Changes in Phenylpropanoid and Trichothecene Production by *Fusarium culmorum* and *F. graminearum* Sensu Stricto via Exposure to Flavonoids

Katarzyna Bińska 1,*, Kinga Stuper-Szablewska 2, Tomasz Kulik 1, Maciej Buśko 2, Dariusz Załuski 3, Sebastian Jurczak 1 and Juliusz Perkowski 2

1 Department of Microbiology and Mycology, University of Warmia and Mazury in Olsztyn, Oczapowskiego 1A, 10-719 Olsztyn, Poland; tomaszkulik76@gmail.com (T.K.); seba.jurczak@gmail.com (S.J.)
2 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.)
3 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.binska@uwm.edu.pl; Tel.: +48-895-233-834

Received: 13 February 2018; Accepted: 3 March 2018; Published: 5 March 2018

**Abstract:** Flavonoids are a group of hydroxylated polyphenolic compounds widely distributed in the plant kingdom. Biosynthesis of these compounds involves type III PKSs, whose presence has been recently predicted in some fungal species through genome sequencing efforts. In this study, for the first time it was found that Fusaria produce flavonoids on solid YES medium. Naringenin, as the central precursor of all flavonoids, was produced at highest quantities, followed by quercetin, kaempferol, apigenin and luteolin. In plants, flavonoids are involved in the protection of cereals to a wide range of stresses, including host defense against Fusaria. Under in vitro conditions, strains of *Fusarium culmorum* and *F. graminearum* sensu stricto were incubated at levels of flavonoids close to amounts produced by cereals in response to fungal infection. The amounts of exogenous naringenin, apigenin, luteolin, kaempferol and quercetin were reduced and converted by fungi to the other flavonoid derivatives. Treatment of fungi with naringenin derivatives led to the inhibition of naringenin production. Correspondingly, the production of fungal-derived phenolic acids decreased in flavonoid treated samples, although this effect appeared to be dependent on the strain, flavonoid molecule and its concentration. Fusaria showed high variability in trichothecene production in response to flavonoids. With emphasis on quercetin, mycotoxin accumulation in the media was significantly decreased by luteolin, kaempferol, naringenin and apigenin. However, in some cases, apigenin led to the increase of mycotoxin content in the media. Gene expression experiments of *Tri* genes responsible for trichothecene biosynthesis (*Tri4*, *Tri5* and *Tri10*) proved that the inhibition of mycotoxin production by flavonoids occurred at the transcriptional level. However, the changes in *Tri* transcript levels were not significant in most apigenin and all kaempferol-treated cultures. In this study, a link was established between antioxidant and antiradical properties of flavonoids and their effects on fungi.

**Keywords:** *Fusarium*; flavonoids; naringenin; quercetin; kaempferol; apigenin; luteolin; trichothecenes

**Key Contribution:** Fusaria showed high variability in trichothecene production in response to flavonoids. Fusaria produce flavonoids on solid YES medium.
1. Introduction

The genus *Fusarium* encompasses a wide diversity of fungal pathogens of high agricultural, economic and medical importance [1]. In cereal production, Fusaria cause severe diseases associated with significant yield losses, including root and crown rot, Fusarium head blight (FHB) in barley and wheat and Fusarium ear rot (FER) in maize. In addition to reducing yields, Fusaria also cause quality deterioration by contamination of the grain with mycotoxins such as trichothecenes, which are a major health concern for humans and animals [2].

Synthetic fungicides, principally from theazole class, play a prominent role in controlling Fusaria in the field. However, the efficacy of azoles for disease control often remains contradictory, presumably due to the difficulty of timing and targeting fungicides to heads [3] and the apparently increased resistance of fungi to this fungicide class [4]. In addition, azole residues can disperse and persist in the environment due to repeated use of these fungicides [5–7], which have a considerable impact on ecosystem health and functionality [8].

The risk of human health and the environment has been lately of significant concern. In order to face it the intensive research into development of novel and environmentally friendly disease management strategies has been proposed [9]. Previous studies have indicated that plant-derived phenylpropanoids may represent a break-through in the protection of crops against fungal pathogens [10,11]. This group of plant secondary metabolites is synthesized through the phenylpropanoid pathway and can be divided into two groups: Flavonoid phenylpropanoids (flavones, flavonols, flavanols, and flavanones) and non-flavonoid phenylpropanoids (stilbenes, lignans and phenolic acids) [12]. The first group encompasses nearly one hundred putative flavonoids which have been identified through metabolomic approaches to play a role in resistance mechanisms against Fusaria. Many of these metabolites correspond to glucoside derivatives of kaempferol and quercetin belonging to the flavonol class. In addition, several compounds of the flavanol, flavanone (naringenin), flavone (apigenin and vitexin derivatives) and anthocyanin classes were also highlighted [13].

The role of flavonoids in plant defense has been previously reviewed in Treutter [14,15], Mierziak et al. [16], Gauthier et al. [13] and Atanasova-Penichon et al. [12]. Most of these compounds are bioactive and contribute to defense mechanisms against abiotic and biotic stresses, environmental interactions [17,18] and play an important role in physiological regulations and signaling [19]. While the production of these secondary metabolites has been well documented in plants, much less is known about the biosynthesis of these compounds by fungi including plant pathogenic species [20].

