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Effect of Naturally Occurring Compounds on Fumonisin Production and *fum* Gene Expression in *Fusarium verticillioides*

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Abstract: *Fusarium verticillioides*, one of the most common pathogens in maize, is responsible for yield losses and reduced kernel quality due to contamination by fumonisins (FBs). Two *F. verticillioides* isolates that differed in their ability to produce FBs were treated with a selection of eight natural phenolic compounds with the aim of identifying those that were able to decrease toxin production at concentrations that had a limited effect on fungal growth. Among the tested compounds, ellagic acid and isoeugenol, which turned out to be the most effective molecules against fungal growth, were assayed at lower concentrations, while the first retained its ability to inhibit toxin production in vitro, the latter improved both the fungal growth and FB accumulation. The effect of the most effective phenolic compounds on FB accumulation was also tested on maize kernels to highlight the importance of appropriate dosages in order to avoid conditions that are able to promote mycotoxin biosynthesis. An expression analysis of genes involved in FB production allowed more detailed insights into the mechanisms underlying the inhibition of FBs by phenolic compounds. The expression of the *fum* gene was generally down-regulated by the treatments; however, some treatments in the low-producing *F. verticillioides* strain up-regulated *fum* gene expression without improving FB production. This study showed that although different phenolic compounds are effective for FB reduction, they can modulate biosynthesis at the transcription level in opposite manners depending on strain. In conclusion, on the basis of in vitro and in vivo screening, two out of the eight tested phenols (ellagic acid and carvacrol) appear to be promising alternative molecules for the control of FB occurrence in maize.

Keywords: maize; *Fusarium verticillioides*; phenolic inhibitors; fumonisins; *fum* gene expression

1. Introduction

Fusarium verticillioides is the most common pathogenic fungus colonizing maize in Italy, and it is responsible for severe ear rot and the accumulation of mycotoxins in kernels. Fumonisins (FBs) are the main mycotoxins produced by *F. verticillioides*, and they represent a concern due to their toxicological implications, as their consumption can lead to serious disorders and mycotoxicosis in livestock [1] and cancer in humans [2]. Several approaches have been considered in order to reduce human and animal exposure to these mycotoxins, but pre-harvest control of this pathogen remains a goal for reducing FBs [3]. Control strategies against *Fusarium* spp. based on the use of synthetic fungicides could negatively affect the environment [4] and cause selective pressure on populations of these fungi, thus promoting the emergence of resistant strains [5]. For this reason, alternative management approaches, such as the development of natural fungicides or inhibitors of mycotoxin biosynthesis, appear to be promising.

Biological control agents can be used as sustainable alternative options in crop protection. Natural molecules that are able to reduce mycotoxin production could represent a further strategy to be investigated [6,7]. Naturally occurring phenolic compounds are metabolites that have become objects of increasing interest due to their biological properties against mycelial growth, germination, and production of conidia [8]; some of them are also involved in host resistance response [9].

Structurally, phenolic compounds are characterized by the presence of a hydroxyl group attached to an aromatic ring structure, and they can range from simple phenols to polyphenols [10,11]. Phenolic compounds activate mechanisms that interfere with pathogen growth and mycotoxin production, and it has mainly been suggested they can act through the modification of fungal lipid membrane permeability to cause the leakage of ions [8,12], acidification of cellular pH, inhibition of biosynthetic enzymes, or modification of transcriptional regulation [13–15]. Further, other studies suggested that the antioxidant properties of these compounds could modulate mycotoxin production due to the quenching of the free radicals, which can reduce the upstream signals, such as ROS, which triggers or enhances biosynthesis of toxins by fungi [16,17]. Because a modification of the oxidative signals required to initialize mycotoxin synthesis [18,19] is expected after treatment, the expression of three genes involved in FB biosynthesis (*fum1* and *fum19*) and regulation (*fum21*) was surveyed. From a set of eight compounds (apocynin, caffeic acid, carvacrol, ellagic acid, ferulic acid, isoeugenol, propyl gallate, and vanillic acid), the present work aimed to identify those that had the best performance in interfering with the FB biosynthetic pathway, but with a low fungicidal effect in order to limit the risk of selection of more tolerant fungal populations. The data obtained in the present study provide insight into natural compounds that can display FB inhibitory capabilities and can function as alternative molecules for the control of *Fusarium verticillioides* and fumonisins.

2. Materials and Methods

2.1. Fungal Strains

A pool of *Fusarium* spp. colonies was isolated from maize kernels grown in Veneto by using the serial dilution method. Colonies that were morphologically identified as *F. verticillioides* [20]) were molecularly confirmed according to Mulè [21]. The cultures were subcultured on PDA (potato dextrose agar; BD/Difco, Sparks, MD, USA) for short-term storage or on SNA (Spezieller Nährstoffarmer agar) for long-term storage and conidia production. Single-spore cultures were obtained according to Tuite [22]; one culture for each isolate was randomly selected and characterized on maize flour for its potential to produce FBs. Briefly, 10 g of maize flour (FB content <200 ppb) was sterilized in a 94 mm glass Petri dish, soaked with 7.5 mL of sterile water, and inoculated in the center with a conidial suspension (10^6 conidia). The procedure was carried out for each selected *F. verticillioides* strain. The cultures were incubated without shaking for 15 days in the dark at 25 °C before FB quantification. Two *F. verticillioides* strains—termed H (high FB producer; ~18,000 µg/Kg on ground maize) and L (low FB producer; ~3000 µg/Kg on ground maize)—were selected for the studies and are hereafter called *FvH* and *FvL*, respectively. To perform all of the experiments, the cultures were grown for 15 days on SNA (Spezieller Nährstoffarmer agar), and a conidial suspension was prepared with sterile water for *FvH* and *FvL* to obtain a final concentration of 10^6 conidia/mL.

2.2. Tested Compounds

Eight naturally occurring phenolic compounds (Figure 1) were selected to assess the effects of these chemicals on fungal growth and FB production. The selection of the compounds was based on their different antioxidant (TEAC; Trolox equivalent antioxidant capacity) and lipophilic (LogP) properties (Table 1). The selection was further guided by efficacy that the compounds demonstrated against other mycotoxigenic fungi [8,23–25]. The TEAC and logP values (Table 1) of each compound were reported on the basis of

previous calculations [25–27]. The TEAC measures the ability of an antioxidant to scavenge oxidizable species, such as radicals. Lipophilicity represents the affinity of a compound for the lipid phase of plant tissues (membranes, waxes, cutin, suberin, etc.) and is a parameter linked to mobility in plants [28]. To improve solubilization and stability of the phenolic compounds in the growth medium [29], β -cyclodextrin CAVAMAX W7 Pharma (β -CD; Wacker Chemie Italia, Peschiera Borromeo, Italy) was used as a coadjutant in each experiment [30]. All of the compounds were purchased from Sigma Aldrich (Milan, Italy).

