.

A solid substrate was chosen based on the evidence that the physiology and developmental biology of fungi may dramatically change depending on whether they grow on a solid substrate or submerged in liquid media.^{50–52}

Absorbance curves were used to quantify the fungal growth rather than the conventional growth test as the absorbance is a function of the incubation time and the number of spores used in inoculation.⁵⁴

As the *Tri5::gfp* construct is inserted at the native location in the genome, the *TRIS* promoter activity corresponds to the expression of the native *TRIS* gene. Based on the untreated sample (blank control), all detections were started 10 h after inoculation as it is known that during this period, considered as the lag phase, cells are adjusting to new growth conditions (Figure 2). After 19 h, a sharp increase of the growth curve was

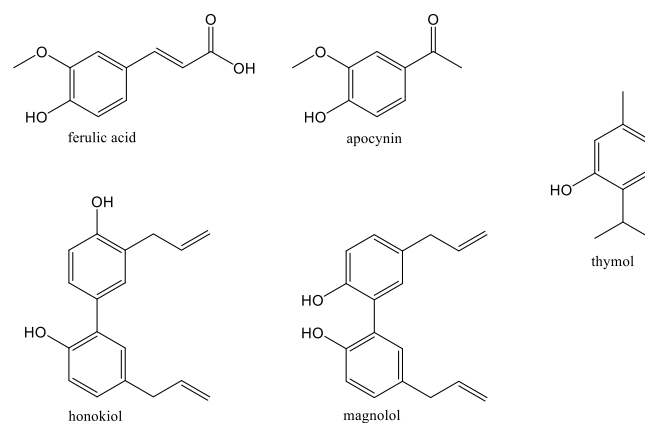


Figure 1. Structure of tested compounds: ferulic acid, apocynin, honokiol, magnolol, and thymol.

observed in the control, indicating the exponential growth phase, which continued to increase. A similar trend was observed with apocynin and ferulic acid growth curves, but a plateau was reached with both compounds after 34 and 37 h, respectively (Figure 2a). After this period, a constant OD was detected for apocynin and ferulic acid, which was considered as