Biosynthesis of flavonoids involves diverse enzymes with specialized activities [21,22]. The first committed step of this pathway is catalyzed by chalcone synthase, belonging to well-studied plant type III PKS. The process begins with the sequential condensation of three acetate units from malonyl-CoA into a 4-coumaroyl-CoA molecule. Subsequently, cyclization reaction leads to the formation of an aromatic tetraketide, naringenin chalcone [23] (Figure 1). Although type III PKSs are widely studied in plants, their presence in some fungal species has mainly been predicted through recent fungal genome sequencing efforts [23,24]. Limited gene expression studies supported the functionality of the putative type III PKSs in *Aspergillus oryzae* [25]. Hypothetical chalcone synthase of *F. graminearum* reference PH-1 strain (NRRL 31084) is available in the GenBank database [26] under accession number XP_011320399, suggesting that the production of flavonoids can be widespread in the genus *Fusarium*.

The main mechanism of action of flavonoids results from their antioxidant properties [27–30], owing to prevention or quenching of reactive oxygen species (ROS), generated by both the pathogen and the plant during infection. The other roles of flavonoids in plant defense are associated with reinforcement of cell walls restricting pathogen access to nutrients [14,15] and the inhibition of the activity of plant cell wall degrading enzymes secreted by fungi [14,15]. Limited in vitro studies showed that flavonoids display molecule-dependent growth inhibitory effects on fungi [31,32], including *Fusarium culmorum* and *F. graminearum* sensu stricto (s.s.) [33–35]. However, their growth inhibitory effect on Fusaria appears to be weaker than phenolic acids [12].
We found that besides phenolic acids, YES + fungal controls exhibited increased accumulation of Toxins 2018 was quantified at the highest levels (comprising 25.8–28.4% of the flavonoids produced by Fusaria).

A more recent study by Bollina and Kushalappa [35] showed that other flavonoids (naringenin and (the first chemical intermediate in trichothecene biosynthesis) to oxygenated trichothecenes [13].

Limited studies also indicated that flavonoids appear to interact directly with fungal secondary metabolism. Flavonoids have been shown to exert an inhibitory effect on mycotoxin production by fungi such as aflatoxin [36,37] and patulin production [38]. Inhibition of trichothecene biosynthesis by flavonoids was revealed by Desjardins et al. [39] and Takahashi-Ando et al. [40], who showed an inhibitory effect of flavones on cytochrome P450 monoxygenase-catalyzing conversion of trichodiene (the first chemical intermediate in trichothecene biosynthesis) to oxygenated trichothecenes [13]. A more recent study by Bollina and Kushalappa [35] showed that other flavonoids (naringenin and quercetin) completely suppress trichothecene production of a single F. graminearum strain at early stages of incubation on artificial media.

The main purpose of our research was to study the changes in phenylpropanoid (flavonoid and phenolic acid) and trichothece profiles via exposure to flavonoids. To achieve it, different strains of F. culmorum and F. graminearum were incubated in the presence of different flavonoid aglycones: naringenin, apigenin, luteolin, kaempferol and quercetin. These compounds were selected because of their contribution to resistance to Fusaria in cereals [34,41,42] and their well-known antioxidant properties [29,30]. Concentrations of flavonoids close to the amounts quantified in kernels after Fusarium infection were also used [43]. The effect of treatment with these compounds was assessed on fungal secondary metabolic profiles. It was found that exogenous flavonoids affected the phenylpropanoid profiles of fungi. Production of flavonoids by Fusaria was also revealed here for the first time. The impact of exogenous flavonoids on both trichothecene accumulation in the media and the expression of Tri genes involved in trichothecene biosynthesis were also analyzed. An attempt was made to establish a link between the antioxidant and antiradical properties of flavonoids and their effects on fungi.

2. Results and Discussion

2.1. Production of Flavonoids by Fusaria

Previous in vitro studies showed that different strains of F. culmorum and F. graminearum s.s. accumulate phenolic acids in the media [44,45]. In this study, we screened fungi for flavonoid production. We found that besides phenolic acids, YES + fungal controls exhibited increased accumulation of flavonoids (Supplementary File S1). Naringenin, as the central precursor of the other flavonoids [46], was quantified at the highest levels (comprising 25.8–28.4% of the flavonoids produced by Fusaria). In plants, naringenin can be converted to apigenin by flavone synthase [47] (Figure 1). Apigenin can be
further hydroxylated by flavonoid 3′ hydroxylase to form luteolin [48]. The presence of both flavones has been detected in fungal cultures. Apigenin comprised 15.9–17.9%, while luteolin reached 9.5–12.2% of flavonoids. Besides flavones, Fusaria produced flavonols (kaempferol and quercetin), which were quantified at higher levels than the first of the aforementioned class of flavonoids. Kaempferol, which is converted from naringenin by flavonol synthase [22], reached 20.7–22.2%. Quercetin, which is synthesized from kaempferol, yielded 21.9–25.3% of flavonoids. In plants, the formation of quercetin involved cytochrome P450 flavonoid monooxygenase associated to a cytochrome P450 reductase, which utilizes O2 and NADPH [49,50].