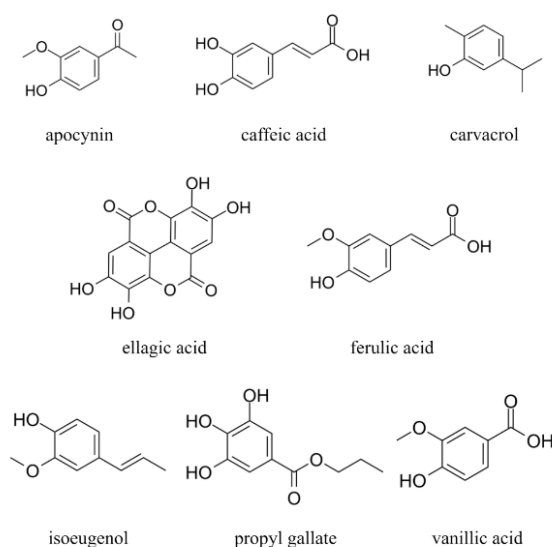


Figure 1. Structure of tested compounds.

Table 1. List of tested compounds listed according to antioxidant activity and lipophilicity.

Compound Name	Type	TEAC ^a	Lipophilicity Log P ^b
Apocynin (APO)	Acetophenones	0.41	0.83
Caffeic acid (CAF)	Cinnamic Acids	1.25	1.15
Carvacrol (CAR)	Phenylpropanoids	0.29	3.37
Ellagic acid (ELL)	Hydroxylated Biphenyls	3.05	1.05
Ferulic acid (FER)	Cinnamic Acids	1.45	1.42
Isoeugenol (ISO)	Phenylpropanoids	0.72	2.52
Propyl gallate (PGA)	Benzoic Acids	3.32	1.51
Vanillic acid (VAN)	Benzoic Acids	0.76	1.24

^a TEAC, Trolox equivalent antioxidant capacity; ^b Log P = lipophilicity estimated by theoretical calculations.

2.3. In Vitro Activity

To evaluate the compounds' activity, MYRO liquid medium with a pH of 6.5 [31] was prepared and autoclaved. The supplementation of the compounds was carried out as previously reported [25,32], with minor modifications. Briefly, the amount of β -CD that was necessary to obtain a final concentration of 3 mM was dissolved under stirring for 1 h in degassed MYRO medium. Next, single phenolic compounds were separately added to the β -CD-MYRO solution to obtain final concentrations of 1 mM each. Each mixture was agitated at 250 rpm for 6 h at 25 °C to promote the reversible inclusion of compounds in the β -CD cavity and aliquoted in sterile flasks (50 mL). The flasks were supplemented with

only water or 3.0 mM β -CD (without the tested compounds) to be used as controls. Conidial suspensions (10^5 conidia per flask) of 100 μ L of *FvH* and *FvL* were inoculated, and the cultures were grown at 25 °C in the dark for 10 days. On the basis of the results achieved at 1.0 mM, the most inhibitory compounds (ELL and ISO) were further investigated to identify lower concentrations (0.5 and 0.25 mM) that were able to decrease the FB content while preserving fungal growth. As a consequence of the decrease in the antioxidant dosage, the β -CD was also changed by reducing its concentration (1.5 and 0.75 mM), thus maintaining a molarity ratio between the β -CD and phenols of 3:1.

2.4. Mycelial Growth Estimation and Fumonisin Quantification

Ten-day-old fungal biomasses were separated from the liquid medium, rinsed thoroughly with tap water, collected on filter paper, and then oven-dried (DRY-Line 112 Prime, VWR Int. Ltd., Leicestershire, England) to obtain the dry weight (105 °C for 24 h). Because phenolic compounds can influence fungal growth and the secondary metabolism—and can even interact with the pH value—treated and untreated cultures were monitored at 0, 1, 3, 5, 7, and 10 days after inoculation for their pH. Liquid broths were harvested and stored at −20 °C for FB analysis. The FB accumulation was analyzed with a lateral flow test (Fumo-V strip, VICAM, Milford, MA, USA) with small modifications to the manufacturer's instructions. Briefly, 5 mL of culture broth separated from the mycelia was mixed with 20 mL of extraction buffer and vortexed for 2 min. An aliquot of each extracted solution (100 μ L) was added to an immunostrip and quantified using a Vertu® lateral flow reader (VICAM, Milford, MA, USA). The limit of detection certified by the manufacturer for this kit was equal to 0.2 ppm. Due to the limitations of ELISA-based methods, the validation of the results was performed with FB-certified samples (LC-MS determination; NEOTRON spa, Modena, Italy).

2.5. In Vivo Activity in Maize Kernels

Based on the in vitro trials, four effective compounds (CAR, ELL, ISO, and PGA) were investigated for their efficacy in FB-free living maize kernels (<100 mg/kg). FER treatment, which was not effective in in vitro cultures at 1.0 mM concentration, was included to verify whether this lack of efficacy was maintained in a different matrix. To prevent mycotoxin production by endophytic *F. verticillioides*, the experiments were conducted on externally and internally sterilized kernels [33]. For the in vivo treatments, the phenolic compounds were used at 1.0 and 3.0 mM and complexed with 3.0 or 9.0 mM of β -CD, respectively, to maintain the proportions with the β -CD used. Fifty grams of kernels were aseptically added to flasks containing 100 mL of compound solution and agitated for 3 h at 100 rpm at 25 °C. The kernels were collected by discarding the solutions, and 20 seeds per plate were placed in 90 mm plates, inoculated with 100 μ L of spore suspension (10^6 spores/mL), and incubated at 25 °C for 10 days without shaking. The kernels were harvested, oven-dried (~40 °C for 24 h), and milled to flour for FB analysis with the lateral flow test according to the manufacturer's instructions.

