




Article

# Resistance-Related L-Pyroglutamic Acid Affects the Biosynthesis of Trichothecenes and Phenylpropanoids by *F. graminearum* Sensu Stricto

Katarzyna Bilka <sup>1,\*</sup>, Kinga Stuper-Szablewska <sup>2</sup>, Tomasz Kulik <sup>1</sup>, Maciej Buśko <sup>2</sup>,  
Dariusz Załuski <sup>3</sup> and Juliusz Perkowski <sup>2</sup>

<sup>1</sup> Department of Botany and Nature Protection, University of Warmia and Mazury in Olsztyn Plac Łódzki 1, 10-727 Olsztyn, Poland; tomaszkulik76@gmail.com

<sup>2</sup> Department of Chemistry, Poznan University of Life Sciences, Wojska Polskiego 75, 60-637 Poznan, Poland; kstuper@up.poznan.pl (K.S.-S.); mabu@up.poznan.pl (M.B.); julperk@up.poznan.pl (J.P.)

<sup>3</sup> Department of Plant Breeding and Seed Production, University of Warmia and Mazury in Olsztyn, Plac Łódzki 3, 10-727 Olsztyn, Poland; dariusz.zaluski@uwm.edu.pl

\* Correspondence: katarzyna.bilka@uwm.edu.pl; Tel.: +48-895-234-724

Received: 31 October 2018; Accepted: 22 November 2018; Published: 24 November 2018



**Abstract:** Fungicide application remains amongst the most widely used methods of fungal control in agroecosystems. However, the extensive use of fungicides poses hazards to human health and the natural environment and does not always ensure the effective decrease of mycotoxins in food and feed. Nowadays, the rising threat from mycotoxin contamination of staple foods has stimulated efforts in developing alternative strategies to control plant pathogenic fungi. A substantial effort is focused on the identification of plant-derived compounds inhibiting mycotoxin production by plant pathogenic fungi. L-Pyroglutamic acid has recently been suggested as playing a role in the response of barley to toxigenic *Fusaria*. Considering the above, we studied the response of various strains of *F. graminearum* sensu stricto to different levels of L-pyroglutamic acid on solid YES (yeast extract sucrose) media. L-Pyroglutamic acid decreased the accumulation of trichothecenes in all examined strains. Gene expression studies addressing *Tri* genes (*Tri4*, *Tri5*, and *Tri10*), which induce the biosynthesis of trichothecenes, revealed the production of mycotoxins by L-pyroglutamic acid to be inhibited at the transcriptional level. Besides inhibitory effects on mycotoxin production, L-pyroglutamic acid exhibited variable and concentration-related effects on phenylpropanoid production by fungi. Accumulation of most of the fungal-derived phenolic acids decreased in the presence of 100 and 400 µg/g of L-pyroglutamic acid. However, a higher dose (800 µg/g) of L-pyroglutamic acid increased the accumulation of *trans*-cinnamic acid in the media. The accumulation of fungal-derived naringenin increased in the presence of L-pyroglutamic acid. Contrasting results were obtained for quercetin, apigenin, luteolin, and kaempferol, the accumulation of which decreased in the samples treated with 100 and 400 µg/g of L-pyroglutamic acid, whereas the highest L-pyroglutamic acid concentration (800 µg/g) seemed to induce their biosynthesis. The results obtained in this study provide new insights for breeders involved in studies on resistance against *Fusaria*.

**Keywords:** *Fusarium*; L-pyroglutamic acid; trichothecenes; phenolic acids; flavonoids; gene expression

**Key Contribution:** L-Pyroglutamic acid inhibited *Tri* gene (*Tri4*, *Tri5* and *Tri10*) expression and decreased the accumulation of trichothecenes in *F. graminearum* s.s. strains. L-Pyroglutamic acid exhibited variable and concentration-related effects on phenylpropanoid production by *F. graminearum* s.s.

## 1. Introduction

A common ascomycete fungus, *Fusarium graminearum* sensu stricto (s.s.) remains a severe cereal pathogen worldwide. On small-grain cereals such as barley and wheat, this necrotroph can cause two different diseases: FHB (*Fusarium* head blight, referred to also as ear blight or scab) and FRR (*Fusarium* foot and root rot) [1,2]. FHB causes serious yield losses and decline in seed quality via grain contamination with type B trichothecenes [3].

Type B trichothecenes inhibit eukaryotic protein synthesis by binding to the 60S ribosomal unit and by interacting with the peptidyl transferase enzyme [4]. Consumption of contaminated food may cause food poisoning manifested by nausea, vomiting, and diarrhea, as well as headache, dizziness, and fever [5,6]. Higher doses of trichothecenes may damage bone marrow, the thymus, and the lymphoid and epithelial cells of the gastrointestinal mucosa [7]. Trichothecenes are heat-stable and therefore are not fully degraded by the cooking of food or by the processes used in food manufacturing [8,9].

The use of fungicides, principally azoles, is a major method for FHB management [10]. The control of *Fusaria* by fungicides often proves ineffective, mainly because of difficulties in timing and administration of the fungicides to the ear in the critical infection period during anthesis [11]. Furthermore, *F. graminearum* s.s. can develop increased resistance to this class of fungicides [12–16], while residues of massively used azoles can pervade into and persist in the environment, thereby posing high risk to nontarget organisms [17].