2.2. Reduction of Exogenous Flavonoids by Fusaria

The amounts of exogenous flavonoids (400 and 800 µg/g) were significantly reduced in fungal treated cultures, as compared to uninoculated YES + flavonoid controls (Supplementary File S1). This decrease was more evident in samples treated with increased levels (800 µg/g) of flavonoids. Naringenin, the precursor of all flavonoids was efficiently converted by fungal strains to apigenin, luteolin, kaempferol and quercetin. Similar results were obtained by Leonard et al. [49], who found that exogenous supplementation of naringenin increase kaempferol and quercetin amounts in culture media of Escherichia coli. The absence of naringenin, as the first precursor of flavonoid biosynthesis pathway in flavone and flavonol treated samples, may indicate inhibition of chalcone synthase activity by these compounds. Treatment of fungi with apigenin led to an increase of luteolin, kaempferol and quercetin in the media, indicating its further efficient conversion. Corresponding results have been found in samples treated with luteolin and kaempferol. However, it was found that besides the formation of the products resulting from flavonoid conversion, fungi treated with luteolin, kaempferol and quercetin accumulated increased levels of their parent metabolites. Luteolin-treated samples exhibited increased levels of apigenin, while in kaempferol-treated samples increased amounts of both flavones (luteolin and apigenin) were found. Unexpected formation of flavones and kaempferol was also evident in quercetin-treated samples. The formation of the intermediate parent metabolites in the presence of exogenous flavones and flavonols remains largely unclear. One can hypothesize that the increase of the parent intermediates could result from insufficient conversion of fungal-derived apigenin, luteolin and kaempferol, which could be produced by fungi at different stages of incubation. On the other hand, the absence of fungal-derived naringenin does not suggest that the production of these compounds occurred in the presence of flavones and flavonols.

2.3. Exogenous Flavonoids Affect Production of Phenolic Acids by Fusaria

The sum of fungal-derived phenolic acids decreased in flavonoid treated samples, however, this effect appeared to be dependent on the strain, flavonoid molecule and its concentration (Supplementary File S1). Among studied phenolic acids, chlorogenic and sinapic acid did not accumulate in the presence of flavonoids, while p-coumaric, caffeic, ferulic and syringic acid could be detected in samples treated with naringenin and apigenin. Higher diversity and quantity of phenolic acids in naringenin and apigenin-treated samples could be explained by their relatively lower antioxidant activities (Table 1). Interestingly, the accumulation of trans-cinnamic acid in luteolin, kaempferol and quercetin-treated samples suggests their inhibitory effect on converting this first intermediate of the shikimate pathway further.
Table 1. A summary of the structures and characteristics of the studied flavonoids.

| Subclass | Compound | Substitution at Carbon Position | Double Bond C2-C3 | Antioxidant Activity | Antiradical Activity ² |
|----------|----------|---------------------------------|-------------------|---------------------|------------------------|
|          |          |                                 |                   | VCEAC/L             | ABTS (µM TROLOX/100 g) | Protection Factor ¹ (%) |
|          |          | 3' 5 7 3' 4'                    |                   |                     |                        |                        |
| Flavones | Apigenin  | - OH OH - OH                   | +                 | 190.4               | 297.5                  | 0.99                   | 0.7                    |
|          | Luteolin  | - OH OH OH OH OH              | +                 | 483.5               | 589.7                  | 4.24                   | n.t.                   |
| Flavonols| Kaempferol| OH OH OH - OH                 | +                 | 450.7               | 540.3                  | 2.49                   | 93.5                   |
|          | Quercetin | OH OH OH OH OH OH            | +                 | 692.5               | 744.9                  | 11.50                  | 89.8                   |
| Flavanones| Naringenin| - OH OH - OH                 | -                 | 301.7               | 529.4                  | 1.09                   | 6.3                    |

¹—Antioxidant activity of flavonoids measured in lard oil by a Rancimat test (from Yang et al. [51]). ²—Antiradical activities of flavonoids (3.3 × 10⁻⁵ M) in a methanol solution of DPPH (1.6 × 10⁻⁵ M) (from Burda and Oleszek [52]).

2.4. Effect of Flavonoids on Trichothecene Accumulation in the Media

The effect of flavonoids on mycotoxin production was evaluated by quantification of trichothecenes in treated versus non-treated plates (YES + fungal controls). As typical for solid media [53], the strains co-produced trichothecene compounds at different levels. In addition, all strains tested co-produced lower amounts of trichothecenes not characteristic for their chemotypes (Supplementary File S2). The decrease in mycotoxin accumulation was revealed in most flavonoid-treated plates. However, this effect appears to be largely dependent on the strain, the flavonoid compound and its concentration and appears to be dependent on the antioxidant and antiradical capacity of the assayed flavonoid. The most dramatic reduction of trichothecene accumulation was reported for quercetin. This flavonoid showed the highest antioxidant and antiradical properties compared to the remaining flavonoids (Table 1). The reduction of trichothecene accumulation by quercetin ranged from 78.2 to 99.8%. A lower reduction of mycotoxin accumulation was found in plates treated by both luteolin and kaempferol. The former compound shows slightly higher antioxidant activity than the latter (Table 1). Luteolin caused 49.6 to 99.8% reduction of mycotoxin accumulation, while the inhibitory effect of kaempferol ranged from 24.2 to 99%.