2.6. Fum Gene Expression Analysis upon Phenolic Compound Treatment at 1.0 mM

The fungal strains were treated with a set of phenolic compounds (CAR, ELL, FER, ISO, and PGA) as previously described, and were then grown for 7 days. FER was included to evaluate the effect of this compound at the transcription level; in vitro, it showed an almost opposite effect on *FvH* and *FvL*. Further, with an under-dosed test, the gene expression was monitored for the ELL treatment with 0.25 mM, and the β -CD was changed by reducing its concentration to 0.75 mM. Fungal mycelia were harvested, rinsed thoroughly, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and an RNA aliquot (1 μ g) was treated with DNase (PerfeCTa® DNase I, Quanta BioSciences,

Gaithersburg, MD, USA) and reverse transcribed into cDNA using M-MLV reverse transcriptase and 25 µmol/L random primers according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The gene expression of the set of biosynthetic genes (*fum1*, *fum19*, and *fum21*) involved in FB synthesis was analyzed using quantitative real-time PCR (qRT-PCR). One-tenth of the cDNA was used to optimize the PCR amplification and primer efficiency. qRT-PCR was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A total of 1 µL of a cDNA dilution (corresponding to 25 ng of total RNA) was amplified in 25 µL of a reaction mixture containing Power SYBR green PCR master mix (Applied Biosystem, Foster City, CA, USA) and 200 µM of each primer (*tub2*, *fum1*, and *fum19*) [34] and *fum21* [35], as shown Supplementary Materials Table S1. The amplification conditions consisted of 45 cycles as follows: 95 °C for 15 s, annealing for 30 s at 58 °C, and extension at 72 °C for 35 s, followed by a final extension step of 4 min at 72 °C. All of the reactions were performed with three technical replicates. β -tubulin was used as a reference gene to normalize the quantitative expression data. The relative expression of the qRT-PCR products was determined according to the method of Pfaffl [36] with the Q-Gene software [37]. Due to the normalization of the data, the figures reported the fold change calculated with respect to the same target in the untreated control. The gene expression for the untreated control was always set to 1.0.

2.7. Statistical Analysis

Data were obtained through three independent experiments to determine the effects on fungal growth and FB accumulation, as well as two independent experiments for the effects on gene expression. Each treatment was performed with four replicates. If required, the results of the different experiments were analyzed after normalization against their respective untreated controls. A comparison of the fungal growth, FB content, and gene expression profiles of both strains was performed with the Kruskal–Wallis test (non-parametric rank comparison) followed by Dunn's test (nonparametric multiple comparisons). All of the calculations that included unmeasurable values (<LOD, defined by the manufacturer as ≤ 200 µg/kg) were conventionally considered contaminated by the half of the LOD value equal to 100 µg/kg. The correlations between the gene expression levels and FB production as a consequence of the treatments were analyzed with Spearman's rank correlation. Analyses were performed with the XLStat 2016 software package (Addinsoft, NY, USA).

3. Results

3.1. In Vitro Effect of Phenolic Compounds on *FvH* Growth and Fumonisin Production

The compounds tested at 1.0 mM exhibited different levels of inhibition of fungal growth and FB production by *FvH* (Table 2). ISO was the most effective inhibitor of fungal growth, leading to a relative decrease (RD) of almost 81.2%, followed by ELL (46.9% RD) and FER (27.8% RD). Concerning FB accumulation, the ISO and ELL treatments inhibited FB production below the limit of detection (LOD), while the other three compounds (PGA, VAN, and CAR) ranged from 40.3% to 31.8%. Because the inhibitory effect against the strain with the high FB production level was considered more valuable than that against the low-producing strain, these five phenolic compounds were selected to assess their effects on mycotoxin synthesis at the gene expression level. By monitoring the pH in the treated cultures (Supplementary Materials Table S2), the values observed after the treatments were very similar to those of the control, with small or localized deviations (CAF 0–5 dpi; CAR 3–10 dpi). The only compound that was able to consistently modify the pH values was ISO, which showed, starting at 1 dpi, pH values higher (+0.3 log pH unit) than the pH observed in the untreated control.

3.2. In Vitro Effect of Phenolic Compounds on *FvL* Growth and Fumonisin Production

The response of *FvL* to the tested compounds (1.0 mM) corresponded to a generalized biomass decrease that ranged from 14% to 55%, and only the CAF, PGA, and VAN treatments did not affect growth (Table 2). As occurred for *FvH*, the ELL and ISO treatments produced the highest growth reductions (54.5% and 38.1% RD, respectively), followed by APO (36.7% RD), FER (20.5% RD), and CAR (17.5% RD). Concerning FB accumulation, six out of the eight treatments decreased mycotoxin content, with the largest reductions induced by ELL (97.2% RD), APO (86.5% RD), and FER (70.1% RD). Differently from *FvH*, the non-complexed β -CD reduced both mycelial growth and FB accumulation by 14.0% and 57.9%, respectively, when compared to the untreated control. An additional experiment was carried out to verify if the non-complexed β -CD was able to sequester FBs in a liquid medium and contaminated maize flour. The results showed the inability of this adjuvant to remove FBs from these substrates (data not shown). Similarly to the observations for *FvH*, the pH values observed after the treatments were very similar to those of the controls (Supplementary Materials Table S3), with small or localized deviations (CAF 0–5 dpi; CAR 3–10 dpi). In this case, the ISO treatment also modified the pH over a time course of 3 to 7 dpi by increasing the values of the +0.3 log pH units with respect to untreated control.

Table 2. In vitro effect of phenolic compounds (1.0 mM) on fungal growth and fumonisin production.

<i>FvH</i>	Dry Fungal Biomass (rel. Yield $\pm$ SE)	Effect on Biomass †	FBs Content (rel. Yield $\pm$ SE)	Effect on FBs †
Control	100 ^{ab}	-	100 ^{ab}	
β -CD	99.8 $\pm$ 1.2 ^{ab}	-	123.5 $\pm$ 3.8 ^a	-
APO	80.3 $\pm$ 1.1 ^{abc}	-	76.6 $\pm$ 0.9 ^{bc}	-
CAF	94.1 $\pm$ 1.2 ^{ab}	-	90.0 $\pm$ 5.0 ^{bc}	-
CAR	93.6 $\pm$ 4.2 ^{abc}	-	68.2 $\pm$ 2.2 ^c	-31.8%
ELL	53.1 $\pm$ 3.4 ^{cd}	-46.9%	n.a. ^d	-99.2%
FER	72.2 $\pm$ 2.1 ^c	-27.8%	136.1 $\pm$ 3.9 ^{ab}	-
ISO	18.8 $\pm$ 0.7 ^d	-81.2%	n.a. ^d	-99.2%
PGA	106.8 $\pm$ 8.0 ^a	-	59.7 $\pm$ 4.8 ^c	-40.3%
VAN	103.4 $\pm$ 6.1 ^a	-	70.0 $\pm$ 0.5 ^c	-30.0%
<i>FvL</i>	Dry Fungal Biomass (rel. Yield $\pm$ SE)	Effect on Biomass †	FBs Content (rel. Yield $\pm$ SE)	Effect on FBs †
Control	100 ^a	-	100.0 ^a	
β -CD	86.0 $\pm$ 3.8 ^b	-14.0%	42.1 $\pm$ 0.6 ^{bc}	-57.9%
APO	63.3 $\pm$ 0.9 ^{cd}	-36.7%	13.5 $\pm$ 0.8 ^{de}	-86.5%
CAF	93.0 $\pm$ 1.7 ^{ab}	-	79.4 $\pm$ 1.5 ^{ab}	-
CAR	82.5 $\pm$ 2.2 ^{bc}	-17.5%	41.5 $\pm$ 0.1 ^{de}	-58.5%
ELL	45.5 $\pm$ 4.1 ^d	-54.5%	n.a. ^e	-97.2%
FER	79.5 $\pm$ 3.9 ^c	-20.5%	21.9 $\pm$ 0.8 ^{de}	-78.1%
ISO	61.9 $\pm$ 2.1 ^{cd}	-38.1%	49.1 $\pm$ 0.4 ^{cd}	-50.9%
PGA	115.3 $\pm$ 4.8 ^a	-	46.2 $\pm$ 0.8 ^{cd}	-53.8%
VAN	95.5 $\pm$ 0.5 ^{ab}	-	78.6 $\pm$ 0.8 ^{ab}	-