In recent years, accelerated efforts have been undertaken into the development of environmentally friendly disease management approaches [18]. Breeding of cultivars exhibiting high resistance against *Fusaria* remains the most promising strategy. Thus, in planta studies have been conducted to identify metabolites associated with resistance to fungal pathogens [19–24]. Other, simultaneous efforts have included in vitro studies evaluating the effect of these metabolites on fungal secondary metabolism. To date, phenylpropanoids have attracted the greatest attention in both in planta and in vitro studies [2,3,25–30]. The latter ones have shown that phenylpropanoids display molecule-dependent inhibitory effects on mycotoxin production by fungi [2,3,27], including *F. graminearum* s.s. [28–30].

In this study, we characterized the impact of L-pyroglutamic acid on the secondary metabolism of *F. graminearum* s.s. This organic acid was previously identified as a resistance-related (RR) metabolite in barley in response to *Fusarium* infection [21,22,31]. L-Pyroglutamic acid is an analogue and a potential precursor of glutamate [32], which is essential for the biosynthesis of glutathione. The latter is known as a powerful antioxidant [33]. Previous studies have shown that L-pyroglutamic acid exhibits both antibacterial [34] and antifungal activity against phytopathogenic *Pseudoperonospora cubensis* and *Phytophthora infestans* [35]. To the best of our knowledge, there are no reports characterizing the impact of this compound on *Fusaria*.

We studied the effect of L-pyroglutamic acid on the accumulation of trichothecenes in the culture media as well as on the expression of the *Tri* genes (*Tri4*, *Tri5*, and *Tri10*) which induce their biosynthesis. We found that both gene expression and trichothecene accumulation were inhibited in all studied strains treated with L-pyroglutamic acid. In the present study, we also showed that the treatment with L-pyroglutamic acid impacts fungal phenolic acid and flavonoid profiles. The results obtained in this study provide new insights, especially for breeders involved in studies on resistance against *Fusaria*.

## 2. Results and Discussion

### 2.1. Exogenous L-Pyroglutamic Acid Affects the Production of Phenolic Acids and Flavonoids by Fungal Strains

After 21 days of incubation, fungi that were exposed to 100, 400, or 800 µg/g of L-pyroglutamic acid reduced its concentration by 90–91%, 46–51%, and 56–60%, respectively. The substantial reduction of exogenous L-pyroglutamic acid might be linked to its efficient metabolism by fungi.

L-pyroglutamic acid can be easily converted to glutamate by 5-oxoprolinase [36,37] and has also been found at the N-terminus of many peptides and proteins [32].

Fungal strains used in this study produced phenolic acids and phenylpropanoids on the solid YES (yeast extract sucrose) medium (Supplementary Table S1), which is in agreement with findings from our previous studies [28–30]. After 21 days of incubation with L-pyroglutamic acid, changes were observed in fungal secondary metabolite profiles. The total phenolic acids content decreased by 3.5- to 5-fold (depending on L-pyroglutamic acid concentration) in the fungal cultures exposed to L-pyroglutamic acid. It is worth noting, however, that in the case of two phenolic acids (protocatechuic and *trans*-cinnamic acids), exceptional results were obtained. No significant changes in protocatechuic acid were found between the samples treated with L-pyroglutamic acid and controls (YES + fungal controls). The second exception concerned the higher concentration of *trans*-cinnamic acid after incubation with 800 µg/g of L-pyroglutamic acid. Nonoxidative deamination of L-phenylalanine to *trans*-cinnamic acid by phenylalanine ammonia-lyase (PAL; E.C 4.3.1.5) is a key step of phenylpropanoid biosynthesis. Accumulation of *trans*-cinnamic acid was also identified by Kulik et al. [29] after *Fusarium* strains' treatment with chlorogenic acid and by Bilska et al. [30] after their treatment with luteolin, kaempferol, and quercetin. The higher concentration of *trans*-cinnamic acid may indicate that the activity of PAL responsible for phenylpropanoid production was not completely inhibited by L-pyroglutamic acid. Increased accumulation of *trans*-cinnamic acid may also suggest that L-pyroglutamic acid has an inhibitory effect on enzymes converting this first intermediate of all phenylpropanoids further.

In this study, all three concentrations of L-pyroglutamic acid induced the biosynthesis of naringenin. Accumulation of other flavonoids (quercetin, apigenin, luteolin, and kaempferol) was inhibited in the samples treated with two lower concentrations of L-pyroglutamic acid (100 and 400 µg/g), whereas the highest acid concentration (800 µg/g) seemed to induce their biosynthesis. The sum of flavonoids increased almost 2.5-fold after incubation with 800 µg/g of L-pyroglutamic acid. It is unclear why the exposure to lower amounts of L-pyroglutamic acid suppressed the production of some flavonoids by fungal strains. However, the increased concentration of naringenin and other flavonoids may be indicative of *trans*-cinnamic acid conversion to naringenin chalcone, which is a precursor of naringenin (Figure 1). It seems that L-pyroglutamic acid stimulates the activity of enzymes responsible for naringenin biosynthesis: chalcone synthase (CHS) and chalcone isomerase (CHI) (Figure 1), whereas the activity of enzymes catalyzing the conversion of phenolic acids could be inhibited.