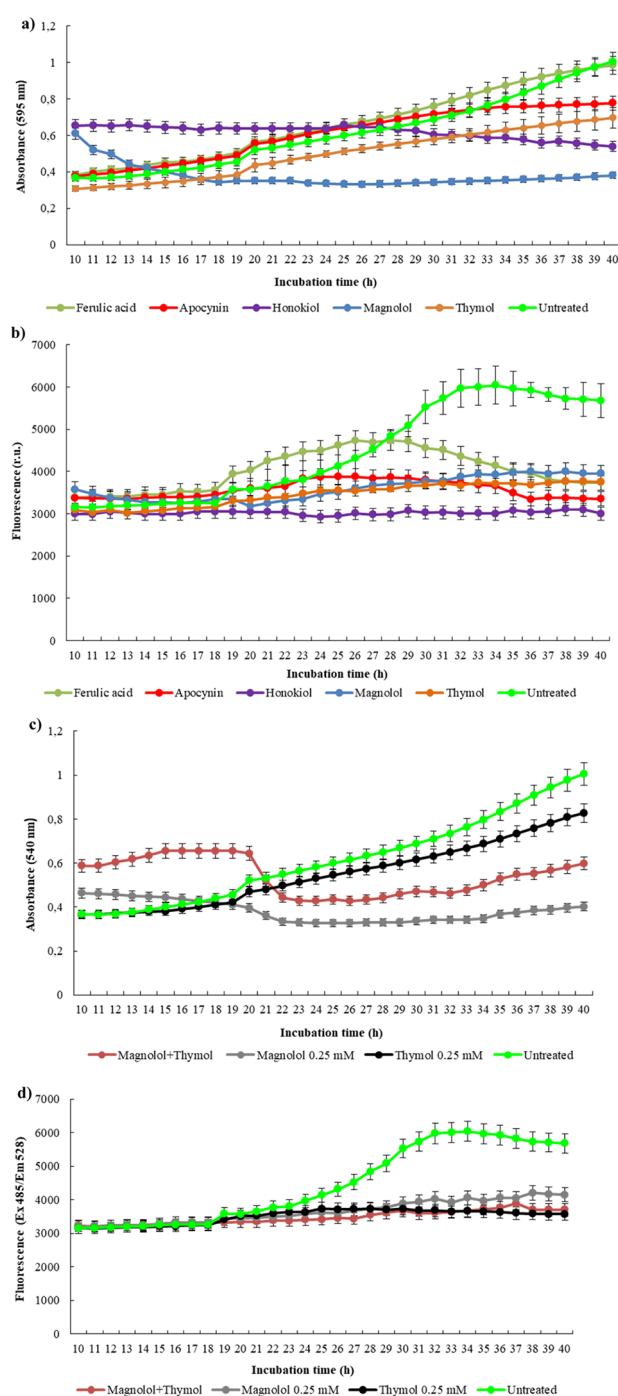


Figure 2. Monitoring fungal growth and expression of *Tris::gfp* in *F. graminearum* in vitro amended with phenolic compounds. Untreated assay as a negative control for each graph. (a) Hyphal growth analyzed by OD₅₄₀ measurements (absorbance) with magnolol, honokiol, ferulic acid, and apocynin at 0.5 mM, respectively, at 24 °C up to 40 h. (b) *TRIS* expression quantified with the levels of GFP fluorescence [performed simultaneously with (a)] measurements. The *FgTris::gfp* strain grown on solid FMM with 5 mM ORN (inducing conditions) at 24 °C up to 40 h. (c) Hyphal growth analyzed by OD₅₄₀ measurements (absorbance) with the equimolar magnolol + thymol mixture at 0.5 mM and magnolol and thymol at 0.25 mM, respectively, at 24 °C up to 40 h. (d) *TRIS* expression quantified with the levels of GFP fluorescence performed simultaneously with (c) measurements. The *FgTris::gfp* strain was grown on solid FMM with 5 mM ORN (inducing conditions) at 24 °C up to 40 h.

an indication of a stationary phase, since there was no measurable increase in fungal growth.

Conversely, magnolol and honokiol treatment at 0.5 mM resulted in fungal growth inhibition over the complete time course, showing an almost flattened curve (Figure 2a). The fungal growth inhibition was more evident in magnolol than in honokiol, albeit the latter showed a slowly but constantly decreasing curve detectable after 26 h.

Hyphal growth was inhibited by the addition of magnolol down to a concentration of 0.25 mM. Unexpectedly, a slight decline in the time–growth curve was observed after 19 h and continued almost stationary up to 40 h, indicating the death of fungal hyphae (Figure 2c).

An almost similar shape of the time–growth curve was recorded with magnolol, whereas a lower value of absorbance was detected in the presence of an equimolar mixture of magnolol and thymol at a final concentration of 0.5 mM (Figure 2c). Hyphal growth was strongly affected upon addition of the combination of magnolol + thymol, with a slightly different curve shape. Conversely, the time–growth curve of thymol at 0.25 mM showed the same trend of the untreated control (Figure 2c) with a negligible reduction in the exponential growth phase, hence confirming a different effect of thymol on mycelium when compared to magnolol at the same concentration. Therefore, an equimolar mixture of magnolol and thymol seems to be the most effective treatment in terms of antifungal activity.

In parallel to growth monitoring, *FgTris::gfp* fluorescence data were also acquired. The untreated sample showed a standard *TRIS* expression reaching the maximum value between 32 and 35 h (Figure 2b). During this period, all compounds assayed at 0.5 mM (ferulic acid, magnolol, honokiol, and apocynin) affected *TRIS* expression, albeit with different intensities. These results confirmed those achieved by previous in vitro assays, where ferulic acid and apocynin were amended at the same concentration in Vogel's liquid medium.²⁷ It should be considered that between 32 and 35 h, the exponential growth phase of the strain *FgTris::gfp* in *Fusarium* minimal medium (FMM) amended with ferulic acid and apocynin at 0.5 mM was not affected by these compounds, whereas no fungal growth was observed in the presence of magnolol and honokiol at the same concentration, as confirmed by a low value of absorbance (Figure 2a,b). *FgTris::gfp* fluorescence was almost undetectable in the presence of an equimolar mixture of magnolol + thymol at a final concentration of 0.5 mM, even in the presence of magnolol or thymol alone at 0.25 mM (Figure 2d). While for magnolol alone at 0.25 mM and for the equimolar mixture of magnolol + thymol, such an inhibitory effect reflects the strong antifungal activity of magnolol; in fact, in the case of thymol alone at 0.25 mM, a strong inhibition of fluorescence was not accompanied by a reduction of fungal growth, similar to what observed for ferulic acid and apocynin.