Values were expressed as percentage relative to the level observed in the control culture for *FvH* (biomass: 153.0 mg; FBs: 12,950 μ g/kg) and *FvL* (biomass: 143.6 mg; FBs: 3650 μ g/kg). FB values were normalized on fungal dry weight. Values with different letters in the rows are significantly different ($p < 0.05$). Statistical analyses were performed separately per strain. n.a. not applicable due to FBs content <LOD. †Effect on biomass and FBs was calculated as a percentage change with respect to untreated control data only for the statistically different treatments.

3.3. In Vitro Effects of Reduced ELL and ISO Dosages on *FvH* and *FvL*

ELL and ISO treatments with a stronger inhibitory capacity were further investigated to identify lower concentrations (0.5 or 0.25 mM) that were able to reduce FB biosynthesis while preserving fungal growth (Table 3). The data obtained on *FvH* for the ELL treatment showed that, differently from the 1.0 and 0.5 mM treatments, the biomass obtained after the 0.25 mM treatment was not different from that of the control, while the FB content remained significantly lower (<LOD). Conversely, for *FvL*, the 0.25 mM ELL treatment inhibited fungal growth by 53.8% compared to the 61.3% obtained with the 0.5 mM treatment; as a result, the FB values were once more below the LOD. Concerning the ISO treatments, while the 0.25 mM treatment had no significant effects on *FvH* growth, a significant concomitant increase in mycotoxin biosynthesis (+109.9%) occurred. Concerning *FvL*, the biomass increased with the dosage treatments from 89.5% to 104.9%, and a concomitant increase in FB content was measured at 0.5 mM (+149.6%).

Table 3. In vitro effect of ELL and ISO treatments (1.0, 0.5, and 0.25 mM) on fungal growth and fumonisin production.

<i>FvH</i>		Dry Fungal Biomass (rel. Yield $\pm$ SE)	Effect on Biomass $\pm$	FBs Content (rel. Yield $\pm$ SE) $\pm$	Effect on FBs $\pm$
Control		100 ^{ab}	-	100 ^b	-
ELL	1.0 mM	48.4 $\pm$ 5.4 ^c	-51.6%	n.a. ^d	-99.5%
	0.5 mM	46.4 $\pm$ 5.6 ^c	-53.6%	n.a. ^d	-99.5%
	0.25 mM	96.1 $\pm$ 3.9 ^b	-	n.a. ^d	-99.5%
ISO	1.0 mM	19.7 $\pm$ 0.9 ^d	-80.3%	n.a. ^d	-99.5%
	0.5 mM	39.6 $\pm$ 0.8 ^{cd}	-60.4%	55.9 $\pm$ 4.9 ^{bc}	-
	0.25 mM	120.9 $\pm$ 2.9 ^a	-	209.9 $\pm$ 15.4 ^a	+109.9%
<i>FvL</i>		Dry Fungal Biomass (rel. Yield $\pm$ SE)	Effect on Biomass $\pm$	FBs Content (rel. Yield $\pm$ SE) $\pm$	Effect on FBs $\pm$
Control		100 ^b		100 ^b	-
ELL	1.0 mM	39.9 $\pm$ 0.6 ^d	-60.1%	n.a. ^d	-97.5%
	0.5 mM	38.7 $\pm$ 2.3 ^d	-61.3%	n.a. ^d	-97.5%
	0.25 mM	46.2 $\pm$ 1.2 ^{cd}	-53.8%	n.a. ^d	-97.5%
ISO	1.0 mM	62.9 $\pm$ 7.0 ^{bc}	-	55.5 $\pm$ 6.2 ^{cd}	-44.5%
	0.5 mM	204.9 $\pm$ 16.6 ^a	+104.9%	249.6 $\pm$ 10.1 ^a	+149.6%
	0.25 mM	189.5 $\pm$ 15.9 ^a	+89.5%	177.1 $\pm$ 12.4 ^{ab}	-

Values were expressed as percentage relative to the level observed in the respective control for *FvH* (biomass: 168.2 mg; FBs: 16,720 μ g/kg) and *FvL* (biomass: 145.4 mg; FBs: 3930 μ g/kg). Values with different letters in the rows are significantly different ($p < 0.05$). Statistical analyses were performed separately per strain. n.a. not applicable due to FBs content <LOD. $\pm$ Effect on biomass and FBs was calculated as a percentage change with respect to untreated control data only for the statistically different treatments.

3.4. In Vivo Effect of Phenolic Compounds on Growth and FB Production by *FvH*

To better estimate the performance of these inhibitors under in vivo conditions, untreated maize kernels and maize kernels treated with the most effective phenolic compounds (1.0 and 3.0 mM) were inoculated with *FvH* (Table 4). The results obtained for each phenolic compound showed that the two concentrations were able to affect FB production in opposite ways. The treatment at 3.0 mM partly matched with the data obtained in vitro at 1.0 mM, resulting in a significant decrease in FB level as a result of ELL (81.2% RD) and ISO (76.9% RD), as well as CAR (96.6% RD) and PGA (68.6% RD). On the other hand, in addition to a significant mycotoxin decrease with the 1.0 mM treatments of ELL and CAR (56.5% and 51.4% RD, respectively), FER and ISO drastically induced FB accumulation by 265.7% to 487.8%, respectively. A significant reduction in FB production was observed with a high concentration of the β -CD treatment (9.0 mM), showing that this

compound—in its free form—can have an effect on FB biosynthesis, depending on concentration.

Table 4. Effect of selected phenolic compounds on FBs production in maize kernels.