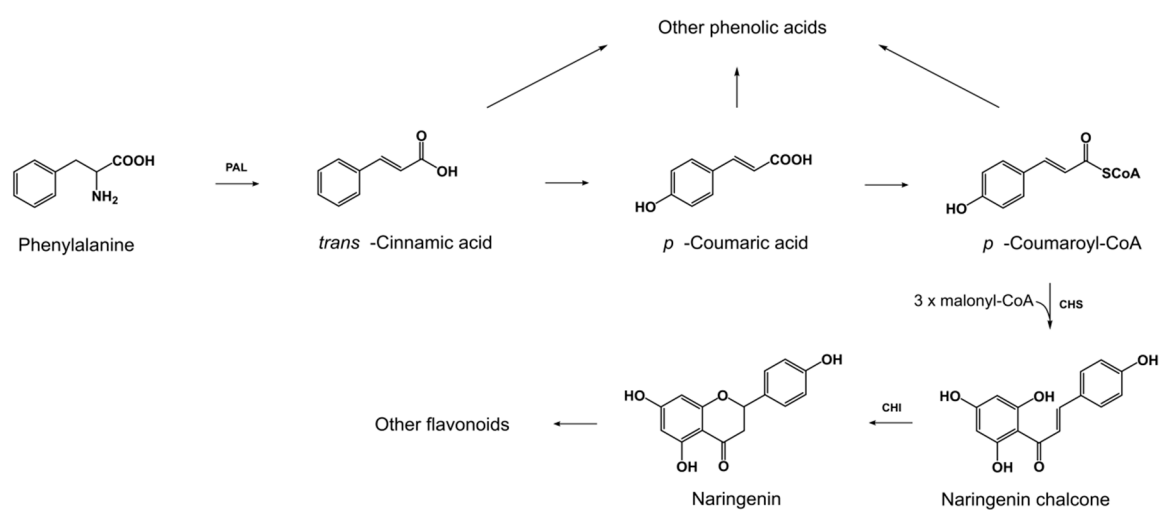


Figure 1. Scheme of the biosynthesis of selected phenylpropanoids.

## 2.2. Effect of L-Pyroglutamic Acid on the Accumulation of Trichothecenes in Culture Media

The effect of L-pyroglutamic acid on the production of trichothecenes by fungal strains was established based on trichothecene counts determined on the treated and nontreated plates (YES + fungal controls). L-Pyroglutamic acid had strong inhibitory effects on trichothecene accumulation in the case of all three studied *F. graminearum* s.s. strains; however, the degree of toxin inhibition was strain-dependent (Table 1). *F. graminearum* s.s. strains kept at 100, 400, or 800 µg/g of L-pyroglutamic acid reduced the trichothecene concentration by 83–89%, 56–93%, and 67–97%, respectively.

**Table 1.** Trichothecene levels and relative quantification (RQ) of *Tri* transcripts of fungal strains incubated with different levels of L-pyroglutamic acid.

L-Pyroglutamic Acid Concentration	Strain	<i>Tri</i> Genotype	Trichothecene Levels (mg/kg) ( <i>n</i> = 3 in Each Condition)					RQ ( <i>n</i> = 6 in Each Condition)			Relative Radial Growth ( <i>n</i> = 3 in Each Condition)	
			DON	3-AcDON	15-AcDON	NIV	Total	<i>Tri4</i>	<i>Tri5</i>	<i>Tri10</i>		
YES + Fungal Controls	CBS 138561	15ADON	1.43 ± 0.7		1.47 ± 0.85			2.9				100
	CBS 119173	3ADON	49.55 ± 3.57	12.24 ± 0.76			61.79					100
	MUCL 53455	NIV				4.41 ± 0.18	4.41					100
100 µg/g	CBS 138561	15ADON	0.47 ± 0.02 (b)		0.03 ± 0.001 (b)		0.5	0.76 (0.635–0.905)	NS	NS		106.28 ± 5.02 (NS)
	CBS 119173	3ADON	5.05 ± 0.3 (d)	1.54 ± 0.03 (d)			6.59	0.349 (0.271–0.458)	NS	NS		95.63 ± 0.44 (NS)
	MUCL 53455	NIV				0.49 ± 0.04 (c)	0.49	NS	NS	NS		89.22 ± 0.43 (b)
400 µg/g	CBS 138561	15ADON	0.17 ± 0.007 (b)		0.02 ± 0.001 (b)		0.19	0.035 (0.028–0.044)	0.34 (0.272–0.429)	0.71 (0.615–0.83)		98.33 ± 2.93 (NS)
	CBS 119173	3ADON	21.14 ± 0.85 (b)	5.95 ± 0.36 (b)			27.09	0.117 (0.088–0.148)	0.421 (0.28–0.637)	0.123 (0.098–0.1)		107.42 ± 2.62 (NS)
	MUCL 53455	NIV				0.6 ± 0.05 (bc)	0.6	0.558 (0.420–0.777)	0.645 (0.531–0.786)	NS		92.24 ± 2.59 (ab)
800 µg/g	CBS 138561	15ADON	0.08 ± 0.003 (b)		0.02 ± 0.001 (b)		0.1	0.065 (0.055–0.08)	0.25 (0.213–0.3)	0.427 (0.368–0.495)		94.14 ± 6.28 (NS)
	CBS 119173	3ADON	15.00 ± 0.3 (c)	4.4 ± 0.09 (c)			19.4	0.072 (0.003–0.456)	0.215 (0.143–0.325)	0.04 (0.008–0.097)		108.73 ± 11.79 (NS)
	MUCL 53455	NIV				0.79 ± 0.08 (b)	0.79	0.451 (0.362–0.563)	0.23 (0.161–0.342)	NS		82.76 ± 2.59 (b)
<b>Degree of mycotoxin inhibition:</b>		<25%	25–50%	50–75%	>75%							

(a), (b), (c), and (d) lettering indicates homogenous groups at *p* < 0.05 followed by the Tukey test; NS—not significant; DON- deoxynivalenol; NIV- nivalenol; 3-AcDON- 3-acetyldeoxynivalenol; 15-AcDON- 15-acetyldeoxynivalenol.