Naringenin displayed reduction of trichothecene accumulation ranging from 40.9 to 99.7%, except of one strain (MUCL 53469), where this reduction ranged from 10.2 to 40.3%. The inhibiting effect of naringenin is close to results obtained for luteolin, which is rather unexpected when comparing their antioxidant properties (Table 1). It is hypothesized that a high reduction of mycotoxin content by naringenin might result from early conversion of naringenin to luteolin, kaempferol and quercetin by fungi (Supplementary File S1), which more effectively interfere with mycotoxin production. However, high reduction of mycotoxin content by naringenin may be explained by increased growth inhibition reported during the early stage of incubation of fungi on the media (Supplementary File S2). The initial stage of fungal incubation appears to be critical for the total mycotoxin accumulation in the media due to the early activation and high expression of Tri genes in the first days of fungal incubation [54]. Changes in growth rate could also partially explain the observed weak inhibition of mycotoxin production in apigenin treated plates. It was found that fungal growth was significantly stimulated in 3 of 12 samples treated with apigenin. In 2 of 3 growth-stimulated samples (CBS 173.31 treated with 400 and 800 µg/g) a 13.7 to 289.4% increase in the sum of trichothecenes was found, as compared to YES + fungal control.

It is intriguing that two structurally similar compounds (apigenin and naringenin) exhibited quite opposite effects on fungal growth. Apigenin derives from naringenin and differs from the latter by forming a double bond in ring C of the flavonoid structure [47,48] (Table 1). To date, information from literature on mechanisms underlying different biological activities of these flavonoids has been mainly limited to describing their action on human targets [55]. Studies of Zhang et al. [56] showed that this single bond is responsible for large differences in binding affinities of these two flavonoid compounds. It has been demonstrated that single bond rendered naringenin is more flexible to bind to human serum transferrin (Tf) (a single-chain iron-binding blood plasma glycoprotein) [56]. It is hypothesized that the increased binding affinity of naringenin to specific fungal targets may explain its observed
higher growth inhibitory effect on fungi. The link between fungal growth changes and mycotoxin accumulation could not be confirmed based on luteolin and kaempferol treated cultures where, in all 7 growth-stimulated samples, a decrease in mycotoxin was reported. The weakest inhibiting effect of apigenin on mycotoxin production is, however, in line with its lowest antioxidant and antiradical properties as compared to other flavonoids tested (Table 1).

2.5. Effect of Flavonoids on the Expression of Tri Genes

The effect of flavonoids on mycotoxin production was tested at the transcriptional level by quantification of Tri4, Tri5 and Tri10 transcripts on day 3 of fungal incubation. Gene expression results are expressed as the relative transcript levels (Supplementary File S2).

Desjardins et al. [39] and Takahashi-Ando et al. [40] provided evidence that flavones are inhibitors of cytochrome P450 monooxygenase responsible for the oxygenations of trichodiene at early stages of trichothecene biosynthesis. P450 monooxygenase is encoded by Tri4, whose expression, as shown in this study, was drastically reduced in 29 of 60 flavonoid-treated samples. The current results also showed that flavonoids not only inhibit expression of Tri4, but also Tri5 gene encoding trichodiene synthase, a terpene cyclase which converts farnesyl pyrophosphate to trichodiene, the hydrocarbon precursor of trichothecenes [57]. Inhibition of Tri5 expression might be explained by suppressed expression of the Tri10 gene, which coordinates expression of four trichothecene pathway-specific genes (Tri4, Tri5, Tri6, and Tri101) and the isoprenoid biosynthetic gene for farnesyl pyrophosphate synthetase (FPPS) [58]. However, it should be noted that inhibition of Tri5 activity occurred in most, but not all, samples with reduced expression of Tri10 (Supplementary File S2). Except for kaempferol treated samples, the effect of flavonoids on Tri gene expression corresponds to the observed differences in mycotoxin profiles of the studied samples.

Among the tested flavonoids, quercetin (with the relatively highest antioxidant and antiradical properties) showed the strongest decrease of Tri transcript levels, in some cases leading to the complete inhibition of Tri gene expression. Both luteolin and naringenin showed strong inhibitory effect on Tri gene activity, although their effects were largely dependent on the strain assayed. Among the tested compounds, apigenin exhibiting the lowest antioxidant and antiradical properties displayed the weakest inhibitory effect on mycotoxin production at a transcriptional level. Significant inhibition of Tri gene expression was found in only 4 of 12 apigenin-treated samples (Supplementary File S2).

Surprisingly, the effect of kaempferol on Tri gene expression was not evident for all studied samples, which is in contrast with its high reduction of trichothecene accumulation in the media (Supplementary File S2). The above result may suggest a different mechanism of action of this compound. Indeed, in contrast to the remaining compounds, kaempferol, has been found to be a more potent hydroxyl than the superoxide radical scavenger [59]. It is hypothesized that the pronounced ability of kaempferol to decrease hydroxyl radical concentration may play a key role in its different activity towards fungal targets. The diverse mechanisms of action of flavonoids have been previously highlighted in Treutter [14,15], who suggested that these compounds might target different components and functions of fungal cells. Such targeting, including binding to enzymes and selected proteins and/or chelation of metals necessary for enzyme activity, as shown in this study, dramatically affects mycotoxin production by fungi with no significant impact on the activity of Tri genes.

In this study, it was found for the first time that flavonoids display variable effect on fungal secondary metabolic profiles. The observed high variability is in line with previous reports demonstrating variable effects of other plant resistance related compounds such as phenolic acids [44,45] and lignans [60] on this group of toxigenic fungi. Notably, the in vitro results presented in this paper are in line with recent in planta studies demonstrating that resistance to Fusarium diseases is closely associated with the promoted biosynthesis of phenylpropanoids, including flavonoids [33,43,61,62]. It is believed that the results obtained in this study may be significant in developing novel strategies to limit mycotoxins contamination of food and feed.
3. Materials and Methods

3.1. Fungal Strains

The six fungal strains used in this study (Table 2) are maintained in 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. Detailed characteristics of the fungal strains are provided in the ToxGen database [63].