	1.0 mM Treatment		3.0 mM Treatment	
	FBs Content (rel. Yield $\pm$ SE) †	Effect on FBs †	FBs Content (rel. Yield $\pm$ SE) †	Effect on FBs †
Control	100 ^c		100 ^a	
β -CD	98.9 $\pm$ 8.8 ^{cd}	-	34.3 $\pm$ 1.3 ^{bc}	-65.7
CAR	48.6 $\pm$ 4.3 ^d	-51.4	3.4 $\pm$ 1.5 ^d	-96.6%
ELL	43.5 $\pm$ 2.2 ^d	-56.5	18.8 $\pm$ 3.6 ^{cd}	-81.2%
FER	365.7 $\pm$ 16.1 ^{ab}	+265.7	72.5 $\pm$ 6.9 ^{ab}	-
ISO	587.8 $\pm$ 3.9 ^a	+487.8	23.1 $\pm$ 1.9 ^{bcd}	-76.9
PGA	157.8 $\pm$ 17.2 ^{bc}	-	31.4 $\pm$ 3.5 ^{bc}	-68.6

Values were expressed as percentage relative to the level observed in the control culture for *FvH* on sterile kernels (FBs: 18,540 μ g/kg). Values with different letters in the rows are significantly different ($p < 0.05$). Statistical analyses were performed separately per treatment. † Effect on FBs was calculated as a percentage change with respect to untreated control data only for the statistically different treatments.

3.5. Effect of Phenolic Compounds on *fum1* Expression

The gene expression analysis of *fum1* revealed an intraspecific variation between the two fungal isolates: the fumonisin high- and low-producing strains. Concerning *FvH* (Figure 2a), the most effective compounds (1.0 mM) in reducing toxin content were ISO and ELL, which caused an almost complete down-regulation of *fum1* expression, followed by CAR, which had a 0.61-fold change (f.c.) compared to the untreated control. FER, which did not have any significant effects on in vitro FB production, did not influence the transcription level of *fum1*. For *FvL* (Figure 2b), ELL, the most effective phenolic compound, downregulated *fum1* expression at 0.03 f.c., followed by FER (0.11 f.c.). Conversely, a strong up-regulation was induced by the ISO (9.48 f.c.) and PGA (5.12 f.c.) treatments, despite the few FBs that were detected. The CAR treatment, which was effective in FB reduction, resulted in a *fum1* expression level that was not different from that of the control. No significant difference in *fum1* expression was observed with β -CD or the control. The statistical analysis revealed that the regulation of *fum1* expression by the tested compounds for *FvH* ($\rho = 0.700$ $p < 0.0001$) and *FvL* ($\rho = 0.455$ $p = 0.001$) correlated with FB production.

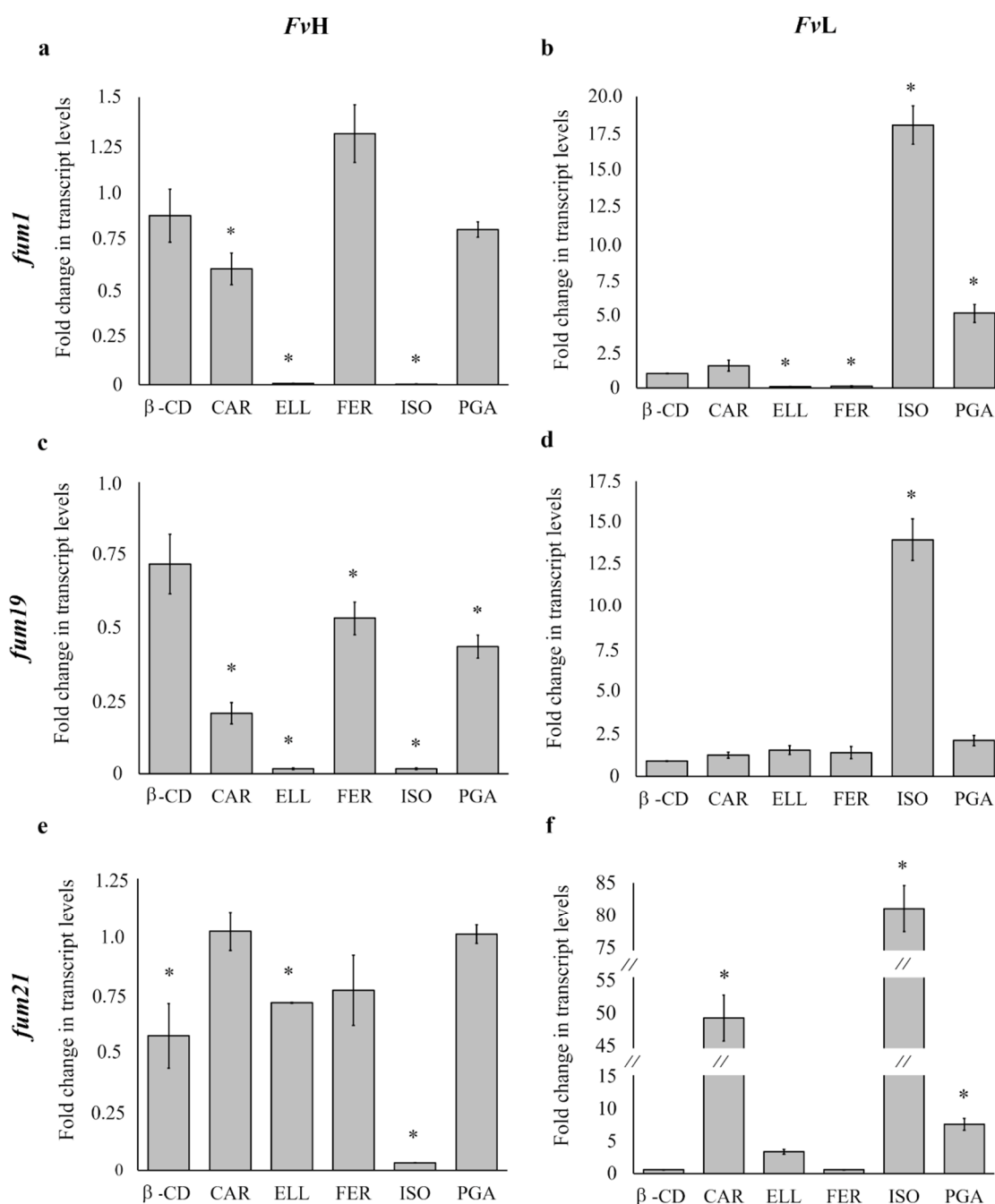


Figure 2. Fold changes on *fum* gene expression observed on *FvH* (figures on the left; **a**: *fum1*; **c**: *fum19*; **e**: *fum21*) and *FvL* (figures on the right; **b**: *fum1*; **d**: *fum19*; **f**: *fum21*) in response to selected 1.0 mM phenolic compound treatments with respect to values observed on untreated cultures. Due to data normalization, the expression level calculated for untreated control was set to 1.0. Bars represent the average value $\pm$ standard error. Bars with asterisks (*) are significantly different to untreated control according to Kruskal–Wallis and Dunn’s test ($p < 0.05$).