Previous studies linked the inhibitory effect of phenylpropanoids to their antioxidant and antiradical properties [29,30,38]. The stronger the antioxidant/antiradical property of the phenylpropanoid, the more efficient the inhibitory effect on mycotoxin production. Indeed, as shown in Table 2, L-pyroglutamic acid displays high antioxidant activity compared to other phenolic acids. It exhibits stronger antioxidant properties than chlorogenic and sinapic acids, which as shown previously, displayed inhibitory effects on mycotoxin production by *Fusaria*. The strong inhibitory effects of L-pyroglutamic acid may also be linked to its high radical-scavenging activity, which appears to be higher than that of *trans*-cinnamic acid (Table 2), exhibiting strong inhibitory effects on trichothecene biosynthesis by *Fusarium* spp. [29].

**Table 2.** Antioxidant capacity (VCEAC/L) and radical scavenging activity (ABTS) of L-pyroglutamic acid and selected phenolic acids.

Acid	VCEAC/L	ABTS ( $\mu\text{mol TROLOX}/100 \text{ g d.m.}$ )
L-Pyroglutamic Acid	421.2	568.1
<i>trans</i> -Cinnamic Acid *	812.3	314.9
Chlorogenic Acid *	12.2	57.1
Sinapic Acid **	121	194.5

\* from Kulik et al. [29]; \*\* from Kulik et al. [28]; VCEAC- vitamin C equivalent antioxidant capacity; ABTS- 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid).

### 2.3. Effect of L-Pyroglutamic Acid on the Expression of *Tri* Genes

The effect of L-pyroglutamic acid on *Tri* gene (*Tri4*, *Tri5*, and *Tri10*) expression was evaluated on day three of incubation (Table 1). Gene expression studies showed the production of mycotoxins by L-pyroglutamic acid to be inhibited at the transcriptional level. L-Pyroglutamic acid inhibited the activity of *Tri* genes; however, this inhibition showed no significant fold-change values ( $P(H1) = 0.001$ ) in most samples treated with 100  $\mu\text{g}/\text{g}$  of L-pyroglutamic acid. Expression of *Tri* genes is regulated by two genes: *Tri10* and *Tri6* [39–41]. *Tri10* positively regulates the transcription of *Tri6* [41], which affects the expression of other *Tri* genes [39,40]. In our study, *Tri10* transcription generally correlated with *Tri4* and *Tri5* expression; however, there were some exceptions. We noticed inhibition of *Tri4* and *Tri5* gene expression in MUCL 53455 strains in the presence of L-pyroglutamic acid, despite no changes observed in *Tri10* expression.

## 3. Conclusions

L-Pyroglutamic acid decreased the accumulation of trichothecenes in all examined strains. Gene expression studies addressing *Tri* genes which induce the biosynthesis of trichothecenes (*Tri4*, *Tri5*, and *Tri10*) revealed the production of mycotoxins by L-pyroglutamic acid to be inhibited at the transcriptional level. Besides inhibitory effects on mycotoxin production, L-pyroglutamic acid exhibited variable and concentration-related effects on phenylpropanoid production by fungi.

## 4. Materials and Methods

### 4.1. Fungal Strains

*Fusarium graminearum* s.s. strains used in this study are summarized in Table 3. Detailed characteristics are provided in the ToxGen database [42]. All strains are maintained in the international fungal collections: Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands), MUCL—MUCL Mycothèque de l'Université catholique de Louvain (Louvain-la-Neuve, Belgium), and ARS Culture Collection, USDA (Peoria, IL, US).

**Table 3.** List of *F. graminearum* s.s. isolates used in this study.

Species	Strain	Trichothecene Genotype	Origin, Host, and Year of Isolation
<i>F. graminearum</i> s.s.	CBS 138561	15ADON	Poland, wheat, 2010
	CBS 119173, NRRL 38369	3ADON	USA, Louisiana, wheat, 2005
	MUCL 53455	NIV	Belgium, corn, 2007

#### 4.2. Medium and Culture Conditions

L-Pyroglutamic acid (dissolved in 10 mL of 96% ethanol) (Sigma–Aldrich, Saint Louis, MO, USA) was added to the YES (yeast extract sucrose) medium to obtain the final concentrations of: 100 µg/g, 400 µg/g, and 800 µg/g. The three acid concentrations corresponded to those used by Kulik et al. [28,29] and Bilska et al. [30]. Three different controls were used in this study: YES-only control (YES medium only), YES + L-pyroglutamic acid control (YES media supplemented with either 100, 400, or 800 µg/g of acid), and three YES + fungal controls (*F. graminearum* s.s. strains incubated on YES media).

Petri dishes (Ø 80 mm) were inoculated from laboratory stock cultures (6–8 weeks old) and incubated at 25 °C (in triplicate) in the dark. Gene expression analyses were performed after 3 days of incubation, while chemical analyses (mycotoxins, flavonoids, and phenolic acids determination) were performed after 21 days of incubation.