Table 2. List of fungal isolates used in this study.

| Species            | Strain          | Trichothecene Genotype | Origin, Host and Year of Isolation |
|--------------------|-----------------|------------------------|------------------------------------|
| F. culmorum        | CBS 173.31, NRRL 26853 | 3ADON                  | Canada, oat, 1927                   |
|                    | MUCL 53469      | 3ADON                  | Belgium, corn, 2007                 |
|                    | CBS 139512      | NIV                    | Poland, wheat, 2003                 |
| F. graminearum s.s.| CBS 119173, NRRL 38369 | 3ADON                  | USA, Louisiana, wheat, 2005         |
|                    | CBS 138561      | 15ADON                 | Poland, wheat, 2010                 |
|                    | MUCL 53455      | NIV                    | Belgium, corn, 2007                 |

3.2. Medium and Culture Conditions

Flavonoid aglycones (Sigma–Aldrich, Saint Louis, MO, USA): Naringenin, apigenin, luteolin, kaempferol and quercetin were dissolved in 10 mL of 96% ethanol and then added to the YES medium to obtain the final concentrations: 400 µg/g and 800 µg/g. The flavonoid concentrations used in this study are close to the amounts produced by plants in the response of Fusarium infection [43]. The flavonoids used in this study exhibited different antioxidant and antiradical capacities, which are apparently defined by their structural characteristics [52] (Table 1).

The study incorporated three different controls: YES-only control (YES medium only), YES + flavonoid controls (YES medium supplemented with either 400 or 800 µg/g of flavonoids) and six YES + fungal controls (fungal strains incubated on YES media). The control samples were supplemented with an identical volume of 96% ethanol. Petri plates (Ø 80 mm) were inoculated from 6–8-week old laboratory stock cultures maintained at 4 °C on PDA slants and incubated at 25 °C (in triplicate) in the dark. For gene expression analysis, plates were incubated for 3 days for each condition, while for chemical analysis (mycotoxin, flavonoid and phenolic acid determination) the plates were incubated for 21 days. Growth inhibition tests were performed at an early stage of the incubation of fungi on PDA medium (till the 6th day after inoculation) as previously described by Ponts et al. [64] and Kulik et al. [60].

3.3. Determination of Flavonoids and Phenolic Acids in the Medium

0.20 g samples for analyses were weighed and placed in sealed 17 mL culture test tubes, where alkaline and acid hydrolysis were conducted. To conduct alkaline hydrolysis, 1 mL of distilled water and 4 mL 2 M aqueous sodium hydroxide was added to the test tubes. Tightly-sealed test tubes were heated in a water bath at 95 °C for 30 min. After cooling (approx. 20 min), the test tubes were neutralized with 2 mL 6 M aqueous hydrochloric acid solution (pH = 2). The samples were then cooled in water with ice. Flavonoids were extracted from the inorganic phase using diethyl ether (2 × 2 mL). The formed ether extracts were continuously transferred to 8 mL vials and acid hydrolysis was then conducted. For this purpose, the aqueous phase was supplemented with a 3 mL 6 M aqueous hydrochloric acid solution. Tightly-sealed test tubes were heated in a water bath at 95 °C for 30 min. After being cooled in water with ice, the samples were extracted with diethyl ether (2 × 2 mL). The produced ether extracts were continuously transferred to 8 mL vials, after which they were evaporated to dryness in a stream of nitrogen. The samples were dissolved in 1 mL of
methanol prior to analyses. The analysis was performed using an Aquity H class UPLC system equipped with an Waters Acquity PDA detector (Waters, Milford, MA, USA). Chromatographic separation was performed on an Acquity UPLC® BEH C18 column (100 mm × 2.1 mm, particle size 1.7 µm) (Waters, Milford, MA, USA). The elution was carried out in a gradient using the following mobile phase composition: A: acetonitrile with 0.1% formic acid, B: 1% aqueous formic acid mixture (pH = 2). The concentrations of flavonoids were determined using an internal standard at wavelengths λ = 320 nm. Compounds were identified based on a comparison of retention time of the analyzed peak with the retention time of the standard and by adding a specific amount of the standard to the analyzed samples and a repeated analysis. The detection level was 1 µg/g. The retention times of the assayed acids are as follows: kaempferol 6.11 min, luteolin 11.89 min, apigenin 16.43 min, chlorogenic acid 21.56 min, caffeic acid 26.19 min, syringic acid 28.05 min, naringenin 31.22 min, quercetin 39.58 min, p-coumaric acid 40.20 min, ferulic acid 46.20 min, sinapic acid 48.00 min and trans-cinnamic acid 52.40 min, respectively. Fungal-derived phenolic compounds were determined in dried YES-only control, YES + flavonoid controls, six YES + fungal controls and treated fungal cultures after a 21-day incubation period (Supplementary File S1) as previously described in Kulik et al. [45].