3.6. Effect of Phenolic Compounds on *fum19* Expression

The analysis of *fum19* expression with *FvH* (Figure 2c) showed that it was down-regulated by all of the tested compounds (1.0 mM) in the following order: ELL and ISO (0.02 f.c., both), CAR (0.21 f.c.), and PGA (0.43 f.c.). Moreover, although the FER treatment did not result in a significant decrease in FB production, a down-regulation of gene expression (0.53 f.c.) was observed. Conversely, a different behavior was shown in the *fum19* gene expression in *FvL* (Figure 2d); the decrease in FB production was not linked to a down-regulation of the gene expression level, and ISO led to a sharp up-regulation of the

transcription levels (14.04 f.c.). No significant difference was observed in the gene expression of β -CD and the control. The data obtained clearly showed that the *fum19* expression after the treatments was correlated with the FB content for *FvH* (ρ 0.649 p < 0.0001), but this effect was lacking for strain *L* (ρ 0.001 p = 0.986).

3.7. Effect of Phenolic Compounds on *fum21* Expression

Similarly to that of *fum1* and *fum19*, the gene expression of *fum21* was differentially regulated between the strains with the 1.0 mM treatments. *Fum21* in strain *FvH* (Figure 2e) was down-regulated in response to the treatments with ISO (0.03 f.c.) and ELL (0.71 f.c.). The CAR and PGA treatments did not show considerable differences in gene expression, despite the fact that both compounds reduced the FB production. No effect of FER on *fum21* expression was observed. Concerning the *FvL* strain (Figure 2f), with the exception of FER and β -CD, which down-regulated gene expression (0.60 f.c.; both), the other compounds strongly induced gene expression. In particular, ISO, CAR, and PGA increased *fum21* transcription by 79.0, 48.9, and 6.6 times, respectively. These data showed that the treatments with phenolic compounds acted on *fum21* expression in an opposite way with respect to the FB content; therefore, no correlation was observed for *FvH* (ρ 0.037 p = 0.400) and *FvL* (ρ 0.061 p = 0.277).

3.8. Effect of Under-Dosed ELL Treatment on *Fum* Gene Expression

Considering the efficacy of the tested phenolic compounds at different concentrations with respect to mycelial growth and FB production, ELL was further investigated with a gene expression analysis at the concentration of 0.25 mM. For both strains, the 0.25 mM ELL treatment decreased the gene expression (Figure 3). With respect to *FvH*, the treatment significantly reduced the expression of *fum1*, *fum19*, and *fum21* by 0.39, 0.30, and 0.45 times, respectively, thus confirming the data obtained after the ELL treatment at 1.0 mM. Moreover, the decrease in *fum21* expression with *FvL* was similar to that which occurred for *FvH* (0.37 f.c.); on the other hand, the expression of *fum1* and *fum19* was more inhibited by the 0.25 mM treatment (0.02 and 0.01 f.c., respectively). Furthermore, the behavior of *fum19* in *FvL* was different from that obtained with the same treatment at 1.0 mM where the transcription did not appear to be different from that of the control. Similarly, the *fum21* expression in both strains with 0.25 mM of ELL displayed a decrease that was different from that of the standard dosage.

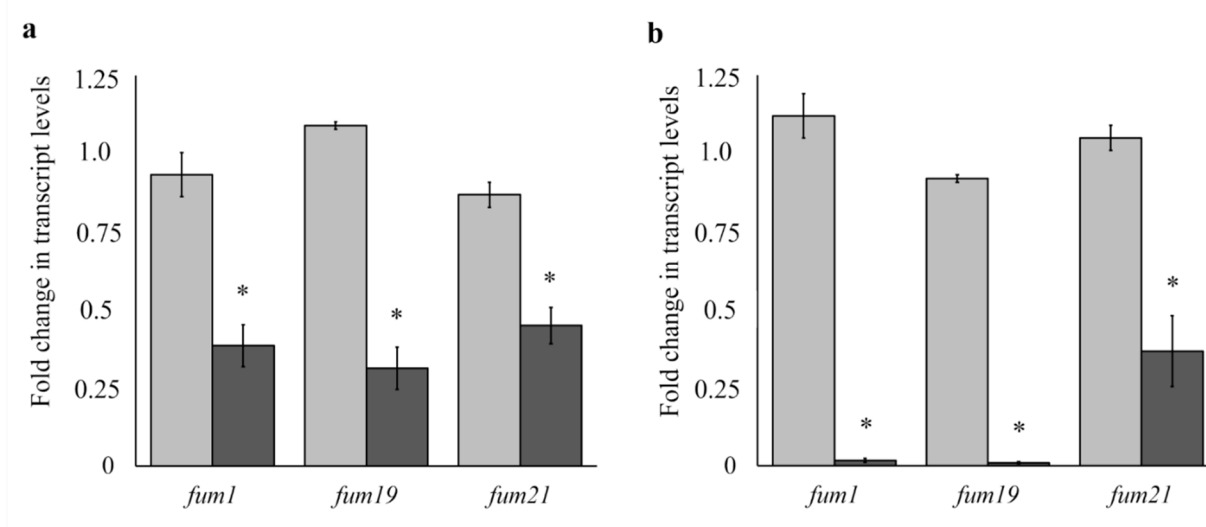


Figure 3. Effect of ELL 0.25 mM treatment on gene expression. Fold change in *fum1*, *fum19*, and *fum21* gene expression by *FvH* (a) and *FvL* (b) in response to β -CD control (0.75 mM; light gray bars) and ELL treatments (0.25 mM; dark gray bars).

Due to data normalization, the expression level calculated for untreated control was set to 1.0. The fold change was calculated according to the expression values observed for each gene on untreated cultures. Bars represent the average value $\pm$ standard error. Bars with asterisks (*) are significantly different to untreated control according to Kruskal–Wallis and Dunn's test ($p < 0.05$).