#### 4.3. Determination of Contents of Phenolic Acids and Flavonoids in the Culture Medium

Fungal-derived phenylpropanoid concentrations were established in dried fungal cultures after 21 days of incubation (Supplementary Table S1), as previously described by Kulik et al. [28,29] and Bilska et al. [30]. Alkaline and acid hydrolysis were conducted in sealed 17-mL culture tubes containing 0.2 g of fungal biomass. An ACQUITY H class UPLC (Ultra performance liquid chromatography) system equipped with a Waters ACQUITY Photodiode Array (PDA) detector (Waters, Milford, MA, USA) was used in the analysis. Concentrations of L-pyroglutamic acid were determined using an internal standard at a wavelength of  $\lambda = 310$  nm. L-Pyroglutamic acid identification was based on a comparison of sample and standard retention times. A specific amount of the standard was added to the analyzed samples. In the next step, the analysis was repeated. The detection level was 1 µg/g. The retention time of L-pyroglutamic acid was 19.05 min.

#### 4.4. Determination of the Antioxidant Capacity (VCEAC/L) and Radical Scavenging Activity (ABTS+) of L-Pyroglutamic Acid

VCEAC/L and ABTS+ assays of L-pyroglutamic acid were conducted according to the methods provided earlier by Kim et al. [43] and Re et al. [44], respectively. The results were expressed as ABTS (µmol TROLOX/100 g d.m.) and mg Vitamin C-equivalent antioxidant capacity per liter (mg VCEAC/L).

#### 4.5. Analysis of Trichothecene Concentrations from Fungal Cultures

GC-MS was used to determine levels of trichothecenes (DON- deoxynivalenol; NIV- nivalenol; 3-AcDON- 3-acetyldeoxynivalenol; 15-AcDON- 15-acetyldeoxynivalenol) in fungal cultures exposed or nonexposed (YES + fungal controls) to various concentrations of L-pyroglutamic acid, as described in a study by Perkowski et al. [45].

#### 4.6. Extraction of Total RNA and Preparation of cDNA

Mycelium from 3-day-old fungal cultures grown on the YES medium exposed or nonexposed (YES + fungal controls) to various concentrations of L-pyroglutamic acid was used for RNA extraction. Six biological replications were prepared for each studied condition. Total RNA was extracted using



a Pure Link<sup>®</sup> RNA MINI KIT (Life Technologies, Carlsbad, CA, USA) following the manufacturer's recommendations. Extraction of RNA and reverse transcription were conducted according to Kulik et al. [46,47]. cDNA samples were stored at  $-25\text{ }^{\circ}\text{C}$ .

#### 4.7. Gene Expression Analysis

In this study, expression of two genes (*Tri4*, *Tri5*) playing a fundamental role in trichothecene biosynthesis [48–52] and expression of the *Tri10* regulatory gene [41] were analyzed. The elongation factor 1-alpha gene (*Ef1 $\alpha$* ) was used as a reference gene. The experiment was conducted in accordance with Kulik et al. [28,29,46,47]. The REST 2009 software [53] was used to normalize the Ct (threshold cycle) values of the target (*Tri4*, *Tri5*, and *Tri10*) genes in control and treated samples, relative to the Ct values obtained for the reference *Ef1 $\alpha$*  gene.

#### 4.8. Statistical Analyses

Statistical analyses were performed using STATISTICA (Data Analysis Software System, ver. 12.5; StatSoft Inc., Tulsa, OK, USA, 2014). Tukey's HSD (honest significant difference) test ( $p < 0.05$ ) was used to show the differences among mycotoxin levels. The production of phenylpropanoids (phenolic acids and flavonoids) was estimated by an analysis of the differences among phenylpropanoid levels between YES-only and YES + fungal controls using the Student's *t*-test at  $p < 0.05$ . This statistical method was also used to test the impact of exogenously applied L-pyroglutamic acid on the accumulation of phenylpropanoids and to analyze the reduction of exogenous L-pyroglutamic acid.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2072-6651/10/12/492/s1>, Supplementary Table S1: Flavonoid and phenolic acid accumulation by *F. graminearum* s.s. strains after 21 d of incubation in the presence of L-pyroglutamic acid.

**Author Contributions:** Conceptualization, T.K.; Formal analysis, K.B., T.K. and D.Z.; Funding acquisition, T.K.; Investigation, K.B., K.S.-S., T.K., M.B., D.Z. and J.P.; Methodology, K.B., K.S.-S., T.K., M.B. and J.P.; Project administration, K.B. and T.K.; Resources, T.K.; Supervision, T.K.; Validation, K.B., K.S.-S., T.K., M.B., D.Z. and J.P.; Visualization, K.B.; Writing—original draft, K.B. and T.K.

**Funding:** This work was funded by the research project: Grant DEC-2013/11/B/NZ9/01788 from the National Science Center, Poland.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## References

1. Nganje, W.E.; Kaitibie, S.; Wilson, W.W.; Leistriz, F.L.; Bangsund, D.A. Economic impacts of Fusarium Head Blight in wheat and barley: 1993–2001. *Agribus. Appl. Econ. Rep.* **2004**, *538*, 1–53.
2. Ponts, N.; Pinson-Gadais, L.; Boutigny, A.L.; Barreau, C.; Richard-Forget, F. Cinnamic-derived acids significantly affect *Fusarium graminearum* growth and in vitro synthesis of type B trichothecenes. *Phytopathology* **2011**, *101*, 929–934. [[CrossRef](#)] [[PubMed](#)]
3. Gauthier, L.; Bonnin-Verdal, M.N.; Marchegay, G.; Pinson-Gadais, L.; Ducos, C.; Richard-Forget, F.; Atanasova-Penichon, V. Fungal biotransformation of chlorogenic and caffeic acids by *Fusarium graminearum*: New insights in the contribution of phenolic acids to resistance to deoxynivalenol accumulation in cereals. *Int. J. Food. Microbiol.* **2016**, *221*, 61–68. [[CrossRef](#)] [[PubMed](#)]
4. Marin, S.; Ramos, A.J.; Cano-Sancho, G.; Sanchis, V. Mycotoxins: occurrence, toxicology, and exposure assessment. *Food Chem. Toxicol.* **2013**, *60*, 218–237. [[CrossRef](#)] [[PubMed](#)]
5. Nakajima, T. Fungicides application against Fusarium Head Blight in wheat and barley for ensuring food safety. In *Fungicides*; Carisse, O., Ed.; InTech: Rijeka, Croatia, 2010; pp. 139–156.
6. Wegulo, S.N. Factors influencing deoxynivalenol accumulation in small grain cereals. *Toxins* **2012**, *4*, 1157–1180. [[CrossRef](#)] [[PubMed](#)]
7. Pestka, J.J. Deoxynivalenol: Toxicity, mechanisms and animal health risks. *Anim. Feed Sci. Technol.* **2007**, *137*, 283–298. [[CrossRef](#)]