3.4. Determination of the Antioxidant Capacity (VCEAC/L) and Radical Scavenging Activity (ABTS⁺) of Flavonoids

VCEAC/L and ABTS⁺ assays of flavonoids were performed as previously described by Kim et al. [65] and Re et al. [66], respectively. For ABTS⁺ generation from ABTS salt, 3 mM of K₂S₂O₈ was reacted with 8 mM ABTS salt in distilled, deionized water for 16 h at room temperature in the dark. The ABTS⁺ solution was then diluted with a pH 7.4 phosphate buffer solution containing 150 mM NaCl (PBS) to obtain an initial absorbance of 1.5 at 730 nm. Fresh ABTS⁺ solution was prepared for each analysis. The reaction kinetics were determined over a 2 h period with readings every 15 min. Reactions were complete in 30 min. Samples and standards (100 µm) were reacted with the ABTS⁺ solution (2900 µm) for 30 min. Trolox was used as a standard. The results were also expressed as mg Vitamin C equivalent antioxidant capacity per liter (mg VCEAC/L).

3.5. Analysis of Trichothecenes from Fungal Cultures

Trichothecenes were determined in fungal cultures treated and non-treated (YES + fungal controls) with different flavonoids by GC-MS as previously described by Perkowski et al. [67].

3.6. Extraction of Total RNA and Preparation of cDNA

The total RNA was extracted from 3-day-old fungal cultures from mycelium grown on YES medium treated and non-treated (YES + fungal controls) with flavonoids. Six biological replications were prepared for each condition. Extraction of RNA and reverse-transcription were performed as previously described in Kulik et al. [60,68]. cDNA samples were stored at −25 °C for transcript quantification.

3.7. Gene Expression Analysis

Tri4 and Tri5 genes which are responsible for the initial stage in the trichothecene biosynthetic pathway were chosen for qPCR analysis, as previously described in Kulik et al. [60,68]. The analysis also included the Tri10 gene responsible for regulation of multiple Tri genes [58]. Gene expression analyses were performed as previously described in Kulik et al. [44,45]. The Ct values of the target Tri4, Tri5, Tri10 and reference Ef1α gene were compared in the control and treated samples and normalized relative to the Ct values obtained for the reference EF1α gene using the REST 2009 software [69].

3.8. Statistical Analyses

The significance of differences among quantities of flavonoids, phenolic acids and mycotoxins and fungal growth was tested using t-Student test at p < 0.05. The t-Student test was also used to evaluate differences in the growth of fungi in flavonoid-treated plates.
Supplementary Materials: The following are available online at www.mdpi.com/2072-6651/10/3/110/s1. Supplementary File S1. Flavonoid and phenolic acid accumulation by fungal strains after 21 days of incubation of fungi in the presence of exogenous flavonoids. Supplementary File S2. RQ (relative quantification) of Tri4, Tri5 and Tri10 transcripts and trichothecene accumulation by fungal strains incubated in the response of different flavonoids.

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

Author Contributions: T.K. planned and designed the research. K.B., K.S.-S., T.K., M.B., D.Z., S.J. and J.P. performed the experiments, conducted fieldwork, and analyzed data, etc. K.B., K.S.-S. and T.K. wrote the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Karlsson, I.; Friberg, H.; Kolseth, A.-K.; Steinberg, C.; Persson, P. Agricultural factors affecting Fusarium communities in wheat kernels. Int. J. Food Microbiol. 2017, 252, 53–60. [CrossRef] [PubMed]
2. Desjardins, A.E. Fusarium Mycotoxins Chemistry. Genetics and Biology; American Phytopathological Society Press: Saint Paul, MN, USA, 2006; ISBN 0-89-54-335-6.
3. Trail, F. For Blighted Waves of Grain: Fusarium graminearum in the Postgenomics Era. Plant Physiol. 2009, 149, 103–110. [CrossRef] [PubMed]
4. Mesterházy, Á.; Tóth, B.; Varga, M.; Bartók, T.; Szabó-Hevér, Á.; Farády, L.; Lehoczki-Krsjak, S. Role of fungicides, application of nozzle types, and the resistance level of wheat varieties in the control of Fusarium head blight and deoxynivalenol. Toxins 2011, 3, 1453–1483. [CrossRef] [PubMed]
5. Kahle, M.; Buerge, I.J.; Hauser, A.; Müller, M.D.; Poiger, T. Azole Fungicides: Occurrence and Fate in Wastewater and Surface Waters. Environ. Sci. Technol. 2008, 42, 7193–7200. [CrossRef] [PubMed]
6. Battaglin, W.A.; Sandstrom, M.W.; Kuivila, K.M.; Kolpin, D.W.; Meyer, M.T. Occurrence of azoxystrobin, propiconazole, and selected other fungicides in US streams, 2005–2006. Water Air Soil Pollut. 2011, 218, 307–322. [CrossRef]
7. Bollmann, U.E.; Tang, C.; Eriksson, E.; Jönsson, K.; Vollertsen, J.; Bester, K. Biocides in urban wastewater treatment plant influent at dry and wet weather: Concentrations, mass flows and possible sources. Water Res. 2014, 60, 64–74. [CrossRef] [PubMed]
8. Álvarez-Pérez, S.; de Vega, C.; Pozo, M.I.; Lenaerts, M.; Van Assche, A.; Herrera, C.M.; Jacquemyn, H.; Lievens, B. Nectar yeasts of the Metschnikowia clade are highly susceptible to azole antifungals widely used in medicine and agriculture. FEMS Yeast Res. 2016, 16, 115. [CrossRef] [PubMed]
9. 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]
10. Boutigny, A.-L.; Richard-Forget, F.; Barreau, C. Natural mechanisms for cereal resistance to the accumulation of Fusarium trichothecenes. Eur. J. Plant Pathol. 2008, 121, 411–423. [CrossRef]
11. 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]
12. 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]
13. Gauthier, L.; Atanasova-Penichon, V.; Chéreau, S.; Richard-Forget, F. Metabolomics to decipher the chemical defense of cereals against Fusarium graminearum and deoxynivalenol accumulation. Int. J. Mol. Sci. 2015, 16, 24839–24872. [CrossRef] [PubMed]
14. Treutter, D. Significance of flavonoids in plant resistance and enhancement of their biosynthesis. Plant Biol. 2005, 7, 581–591. [CrossRef] [PubMed]
15. Treutter, D. Significance of flavonoids in plant resistance: A review. Environ. Chem. Lett. 2006, 4, 147–157. [CrossRef]
16. Mierziak, J.; Kostyn, K.; Kulma, A. Flavonoids as important molecules of plant interactions with the environment. Molecules 2014, 19, 16240–16265. [CrossRef] [PubMed]
17.Hamberger, B.; Ehling, J.; Barbazuk, B.; Douglas, C.J. Comparative genomics of the shikimate pathway in Arabidopsis, Populus trichocarpa and Oryza sativa: Shikimate pathway gene family structure and identification of candidates for missing links in phenylalanine biosynthesis. In Recent Advances in Phytochemistry. Volume 40. Integrative Plant Biochemistry. Romeo, J.T., Ed.; Elsevier Ltd.: Oxford, UK, 2006; pp. 85–113. ISBN 9780080451251.