Transcription analysis of the reporter strain *FgTris::gfp* upon exposure to phenolic compounds showed that the *gfp* expression level is upregulated, albeit not significantly, by the equimolar mixture of magnolol + thymol and thymol at 0.25 mM (Figure 3). This result does not correlate with the plate reader-mediated *gfp* expression even though a moderate fungal growth inhibition was observed in the mixture of magnolol + thymol. We used thymol in combination with magnolol because we speculated that different mechanisms of action would increase the final effect on *TRIS* inhibition. Indeed, the

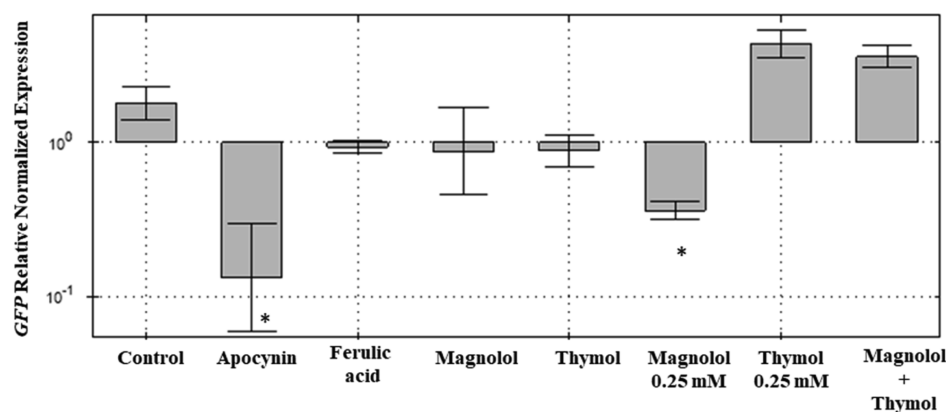


Figure 3. Relative normalized expression analysis by qRT-PCR of *gfp* produced by a *FgTri5::gfp* strain after 7 days growth on FMM with 5 mM ORN and the corresponding treatment at 24 °C. *Compounds are statistically different from the control (untreated) at $p < 0.05$.

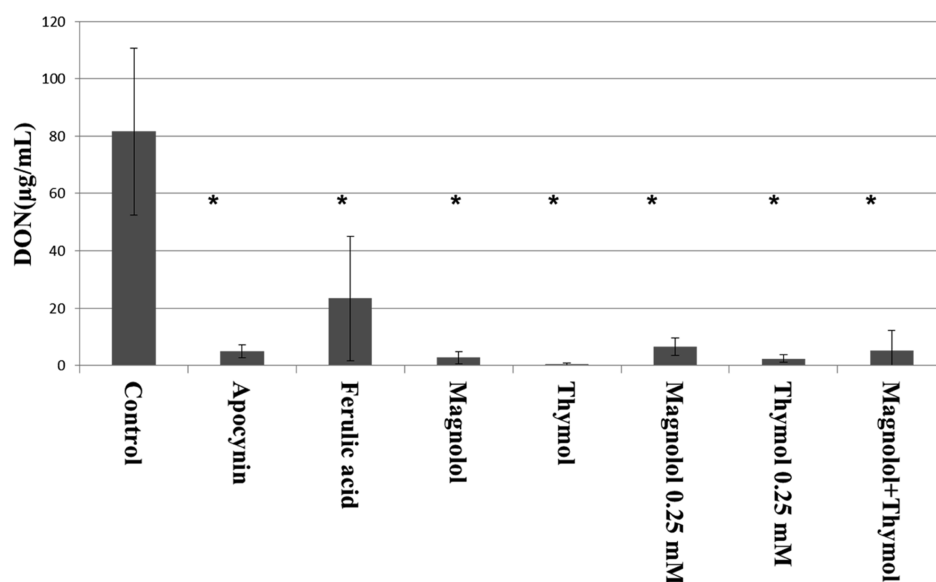


Figure 4. Inhibitory activity of phenolic compounds on DON production by *F. graminearum* wild-type PH-1. Germinated spores were inoculated into FMM with 5 mM ORN and the corresponding treatment and distributed into 96-well plates. After 14 days of incubation at 24 °C, the amount of DON and derivatives was quantified. *Compounds are statistically different from the control (untreated) at $p < 0.05$ and $p < 0.01$.

combination of magnolol + thymol showed the strongest effect in inhibiting *TRI5* expression and total DON production (Figure 4). In contrast, apocynin and magnolol at 0.25 mM downregulated the *gfp* gene expression significantly (Figure 3), as shown also in the previous experiment (Figure 2b). Although ferulic acid, magnolol, and thymol applied at 0.5 mM induced a reduction in the expression level, the difference in the expression was not statistically different from the control. Figure 3 does not contain any expression data related to honokiol because no mycelium growth was detected for this treatment.