8. Hazel, C.M.; Patel, S. Influence of processing on trichothecene levels. *Toxicol. Lett.* **2004**, *153*, 51–59. [[CrossRef](#)] [[PubMed](#)]
9. Bullerman, L.B.; Bianchini, A. Stability of mycotoxins during food processing. *Int. J. Food Microbiol.* **2007**, *119*, 140–146. [[CrossRef](#)] [[PubMed](#)]
10. Paul, P.A.; McMullen, M.P.; Hershman, D.E.; Madden, L.V. Meta-analysis of the effects of triazole-based fungicides on wheat yield and test weight as influenced by *Fusarium* head blight intensity. *Phytopathology* **2010**, *100*, 160–171. [[CrossRef](#)] [[PubMed](#)]
11. Lucas, J.A.; Hawkins, N.J.; Fraaije, B.A. The evolution of fungicide resistance. *Adv. Appl. Microbiol.* **2015**, *90*, 29–92. [[CrossRef](#)]
12. Klux, M.B.; Verreet, J.-A.; Beyer, M. Comparison of the declining triazole sensitivity of *Gibberella zeae* and increased sensitivity achieved by advances in triazole fungicide development. *Crop Prot.* **2007**, *269*, 683–690. [[CrossRef](#)]
13. Chen, C.-J.; Yu, J.-J.; Bi, C.-W.; Zhang, Y.-N.; Xu, J.-Q.; Wang, J.-X.; Zhou, M.-G. Mutations in a  $\beta$ -tubulin confer resistance of *Gibberella zeae* to benzimidazole fungicides. *Phytopathology* **2009**, *99*, 1403–1411. [[CrossRef](#)] [[PubMed](#)]
14. Yin, Y.; Liu, X.; Li, B.; Ma, Z. Characterization of sterol demethylation inhibitor-resistant isolates of *Fusarium asiaticum* and *F. graminearum* collected from wheat in China. *Phytopathology* **2009**, *99*, 487–497. [[CrossRef](#)] [[PubMed](#)]
15. Becher, R.; Hettwer, U.; Karlovsky, P.; Deising, H.B.; Wirsal, S.G.R. Adaptation of *Fusarium graminearum* to tebuconazole yielded descendants diverging for levels of fitness, fungicide resistance, virulence, and mycotoxin production. *Phytopathology* **2010**, *100*, 444–453. [[CrossRef](#)] [[PubMed](#)]
16. Sun, H.-Y.; Zhu, Y.-F.; Liu, Y.-Y.; Deng, Y.-Y.; Li, W.; Zhang, A.-X.; Chen, H.-G. Evaluation of tebuconazole for the management of *Fusarium* head blight in China. *Australasian Plant Pathol.* **2014**, *43*, 631–638. [[CrossRef](#)]
17. Chen, Z.F.; Ying, G.G. Occurrence, fate and ecological risk of five typical azole fungicides as therapeutic and personal care products in the environment: A review. *Environ. Int.* **2015**, *84*, 142–153. [[CrossRef](#)] [[PubMed](#)]
18. Villa, F.; Cappitelli, F.; Cortesi, P.; Kunova, A. Fungal Biofilms: Targets for the development of novel strategies in plant disease management. *Front. Microbiol.* **2017**, *8*, 654. [[CrossRef](#)] [[PubMed](#)]
19. Hamzehzarghani, H.; Paranidharan, V.; Abu-Nada, Y.; Kushalappa, A.C.; Mamer, O.; Somers, D. Metabolic profiling to discriminate wheat near isogenic lines, with quantitative trait loci at chromosome 2DL, varying in resistance to *Fusarium* Head Blight. *Can. J. Plant Sci.* **2008**, *88*, 789–797. [[CrossRef](#)]
20. Paranidharan, V.; Abu-Nada, Y.; Hamzehzarghani, H.; Kushalappa, A.C.; Mamer, O.; Dion, Y.; Rioux, S.; Comeau, A.; Choiniere, L. Resistance-related metabolites in wheat against *Fusarium graminearum* and the virulence factor deoxynivalenol (DON). *Botany* **2008**, *86*, 1168–1179. [[CrossRef](#)]
21. Bollina, V.; Kumaraswamy, G.K.; Kushalappa, A.C.; Choo, T.M.; Dion, Y.; Rioux, S.; Faubert, D.; Hamzehzarghani, H. Mass spectrometry-based metabolomics application to identify quantitative resistance-related metabolites in barley against *Fusarium* head blight. *Mol. Plant Pathol.* **2010**, *11*, 769–782. [[CrossRef](#)] [[PubMed](#)]
22. Kumaraswamy, G.K.; Bollina, V.; Kushalappa, A.C.; Choo, T.M.; Dion, Y.; Rioux, S.; Mamer, O.; Faubert, D. Metabolomics technology to phenotype resistance in barley against *Gibberella zeae*. *Eur. J. Plant Pathol.* **2011**, *130*, 29–43. [[CrossRef](#)]
23. Kumaraswamy, K.G.; Kushalappa, A.C.; Choo, T.M.; Dion, Y.; Rioux, S. Mass spectrometry based metabolomics to identify potential biomarkers for resistance in barley against *Fusarium* Head Blight (*Fusarium graminearum*). *J. Chem. Ecol.* **2011**, *37*, 846–856. [[CrossRef](#)] [[PubMed](#)]
24. Gunnaiah, R.; Kushalappa, A.C. Metabolomics deciphers the host resistance mechanisms in wheat cultivar Sumai-3, against trichothecene producing and non-producing isolates of *Fusarium graminearum*. *Plant. Physiol. Biochem.* **2014**, *83*, 40–50. [[CrossRef](#)] [[PubMed](#)]
25. Boutigny, A.-L.; Barreau, C.; Atanasova-Penichon, V.; Verdal-Bonnin, M.-N.; Pinson-Gadais, L.; Richard-Forget, F. Ferulic acid, an efficient inhibitor of type B trichothecene biosynthesis and *Tri* gene expression in *Fusarium* liquid cultures. *Mycol. Res.* **2009**, *113*, 746–753. [[CrossRef](#)] [[PubMed](#)]
26. Boutigny, A.L.; Atanasova-Pénichon, V.; Benet, M.; Barreau, C.; Richard-Forget, F. Natural phenolic acids from wheat bran inhibit *Fusarium culmorum* trichothecene biosynthesis *in vitro* by repressing *Tri* gene expression. *Eur. J. Plant Pathol.* **2010**, *127*, 275–286. [[CrossRef](#)]