18. Maeda, H.; Dudareva, N. The shikimate pathway and aromatic Amino acid biosynthesis in plants. Annu. Rev. Plant Biol. 2012, 63, 73–105. [CrossRef] [PubMed]

19. Tohge, T.; Watanabe, M.; Hoeftgen, R.; Fernie, A.R. Shikimate and Phenylalanine Biosynthesis in the Green Lineage. Front. Plant Sci. 2013, 4, 62. [CrossRef] [PubMed]

20. Hyun, M.W.; Yun, Y.H.; Kim, J.Y.; Kim, S.H. Fungal and Plant Phenylalanine Ammonia-lyase. Mycobiology 2011, 39, 257–265. [CrossRef] [PubMed]

21. Vogt, T. Phenylpropanoid Biosynthesis. Mol. Plant 2010, 3, 2–20. [CrossRef] [PubMed]

22. Kim, B.G.; Joe, E.J.; Ahn, J.H. Molecular characterization of flavonol synthase from poplar and its application to the synthesis of 3-O-methylkaempferol. Biotechnol. Lett. 2010, 32, 579–584. [CrossRef] [PubMed]

23. Yu, D.; Xu, F.; Zeng, J.; Zhan, J. Type III polycrystalline synthases in natural product biosynthesis. IUBMB Life 2012, 64, 285–295. [CrossRef] [PubMed]

24. Juvvadi, P.R.; Seshime, Y.; Kitamoto, K. Genomics reveals traces of fungal phenylpropanoid-flavonoid metabolic pathway in the filamentous fungus Aspergillus oryzae. J. Microbiol. 2005, 43, 475–486. [PubMed]

25. Seshime, Y.; Juvvadi, P.R.; Fujii, I.; Kitamoto, K. Discovery of a novel superfamily of type III polycrystalline synthases in Aspergillus oryzae. Biochem. Biophys. Res. Commun. 2005, 331, 253–260. [CrossRef] [PubMed]

26. Cuomo, C.A.; Guldener, U.; Xu, J.R.; Trail, F.; Turgeon, B.G.; Di Pietro, A.; Walton, J.D.; Ma, L.J.; Baker, S.E.; Rep, M.; et al. The Fusarium graminearum genome reveals a link between localized polymorphism and pathogen specialization. Science 2007, 317, 1400–1402. [CrossRef] [PubMed]

27. Pietta, P.G. Flavonoids as antioxidants. J. Nat. Prod. 2000, 63, 1035–1042. [CrossRef] [PubMed]

28. Rice-Evans, C. Flavonoid antioxidants. Curr. Med. Chem. 2001, 8, 797–807. [CrossRef] [PubMed]

29. Hernandez, I.; Alegre, L.; van Breusegem, F.; Munne-Bosch, S. How relevant are flavonoids as antioxidants in plants? Trends Plant Sci. 2009, 14, 125–132. [CrossRef] [PubMed]

30. Agati, G.; Azzarello, E.; Pollastri, S.; Tattini, M. Flavonoids as antioxidants in plants: Location and functional significance. Plant Sci. 2012, 196, 67–76. [CrossRef] [PubMed]

31. Padmavathi, M.; Sakhthivel, N.; Thara, K.V.; Reddy, A.R. Differential sensitivity of rice pathogens to growth inhibition by flavonoids. Phytochemistry 1997, 46, 499–502. [CrossRef]

32. Parvez, M.M.; Tomita-Yokotani, K.; Fujii, Y.; Konishi, T.; Iwashina, T. Effects of quercetin and its seven derivatives on the growth of Arabidopsis thaliana and Neurospora crassa. Biochem. Syst. Ecol. 2004, 32, 631–635. [CrossRef]

33. Silva, A.M.S.; Weidenbörner, M.; Cavaleiro, J.A.S. Growth control of different Fusarium species by selected flavonoids and flavonoid mixtures. Mycol. Res. 1998, 102, 638–640. [CrossRef]