4. Discussion and Conclusion

To investigate the in vitro effects of naturally occurring phenols on the growth of *F. verticillioides* and FB production, eight phenolic compounds were tested in two strains that differed in their abilities to produce FBs. The sensitivities of *FvH* and *FvL* to these compounds at the selected concentrations were heterogeneous. Concerning their effect on the fungal growth of the *FvH* strain, the majority of compounds produced no significant difference with respect to the untreated cultures at 1.0 mM, except for ISO, ELL, and FER. In contrast, the response of the *FvL* strain to the phenols exhibited a generalized biomass decrease, and only CAF, PGA, and VAN were ineffective. With respect to FB production, five (CAR, ELL, ISO, PGA, and VAN) out of the eight treatments decreased the FB content produced by the *FvH* strain, with decreases ranging from 30 to 99.2%; six (APO, CAR, ELL, FER, ISO, and PGA) out of the eight treatments decreased the FB content produced by the *FvL* strain, with values ranging from 50.9% to 97.2%. Considering the data on fungal growth and FB production, some compounds were effective against a single strain or both strains, and the small changes in pH due to the supplementation of the phenols could not explain the observed variability. A specific phenolic compound could, therefore, modulate fungal growth and mycotoxin production differently according to the strain. The variations in the effects among the phenolic compounds and the different *Fusarium* isolates could likely be attributed to physical and chemical properties, such as the lipophilicity and antioxidant activity [38], to both fungal genus and strain features [15,39–41], or to the degradation of phenols into less effective molecules [42–44]. Our investigation focused on the identification of natural compounds that were able to inhibit mycotoxin production with a limited fungicidal effect. Despite the strong reduction in biomass at the concentration of 1.0 mM, ELL and ISO can be considered promising for controlling FBs with particular efficacy in a high-producer strain (*FvH*). However, to reduce the fungicidal effect caused by ELL and ISO on the fungal growth at 1.0 mM, they were under-dosed at 0.5 and 0.25 mM. Concerning ELL, each investigated concentration inhibited FB production, and the in vitro fungal growth was restored by diminishing its concentration to 0.25 mM, resulting in a functional concentration for in vitro FB inhibition. The inhibitory effect on FB production after the 1.0 mM ELL treatment was also observed in *F. culmorum* ISPave MCf21 [25]. However, another strain named FcUK99 induced an increase in trichothecene production of 59% at the same concentration [45]. On the contrary, the under-dosed ISO treatments (0.5 and 0.25 mM) promoted FB content in both strains—they maintained a fungicidal effect (*FvH*) or increased the growth of the biomass (*FvL*), depending on strain. These behaviors are different from those observed in *F. verticillioides* MRC4316 [8], in which a high ISO concentration produced a strong growth inhibition, while the 1.0 mM treatment did not alter the FB production [46]. Significant increases in biomass as a consequence of antioxidant treatments were already reported in *F. graminearum* and *F. poae*, where p-hydroxybenzoic acid, vanillic acid, quercetin, and rutin slightly stimulated mycelium growth [47]. Indeed, concerning the increased toxin content, it is not uncommon for a low phenolic concentration to be more inductive than a higher one; in fact, in *F. culmorum*, ISPave MCf21, a eugenol dimer at a concentration of 1.0 mM, promoted 3ADON biosynthesis by 2.24 times compared to the control, whereas a concentration of 0.5 mM promoted toxins by 4.51 times [25]. Concerning the other compounds tested, some chemicals (APO, CAF, VAN, FER, CAR, and PGA) were less effective than expected. The effective reduction observed in the growth and mycotoxin content after APO treatment in *FvL* (36.7 and 86.5, respectively) was similar to the data reported for *F. culmorum* INRA 117 (44% and 100% decreases in biomass and 3-ADON, respectively) [45]; on the other hand, these effects strongly diminished for *FvH* (19.7% and 23.4%). Regarding the fungal growth

and FB content in *FvH* and *FvL* after the CAF and VAN treatments, the results were quite low for both strains, confirming the previous reports for *F. verticillioides* 25N and *F. proliferatum* 73N, where low or no efficacy was observed at the concentration of 500 µg/g in corn (equivalent to a ~3 mM solution) [40]. However, their use should be deeply investigated, because a consistent FB reduction by these compounds was reported in *F. verticillioides* MRC826 [48] and *F. verticillioides* 63 [44], suggesting a strong dependence by the strain. As a further compound, FER is one of the most investigated phenols for mycotoxin control due to its ability to interfere with deoxynivalenol production [9,49]. In our experiments, FER showed a small reduction in fungal growth for both strains (21.5–27.8%), but the FB was decreased by 78.1% only in the low-producing strain (*FvL*), and this was not observed in the high-producing strain (*FvH*). The data obtained from the FER treatments are complex and depend on the concentration; in *F. verticillioides* M7075 and *F. proliferatum* RC 2080, treatments at concentrations of 1–10 mM increased both the growth and FB content [50], with a trend for FB content similar to that observed in *FvH*. These authors also stated that FER treatments at higher concentrations (20–25 mM) were effective in reducing fungal activity, and similar results were obtained by Nesci and colleagues [51] for *Aspergillus* section *Flavi* and its aflatoxin production. Concerning CAR and PGA, to our knowledge, few reports are available for these treatments for toxigenic fungi, and the present data provide evidence for a larger tolerance of both *FvH* and *FvL* toward CAR. On the contrary, application of CAR at 1 mM in *F. verticillioides* MRC 4316 decreased conidial germination (94.7% vitality reduction) in an investigation by Dambolena and colleagues [8]. Similarly, PGA, which showed a weaker effect on the strains that we investigated, caused decreases in biomass (88.0% and 97.7%) and 3-ADON production in *F. culmorum* FcUK99 [45].

A set of five compounds (CAR, ELL, FER, ISO, and PGA) that were found to be the best FB inhibitors in our in vitro experiments was assayed in vivo in sterile kernels. The FB reduction in the kernels was evaluated at two concentrations (1.0 and 3.0 mM). At 1.0 mM, only CAR and ELL were effective as FB inhibitors, unlike the other phenols, which were ineffective or even had negative consequences. However, after treatments at 3 mM, the FB content was strongly reduced by CAR (96%) and ELL (81%). For the other compounds, the FB content appeared to be rather similar to that in the data obtained in vitro. Some authors have indicated that the efficacy of phenolic compounds could be related to their lipophilicity or antioxidant properties [16,52,53]; however, the balance of these two properties has been suggested as a key factor for mycotoxin control [25,54,55]. Instead, ELL and ISO molecules, which are characterized by intermediate chemical properties (ELL: TEAC 3.05, LogP 1.05; ISO: TEAC 0.72, LogP 2.52), strongly influenced the fungal biomass. As previously mentioned, one of the properties that determines absorption and translocation is lipophilicity, which is measured as LogP [28]. Because Yang and colleagues indicated 3.39 as the LogP value with the maximal tissue uptake in maize kernels [56], chemicals with a LogP value that is far from this value should be taken up with lower efficiency, thus modulating the actual concentration of each compound in the kernel tissues. Interestingly, our treatments performed with CAR—which should cross the cell layers with the highest rate allowed by the kernel tissues—in maize (LogP 3.37) caused a significant FB reduction, which was observed at both 1.0 and 3.0 mM, with better results than those obtained in vitro. In contrast, when applied as a fumigant, the same treatment was found to be able to control *F. verticillioides* in experiments in vitro and in naturally infected kernel, but the same concentration failed to control the FB content [57]. The discrepancies obtained in these studies between the in vitro and in vivo conditions could likely be explained by the ready availability of the tested phenols in in vitro experiments, which, when applied to kernels, could be less effective due to a delayed or selective absorption through tissues. Further explanations could involve a possible degradation of the phenolic compounds by the metabolism of living seeds [58] or modifications of their chemical properties due to pH, which can further modify the stability of these molecules [59]. As previously found, phenols with high antioxidant capacities can act by interfering

with redox-sensitive cellular components [60]. Their high lipophilicity values can alter their ability to interact with the polar groups of membranes [54]. For these reasons, the inhibition of fungal growth and FB content by these two compounds characterized by quite opposite antioxidant capacities and lipophilicity could likely be due to their different mechanisms of interference. Taken together, these results provide new insights into the efficacy of phenols as inhibitors of FB production, suggesting ELL and CAR as the most promising for their efficacy as inhibitors at lower doses in order to limit the negative antifungal properties. Moreover, under-dosing some compounds can increase mycotoxin production, as was found for the eugenol dimer, showing that some compounds should be carefully evaluated [25].