27. Pani, G.; Scherm, B.; Azara, E.; Balmas, V.; Jahanshiri, Z.; Carta, P.; Fabbri, D.; Dettori, M.A.; Fadda, A.; Dessi, A.; et al. Natural and natural-like phenolic inhibitors of type b trichothecene in vitro production by the wheat (*Triticum* sp.) pathogen *Fusarium culmorum*. *J. Agric. Food Chem.* **2014**, *62*, 4969–4978. [[CrossRef](#)] [[PubMed](#)]
28. Kulik, T.; Stuper-Szablewska, K.; Bilaska, K.; Buško, M.; Ostrowska-Kołodziejczak, A.; Załuski, D.; Perkowski, J. Sinapic acid affects phenolic and trichothecene profiles of *F. culmorum* and *F. graminearum* sensu stricto. *Toxins* **2017**, *9*, 264. [[CrossRef](#)] [[PubMed](#)]
29. Kulik, T.; Stuper-Szablewska, K.; Bilaska, K.; Buško, M.; Ostrowska-Kołodziejczak, A.; Załuski, D.; Perkowski, J. *trans*-Cinnamic and chlorogenic acids affect the secondary metabolic profiles and ergosterol biosynthesis by *Fusarium culmorum* and *F. graminearum* sensu stricto. *Toxins* **2017**, *9*, 198. [[CrossRef](#)] [[PubMed](#)]
30. Bilaska, K.; Stuper-Szablewska, K.; Kulik, T.; Buško, M.; Załuski, D.; Jurczak, S.; Perkowski, J. Changes in phenylpropanoid and trichothecene production by *Fusarium culmorum* and *F. graminearum* sensu stricto via exposure to flavonoids. *Toxins* **2018**, *10*, 110. [[CrossRef](#)] [[PubMed](#)]
31. Bollina, V.; Kushalappa, A.C.; Choo, T.M.; Dion, Y.; Rioux, S. Identification of metabolites related to mechanisms of resistance in barley against *Fusarium graminearum*, based on mass spectrometry. *Plant Mol. Biol.* **2011**, *77*, 355–370. [[CrossRef](#)] [[PubMed](#)]
32. Kumar, A.; Bachawat, A. Pyroglutamic acid: throwing light on a lightly studied metabolite. *Curr. Sci.* **2012**, *102*, 288–297.
33. Forman, H.J.; Zhang, H.; Rinna, A. Glutathione: Overview of its protective roles, measurement, and biosynthesis. *Mol. Aspects Med.* **2009**, *30*, 1–12. [[CrossRef](#)] [[PubMed](#)]
34. Huttunen, E.; Noro, K.; Yang, Z. Purification and identification of antimicrobial substances produced by two *Lactobacillus casei* strains. *Int. Dairy J.* **1995**, *5*, 503–513. [[CrossRef](#)]
35. Zhu, F.; Yuan, C.; Gang, F.; Yang, C.; Wu, W.; Zhang, J. Bioassay—guided isolation of antifungal compounds from *Disporopsis aspersa* (Hua) Engl. ex Diels against *Pseudoperonospora cubensis* and *Phytophthora infestans*. *Chem. Biodivers.* **2018**, *15*, e1800090. [[CrossRef](#)] [[PubMed](#)]
36. Van der Werf, P.; Orłowski, M.; Meister, A. Enzymatic conversion of 5-oxo-L-proline (L-pyrrolidone carboxylate) to L-glutamate coupled with cleavage of adenosine triphosphate to adenosine diphosphate, a reaction in the  $\gamma$ -glutamyl cycle. *Proc. Natl. Acad. Sci. USA* **1971**, *68*, 2982–2985. [[CrossRef](#)]
37. Yang, P.; Chen, Y.; Wu, H.; Fang, W.; Liang, Q.; Zheng, Y.; Olsson, S.; Zhang, D.; Zhou, J.; Wang, Z.; et al. The 5-oxoprolinase is required for conidiation, sexual reproduction, virulence and deoxynivalenol production of *Fusarium graminearum*. *Curr. Genet.* **2018**, *64*, 285–301. [[CrossRef](#)] [[PubMed](#)]
38. Atanasova-Penichon, V.; Barreau, C.; Richard-Forget, F. Antioxidant secondary metabolites in cereals: potential involvement in resistance to *Fusarium* and mycotoxin accumulation. *Front Microbiol.* **2016**, *7*, 566. [[CrossRef](#)] [[PubMed](#)]
39. Proctor, R.H.; Hohn, T.M.; McCormick, S.P.; Desjardins, A.E. *Tri6* encodes an unusual zinc finger protein involved in regulation of trichothecene biosynthesis in *Fusarium sporotrichioides*. *Appl. Environ. Microbiol.* **1995**, *61*, 1923–1930. [[PubMed](#)]
40. Hohn, T.M.; Krishna, R.; Proctor, R.H. Characterization of a transcriptional activator controlling trichothecene toxin biosynthesis. *Fungal Genet. Biol.* **1999**, *26*, 224–235. [[CrossRef](#)] [[PubMed](#)]
41. Tag, A.G.; Garifullina, G.F.; Peplow, A.W.; Ake, C., Jr.; Phillips, T.D.; Hohn, T.M.; Beremand, M.N. A novel regulatory gene, *Tri10*, controls trichothecene toxin production and gene expression. *Appl. Environ. Microbiol.* **2001**, *67*, 5294–5302. [[CrossRef](#)] [[PubMed](#)]
42. Kulik, T.; Abarenkov, K.; Buško, M.; Bilaska, K.; van Diepeningen, A.D.; Ostrowska-Kołodziejczak, A.; Krawczyk, K.; Brankovics, B.; Stenglein, S.; Sawicki, J.; Perkowski, J. ToxGen: An improved reference database for the identification of type B-trichothecene genotypes in *Fusarium*. *PeerJ* **2017**, *5*, e2992. [[CrossRef](#)] [[PubMed](#)]
43. Kim, D.O.; Lee, K.W.; Lee, H.J.; Lee, C.Y. Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *J. Agric. Food Chem.* **2002**, *50*, 3713–3717. [[CrossRef](#)] [[PubMed](#)]
44. Re, R.; Pellerini, N.; Proteggente, A.; Pannala, A.S.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [[CrossRef](#)]
45. Perkowski, J.; Kiecana, I.; Kaczmarek, Z. Natural occurrence and distribution of *Fusarium* toxins in 15 naturally-contaminated barley cultivars. *Eur. J. Plant. Pathol.* **2003**, *109*, 331–339. [[CrossRef](#)]