In order to confirm the results obtained from the two previous analyses, total DON quantification using liquid chromatography–mass spectrometry (LC–MS) was performed. Figure 4 highlights a strong inhibitory effect for most compounds, and differences between treatments were all statistically significant.

Ferulic acid reduced total DON accumulation by about 30% compared to the untreated control. The equimolar solution of magnolol + thymol at the final concentration of 0.5 mM and apocynin at 0.5 mM induced a similar effect, reducing the

DON production by 90%. Magnolol and thymol applied alone at 0.5 and 0.25 mM, respectively, reduced total DON production by more than 95%. Thymol at 0.5 mM proved the most effective mycotoxin inhibitor by completely inhibiting DON biosynthesis.

The combination of an equimolar solution of magnolol + thymol at the final concentration of 0.5 mM suggests an additive effect; in fact, the amount of total DON produced when this combination is administered is the sum of the amounts produced when magnolol and thymol are supplemented separately at 0.25 mM each.

As reviewed by Atanasova-Penichon et al.³⁷ and Schöneberg et al.,⁵⁶ ferulic acid is considered as potent phenolic acid with antifungal activity against different *Fusarium* species, which is in agreement with our results.^{27,46} Ferruz et al.⁴⁵ suggested that the decrease in toxin production in ferulic acid-amended medium could result from a decrease in the level of *TRI* gene expression.

Apocynin, known for its antioxidant, antimicrobial, and anti-inflammatory activities,^{27,57,58} displayed a concentration-dependent inhibitory effect on trichothecene biosynthesis. In

a previous modeling approach, Pani et al.⁴⁶ considered the trichodiene synthase protein *TRIS* as a hypothetical target for phenolic inhibitors of trichothecene biosynthesis, providing an estimation of *F. culmorum TRIS* protein–ligand interactions with apocynin. Our hypothesis was that the low efficient trichothecene inhibition observed could be related to the small size of apocynin, which limits extensive protein–ligand interactions. Moreover, a putative five-membered ring hydrogen bond, featuring the aromatic ring of apocynin, would prevent strong intermolecular interactions with the phenolic –OH group. However, fungal matrices are multi-component systems, where the medium can be considered as both lipidic and emulsion system, therefore lipophilic and hydrophilic interactions should be both considered. Accordingly, the partition capacity of a compound between a lipophilic–hydrophilic phase should be also taken into account. A low log *P* value was evaluated for ferulic acid (1.42) and apocynin (0.83) hampering the full permeability of these compounds through the fungal membrane as magnolol (5.03) and honokiol (5.03) did. Among these compounds, thymol might provide the best compromise between fungal growth and mycotoxin inhibition as it presents an intermediate log *P* value (3.37).

As a result, *F. graminearum* PH-1 showed a variable response to the tested compounds in terms of inhibition of mycelial growth and modulation of mycotoxin production. We presumed that such effects might be evidenced by a different pH of the environment where interactions between inhibitors and fungal enzymes take place. By monitoring medium pH in the control and in substrates amended with the tested compounds, a different trend was observed between 36 and 96 h (Table S1): ferulic acid and apocynin (i.e., trichothecene inhibitors with no effect on vegetative growth at 0.5 mM) showed a significant decrease in pH at 72 h (pH 4.26 and 5.18, respectively), which was similar to the control, and reached a steady value until 96 h. Honokiol and magnolol (both effective fungicides) buffered the medium pH, which was kept at neutral value up to 96 h. Thymol showed a different behavior according to the concentration: at 0.5 mM, thymol showed a buffering ability similar to that provided by magnolol and honokiol until 72 h, and then, acidic pH was recorded at 96 h; at 0.25 mM, instead, the effect was observed 24 h earlier. Interestingly, the equimolar mixture of magnolol + thymol at 0.5 mM provided complete pH buffering of the medium in the range 0–96 h, with complete fungal growth and mycotoxin inhibition. Hence, the measurement of medium pH during fungal growth may represent a further element to highlight compounds displaying inhibitory capacity toward mycotoxin without significant effect on vegetative growth.