It should be noted that cyclodextrin was considered to be inert without detrimental interferences in fungal metabolism [25,61]. However, in the present study, 3 mM β -CD reduced the in vitro FB content in the *FvL* strain. On the other hand, when its concentration was increased to 9.0 mM, such as in the in vivo experiment, β -CD was also effective against the high-producing strain (*FvH*). Although the results of the in vivo and in vitro experiments did not fully overlap, this behavior could suggest a dose- and strain-dependent response. However, no clear activity can be proposed for this adjuvant; indeed, the FER treatments were ineffective in spite of the presence of 3.0 mM β -CD; therefore, no direct effects were observed for the FER- β -CD complex. This aspect could indicate that, when in free form, cyclodextrin might interact with some metabolites, sequester them, or create less favorable environments for FB biosynthesis.

The effect of phenolic compound treatments in fungi can include the impairment of cellular ionic homeostasis and redox potential, the quenching of free radicals, and the inhibition of redox-sensitive enzymes [17,62,63]. Because most of these aspects are also involved in mycotoxin initialization and production, the expression of a set of representative *fum* genes involved in the FB biosynthetic pathway was analyzed after the treatments. Similarly to the mycotoxin content, where a large variability was observed between strains with respect to FB production, the gene expression also demonstrated a similar variability. Interestingly, ISO was the only compound that coherently decreased or increased the expression of the three investigated genes—*fum1*, *fum19*, and *fum21*—in *FvH* and *FvL*, respectively. *Fum1* encodes for a polyketide synthase—the first step required for the biosynthesis of FBs [64]—and its inhibition or knock-out leads to the stopping of FB synthesis [65,66]. Concerning the gene expression of *fum1* in *FvH*, the levels were strongly down-regulated by ISO, ELL, and CAR, and this correlated the low FB accumulation in the in vitro culture. A negative effect on *fum1* gene expression and FB production was observed [67] after treatments with naturally occurring phenolic compounds extracted from *Cuminum cyminum* essential oil in a *Fusarium verticillioides* strain [68]. In contrast, the *fum1* expression in *FvL* was down-regulated in response to ELL and FER, while a strong up-regulation was observed after the ISO and PGA treatments. Although the *fum* genes in *FvL* were up-regulated during these specific interactions, the FB content accumulated at very low levels. Similar results linking small amounts of toxins to increased gene transcription were suggested for the TRI genes in *F. graminearum* PH-1 treated with a low dosage (0.25 mM) of thymol or thymol with magnolol [15]. Our data suggest that some compounds might not only regulate mycotoxin production at the transcription level [43,69,70], but could also be effective at the post-transcription level, as suggested for trichodiene synthase (TRI5) in *F. culmorum* [45]. Indeed, as proposed by Pani and colleagues by working on a TRI5 model, phenolic compounds could interact with functional groups or amino acids that are putatively involved in enzymatic interactions by modifying or modulating its activity [45]. Another gene involved in FB biosynthesis is *fum19*, an ABC transporter that is involved in the extracellular export of fumonisins and is responsive to stress challenges [71–73]. Although several investigations of *fum19* gene [34,74,75] showed that it is not essential for FB production and that its deletion displayed few effects on the synthesis of these toxins [65,76], other experiments suggested a positive correlation

with mycotoxin production [77,78]. In light of the present experiments, a positive correlation between *fum19* expression and FB content was observed for the *FvH* strain, and the trend of *fum19* expression was similar to that observed for *fum1*. Similar conclusions could not be drawn for the *FvL* strain, in which, despite the decrease in mycotoxin accumulation, *fum19* expression appeared to be almost completely unaffected by the treatments, and the only matching treatment was ISO. By working with a *fum19*-deleted *F. proliferatum* strain, previous authors suggested that this gene likely exerts a negative regulatory effect on *fum1* [76]; however, we could not evaluate it, because significant reductions in *fum19* expression did not correspond to increases in *fum1*.

As observed for the previously mentioned genes, *fum21* was also differentially expressed among the different treatments and strains. In the *FvH* strain, only the ISO treatment caused a strong down-regulation of *fum21*, and FB production was completely inhibited. Because *fum21* is a transcription factor that is required for FB production [79], its up-regulation in the *FvL* strain should have caused an enrichment in mycotoxin production; however, despite its up-regulation with the ISO and PGA treatments, FB accumulation was inhibited by these treatments. These findings suggested that phenolic compounds can affect FB accumulation by generally down-regulating *fum* gene expression; however, some treatments in the FB low-producing *F. verticillioides* were able to up-regulate *fum* gene expression without improving FB production. However, apart from the gene cluster for FB biosynthesis, additional genes, including, *FCC1* [80], *ZFR1* [81], *FCK1* [82], *AREA* [83], and *FvVE1* [84], are also involved in FB regulation. These genes might also be responsive to the phenolic compounds that modulate FB production. Interestingly, at 3.0 mM, non-complexed β -CD demonstrated a certain activity against FB biosynthesis by reducing *fum21* expression in *FvH*, but to a limited extent.

The findings of the present work contribute to the knowledge of phenolic compounds as effective molecules for the containment of FBs. These compounds showed diverse efficacy with respect to fungal growth and FB accumulation in in vitro and in vivo experiments, and it was found that the effects of the compounds were also dependent on the strain. This aspect must be taken into consideration for future applications in the open field, as *Fusarium* populations are wide and heterogeneous. However, two out of the eight compounds— in particular CAR and ELL—were shown to reduce FB accumulation in *F. verticillioides* in vitro and in kernels. In conclusion, we identified some natural phenols that are able to significantly reduce FB accumulation; they seem to be promising molecules that can be evaluated as biopesticides under open-field conditions in order to control fumonisin occurrence in maize. As the next step, these phenolic compounds should be tested in maize plants from the silking to the dough stage, as these stages are particularly sensitive to *F. verticillioides* colonization. However, the number and dosage of the treatments need to be evaluated in the field along with agronomic practices.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/11/6/1060/s1. Table S1: List of primers used for FB-related gene expression analysis; Table S2: pH values measured in the high-FB producer strain treated flasks; Table S3: pH values measured in the low-FB producer strain treated flasks.

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