46. Kulik, T.; Łojko, M.; Jestoi, M.; Perkowski, J. Sublethal concentrations of azoles induce *Tri* transcript levels and trichothecene production in *Fusarium graminearum*. *FEMS Microbiol. Lett.* **2012**, *335*, 58–67. [[CrossRef](#)] [[PubMed](#)]
47. Kulik, T.; Buško, M.; Pszczółkowska, A.; Perkowski, J.; Okorski, A. Plant lignans inhibit growth and trichothecene biosynthesis in *Fusarium graminearum*. *Let. Appl. Microbiol.* **2014**, *59*, 99–107. [[CrossRef](#)] [[PubMed](#)]
48. Hohn, T.M.; Desjardins, A.E. Isolation and gene disruption of the *Tox5* gene encoding trichodiene synthase in *Gibberella pulicaris*. *Mol. Plant Microbe Interact.* **1992**, *5*, 249–256. [[CrossRef](#)] [[PubMed](#)]
49. Hohn, T.M.; Desjardins, A.E.; McCormick, S.P. Analysis of *Tox5* gene expression in *Gibberella pulicaris* strains with different trichothecene production phenotypes. *Appl. Environ. Microbiol.* **1993**, *59*, 2359–2363. [[PubMed](#)]
50. Hohn, T.M.; Desjardins, A.E.; McCormick, S.P. The *Tri4* gene of *Fusarium sporotrichioides* encodes a cytochrome P450 monooxygenase involved in trichothecene biosynthesis. *Mol. Gen. Genet.* **1995**, *248*, 95–102. [[CrossRef](#)] [[PubMed](#)]
51. McCormick, S.P.; Alexander, N.J.; Proctor, R.H. *Fusarium Tri4* encodes a multifunctional oxygenase required for trichothecene biosynthesis. *Can. J. Microbiol.* **2006**, *52*, 636–642. [[CrossRef](#)] [[PubMed](#)]
52. Tokai, T.; Koshino, H.; Takahashi-Ando, N.; Sato, M.; Fujimura, M.; Kimura, M. *Fusarium Tri4* encodes a key multifunctional cytochrome P450 monooxygenase for four consecutive oxygenation steps in trichothecene biosynthesis. *Biochem. Biophys. Res. Commun.* **2007**, *353*, 412–417. [[CrossRef](#)] [[PubMed](#)]
53. Pfaffl, M.W.; Horgan, G.W.; Dempfle, L. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **2002**, *30*, e36. [[CrossRef](#)] [[PubMed](#)]



© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).