Even though magnolol and honokiol are structural isomers, honokiol presented a stronger antifungal activity in comparison to magnolol at 0.5 mM against *Fusarium* spp. of clinical and agro-ecological interest;⁵⁹ however, these assays were performed in Petri dishes, where the fungus may display a different behavior compared to the 96-wells adopted in the present work.

Recently, the key role of honokiol as an activator of mitochondrial ROS by mitochondrial dysfunction and depolarization of mitochondrial membrane potential in *Candida albicans* was elucidated.⁶⁰ Honokiol has also the capacity to hinder the high content of pro-oxidant iron ions in fungi by sequestering the ions.⁶¹ According to the literature, magnolol interacts with ergosterol present in the cell

membrane, inducing a partial disruption of its structure.⁶² Moreover, cell wall components such as β -1,3-glucans were proposed as a potential target of magnolol.⁶³

Thymol, a natural monoterpene phenol found primarily in thyme (*Thymus* L.), oregano (*Origanum* L.), and tangerine (*Citrus reticulata* L.) peel, has been reported to have potential antifungal activity against *F. graminearum* due to the cell membrane damage originating from lipid peroxidation and the disturbance of ergosterol biosynthesis.⁶⁴

The molecular structure of thymol is responsible for its strong ability to dissolve and accumulate in the cell membrane, leading to a cell membrane destabilization that has been ascribed to more efficient proton transfer disruption.⁶⁵ Accordingly, disruption of the membrane integrity and alteration of the cell wall were observed when *F. graminearum*,⁶⁴ *Candida* sp.,⁶⁵ and *Saccharomyces cerevisiae*⁶⁶ were treated with thymol. Similarly, Di Pasqua et al.⁶⁷ indicated that thymol alters an array of cellular metabolic pathways in *Salmonella enterica* serovar Thompson.

Zhang et al.⁶⁸ reported that one mechanism of the fungicidal activity of thymol against *Fusarium oxysporum* may be attributed to the inhibition of cell wall biosynthesis and to the activation of the expression level of cell wall degrading enzymes. A survey by Zabka and Pavela⁶⁹ has shown that thymol exerts a fungicidal activity against *Fusarium verticillioides* and *F. oxysporum* with a minimum inhibitory concentration of 108 μ g/mL (0.72 mM) and 115 μ g/mL (0.73 mM), respectively. Gill et al.⁷⁰ also elucidated how a 0.06% active thymol concentration was required to inhibit *F. graminearum* germination in a very challenging 10 s exposure window.

In addition to the efficacy and the low toxicity advantages of thymol, a synergistic and efficacy enhancement effect was observed when it was added to conventional antifungal drugs such as azoles and amphotericin B.⁷¹ In the present work, a combination of the naturally occurring compounds magnolol and thymol, presumably displaying differing modes of action toward the fungal cell, achieved a higher effectiveness.

In conclusion, three different approaches based on high-throughput and transcription analysis and LC–MS were performed in vitro evidencing the strong activity of thymol and a equimolar mixture of thymol + magnolol at 0.5 mM in inhibiting production of DON and its derivatives by the head blight pathogen *F. graminearum*. Studies are in progress with the aim to elucidate how these compounds may interact with the transcriptional machinery or the signaling pathways, leading to *TRIS* expression.

3. METHODS

3.1. Fungal Strains and Culture Conditions. The *F. graminearum* reporter strain *FgTriS::gfp* and the corresponding wild-type PH-1 have been described.³⁴ Conidial suspensions of *FgTriS::gfp* and PH-1 were pre-cultured in the mung bean broth (MBS) for 5 days on a rotary shaker at 24 °C and 180 rpm. MBS was amended with streptomycin (50 μ g/mL) and tetracycline (50 μ g/mL) to prevent bacterial growth. Cultures were filtered, and spores were collected by centrifugation, adjusted to 2.5×10^5 colony-forming units (cfu)/mL in sterile water, and used as inoculum for solid FMM.⁷²

For expression analyses, 10 μ L of 2.5×10^5 cfu/mL were inoculated in solid FMM, with 5 mM L-ornithine (ORN) as a sole nitrogen source.

3.2. Tested Compounds. Ferulic acid, apocynin, honokiol, magnolol, and thymol (Figure 1) were purchased from Sigma-Aldrich (Italy) and Chemos GmbH (Germany) and used with a purity >98%. Lipophilicity of the compounds was estimated using the logarithm of the partition coefficient for *n*-octanol/water ($\log P$), which was calculated using ChemBioDraw Ultra 13.0.

All selected compounds were tested for their ability to inhibit *Tri5::gfp* expression by *F. graminearum* at the concentration 0.5 mM, whereas for magnolol and thymol, a concentration of 0.25 mM was also applied. Moreover, an equimolar mixture of magnolol + thymol at the same final concentration of 0.5 mM was tested. Each compound was resuspended in sterile water and sonicated at room temperature for 1 h at 80 Hz (Branson model 3510 OPTO-LAB).

The evolution of pH in FMM amended with the different compounds was recorded at 0, 14, 36, 72, and 96 h (Table S1).

3.3. Photometric Continuous Reading Platform-Plate Reader Assay. To screen for compounds affecting *F. graminearum* growth and trichothecene biosynthesis gene expression, 100 μ L of solid FMM with the indicated nitrogen source were distributed into 96-well plates (Corning, NY, USA) and supplemented with each compound at a temperature not exceeding 50 °C. Plates were inoculated with 40 μ L of *FgTri5::gfp* conidia, prepared as described before, and incubated at 24 °C in the plate reader for 72 h. Absorbance (OD) and fluorescence were detected and quantified every 60 min in Synergy Mx Multi-Mode Microplate Readers (BioTek, Germany) equipped with GFP (Ex 485/Em 528) filter sets provided by the Bioactive Microbial Metabolites (BiMM) unit, BOKU. The inhibitory activity of fungal growth was measured at a wavelength of 540 nm by the absorbance (OD) of the culture depending on the time of growing incubation and the number of spores used for the inoculation. The untreated sample (compound-free) was considered as a blank control. The fluorescence blanks were regularly inoculated with water. The non-fluorescent wild-type strain (PH-1) was employed to subtract the background fluorescence from the sample data. Data were recorded and analyzed with Gen5™ Data Analysis Software v2.0 and exported to Microsoft Excel for generation of the graphs. Graphs were generated for an incubation time between 10 and 40 h because growth was detected as early as 10 h after inoculation and flattened growth curves were observed from 40 to 72 h. Experiments were repeated at least two times in quadruplicate.

3.4. Gene Expression Analysis in *FgTri5::gfp* by Quantitative Real-Time PCR. For gene expression analysis, 10 μ L of the spore suspension (2.5×10^5 cfu/mL) of the *F. graminearum* reporter strain *FgTri5::gfp* were inoculated in FMM amended with the selected compounds at a final concentration of 0.5 mM and covered by cellophane. Four plates for each compound were prepared and incubated at 24 °C for 7 days. After 84 h, the mycelium was carefully harvested, ground in liquid nitrogen, and stored at -80 °C. Total RNA was extracted from frozen mycelium using the GeneMATRIX Universal RNA purification kit (URx Ltd., Gdansk, Poland) following the manufacturer's protocol. cDNA was synthesized from the total RNA ($A_{260}/A_{280} > 1$) using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The primers used for quantitative real-time PCR (qRT-PCR) were designed using Snapgene 3.3.2 as follows: qRT-gfpFw (5'-GGAGCTTTTCACTGGCGTC-3'); qRT-gfpRev (5'-CCATATGTAGCGTCTCCCTC-3'); 18SFw (5'-

TTGACCCGTTTCGGCACCTTAC-3'); and 18SRev (5'-AAGTTTCAGCCTTGCGACCATAC-3'). The gene 18S has been selected as a reference being the most stable among the four potential reference genes (β -tubulin, actin, 18S, and *g3pdh*) used in preliminary studies (data not shown). The reactions were held using the CFX96 Touch Real-Time PCR detection System Bio-Rad (version 3.1, 2010) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) by starting with 10 ng/ μ L of synthesized cDNA. The PCR program was as follows: 95 °C for 3 min, 40 cycles of 95 °C for 10 s and 58 °C for 1 min. The melting curve was defined by 0.5 °C increments of the slow ramp rate between 55 and 95 °C after the RT-PCR cycles. The RT-qPCR experiment was repeated three times, with each sample having three replicates. The relative expression level of the genes was calculated using the $2^{-\Delta\Delta C_T}$ method,⁷³ and the standard deviation (SD) was calculated from the three biological replicates.

3.5. Sample Preparation for DON Analysis. Mycotoxins (DON and its acetylated forms) were extracted from the growing mycelia 14 days post inoculation. Six mycelial plugs from actively growing mycelium in the 96-well plates, prepared and incubated as previously described, were transferred into 2 mL microcentrifuge tubes (Eppendorf, Germany). Then, 1 mL extraction solvent [acetonitrile/water/acetic acid (v/v/v), 79:20:1] was added to the tubes and shaken in an orbital shaker (Typ LSR-V, Adolf Kühner AG, Birsfelden, Switzerland) for 2 h at 180 rpm. Subsequently, supernatants were transferred to chromatography vials and diluted 1:10 with extraction solvent.

3.6. LC-MS Analysis. Mycotoxin separation was performed using a 1200 series HPLC liquid chromatographic system (Agilent Technologies, USA). A Phenomenex Gemini C18 (150 mm \times 2.1 mm, 5 μ m, 100 Å) was used for chromatographic separation. The flow rate was 0.400 mL/min during a 15 min period with an injection volume of 5 μ L. Mobile phases were: 5 mM ammonium acetate with 0.1% acetic acid (A) and methanol with 0.2% acetic acid (B). A linear gradient elution of solvents was applied with the following program: 0 min, 80% B; 4 min, 45% B. The column was equilibrated for 6 min prior to each analysis. The chromatographic system was coupled to a OrbiTrap high-resolution mass spectrometer (Q Exactive) equipped with the heated electrospray ionization probe HESI-II (Thermo Fisher Scientific, Bremen, Germany) operating in both positive and negative ion modes. Parameters of the ion source were as follows: spray voltage, 3.5 kV; sheath gas flow rate 45 (arbitrary units); auxiliary gas, 10 (arbitrary units); sweep gas, 3 (arbitrary units); and capillary temperature at 320 °C. Full MS acquisition was performed with resolution power 70,000 full width at half-maximum with mass accuracy of 5 ppm. The MS parameters were: AGC target 3e6, maximum injection time 200 ms, and scan range 100–1000 *m/z*.

The Xcalibur 3.1.66 software (Thermo Fisher) was used to control the instruments and to process the data. Mass deviation were calculated as parts per million [(calculated mass - experimental mass/calculated mass)] and were found to be below 5 ppm. Peaks were identified on the basis of their retention time relative to external standards (t_R) and high-resolution MS spectra. All quantitative data were acquired in the full MS scan mode; if a target compound was present, its precursor ion scan triggered a parallel reaction monitoring mode.

3.7. Statistical Analysis. The results obtained from separate experiments, except for the gene expression data, were represented as the mean and SD of at least three replicated measurements. The significant differences between treatments were statistically evaluated by SD and one-way analysis of variance (ANOVA) using Minitab for Windows, release 17. The data between two different treatments were compared statistically by ANOVA, followed by Tukey test if the ANOVA result was significant at $p < 0.05$.

Gene expression analysis was carried out by applying a non-parametric ANOVA followed by the Friedman test.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.0c04260>.

Measurement of pH in liquid FMM amended with ORN (5 mM) and different phenolic compounds after 14, 36, 72, and 96 h post inoculation (h) with *F. graminearum* wild-type PH-1 at 24 °C and summary table of the tested compound responses relative to the control (untreated) in terms of inhibition of the mycelial growth, *Tri5::gpf* expression, modulation of mycotoxin production, and buffering ability; percentages calculated at 32 h when the untreated fluorescence curve reached the maximum value, and, in terms of OD, the fungi in the exponential growth phase (PDF)

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<https://pubs.acs.org/doi/10.1021/acsomega.0c04260>

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors gratefully acknowledge Roman Labuda, Michael Sulyok, and Hannes Gratzl for assistance with the preliminary mycotoxin analysis experiment. This study has been supported by the following projects: CNR-CONICET Bilateral project 2016–2018; RASSR73282 “IDEASS: Approcci innovativi nella difesa delle colture agrarie: studi in silico, selezione e sintesi di composti a ridotta tossicità per il contenimento di funghi”; CUP: J86C16000070009 SSPIT: Selection of sustainable phenolic inhibitors of trichothecene biosynthesis: in vitro studies and computational insight into the structure–activity relationship.” BiMM is supported by grant #K3-G-2/026-2013 of the Lower Austria Science Fund NFB. S.O. acknowledges financial support from SIPCAM Italia S.p.A and from Erasmus + Traineeship Program 2017/2018.

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