34. Bollina, V.; Kumarswamy, 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]

35. Bollina, V.; Kushalappa, A.C. In vitro inhibition of trichothecene biosynthesis in Fusarium graminearum by resistance-related endogenous metabolites identified in barley. Mycology 2011, 2, 291–296. [CrossRef]

36. Chitarrini, G.; Nobili, C.; Pinzari, F.; Antonini, A.; de Rossi, P.; del Fiore, A.; Procacci, S.; Tolaini, V.; Scala, V.; Scarpiti, M.; et al. Buckwheat achenes antioxidant profile modulates Aspergillus flavus growth and aflatoxin production. Int. J. Food Microbiol. 2014, 189, 1–10. [CrossRef] [PubMed]

37. Norton, R.A. Inhibition of aflatoxin B1 biosynthesis in Aspergillus flavus by anthocyanidins and related flavonoids. J. Agric. Food Chem. 1999, 47, 1230–1235. [CrossRef] [PubMed]

38. Salas, M.P.; Reynoso, C.M.; Celiz, G.; Daz, M.; Resnik, S.L. Efficacy of flavonones obtained from citrus residues to prevent patulin contamination. Food Res. Int. 2012, 48, 930–934. [CrossRef]

39. Desjardins, A.E.; Plattner, R.D.; Spencer, G.F. Inhibition of trichothecene toxin biosynthesis by naturally-occurring shikimate aromatics. Phytochemistry 1988, 27, 767–771. [CrossRef]

40. Takahashi-Ando, N.; Ochiai, N.; Tokai, T.; Ohsato, S.; Nishiuchi, T.; Yoshida, M.; Fujimura, M.; Kimura, M. A screening system for inhibitors of trichothecene biosynthesis: Hydroxylation of trichodiene as a target. Biotechnol. Lett. 2008, 30, 1055. [CrossRef] [PubMed]
Toxins 2018, 10, 110

41. 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]

42. Gunnaiah, R.; Kushalappa, A.C.; Duggavathi, R.; Fox, S.; Somers, D.J. Integrated metabolo-proteomic approach to decipher the mechanisms by which wheat QTL (Fhb1) contributes to resistance against Fusarium graminearum. *PLoS ONE* 2012, 7, e40695. [CrossRef] [PubMed]

43. Bu´ sko, M.; Góral, T.; Ostrowska, A.; Matysiak, A.; Walenty-Góral, D.; Perkowski, J. The effect of Fusarium inoculation and fungicide application on concentrations of flavonoids (apigenin, kaempferol, luteolin, naringenin, quercetin, rutin, vitexin) in winter wheat cultivars. *Am. J. Plant Sci.* 2014, 5, 3727–3736. [CrossRef]

44. Kulik, T.; Stuper-Szablewska, K.; Bilska, K.; Bu´ sko, M.; Ostrowska-Kołodziejczak, A.; Zaluski, D.; Perkowski, J. Sinapic Acid Affects Phenolic and Trichothecene Profiles of *F. culmorum* and *F. graminearum* Sensu Stricto. *Toxins* 2017, 9, 264. [CrossRef] [PubMed]

45. Kulik, T.; Stuper-Szablewska, K.; Bilska, K.; Bu´ sko, M.; Ostrowska-Kołodziejczak, A.; Zaluski, 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]

46. Jiang, H.; Wood, K.V.; Morgan, J.A. Metabolic Engineering of the Phenylpropanoid Pathway in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 2005, 71, 2962–2969. [CrossRef] [PubMed]

47. Lee, H.; Kim, B.G.; Kim, M.; Ahn, J.H. Biosynthesis of Two Flavones, Apigenin and Genkwanin in *Escherichia Coli*. *J. Microbiol. Biotechnol.* 2015, 25, 1442–1448. [CrossRef] [PubMed]

48. Marín, L.; Gutiérrez-del-Río, I.; Yagüe, P.; Manteca, Á.; Villar, C.J.; Lombó, F. De Novo Biosynthesis of Apigenin, Luteolin, and Eriodictyol in the Actinomycete *Streptomyces albus* and Production Improvement by Feeding and Spore Conditioning. *Front. Microbiol.* 2017, 8, 921. [CrossRef] [PubMed]

49. Leonard, E.; Yan, Y.; Koffas, M.A.G. Functional expression of a P450 flavonoid hydroxylase for the biosynthesis of plant-specific hydroxylated flavonols in Escherichia coli. *Metab. Eng.* 2006, 8, 172–181. [CrossRef] [PubMed]

50. Stahlhut, S.G.; Siedler, S.; Malla, S.; Harrison, S.J.; Maury, J.; Neves, A.R.; Forster, J. Assembly of a novel biosynthetic pathway for production of the plant flavonoid fisetin in *Escherichia coli*. *Metab. Eng.* 2015, 31, 84–93. [CrossRef] [PubMed]

51. Yang, J.-G.; Liu, B.-G.; Liang, G.-Z.; Ning, Z.-X. Structure-Activity Relationship of Flavonoids Active against Lard Oil Oxidation Based on Quantum Chemical Analysis. *Molecules* 2009, 14, 46–52. [CrossRef] [PubMed]

52. Burda, S.; Oleszek, W. Antioxidant and Antiradical Activities of Flavonoids. *J. Agric. Food Chem.* 2001, 49, 2774–2779. [CrossRef] [PubMed]

53. Alexander, N.J.; McCormick, S.P.; Waalwijk, C.; van der Lee, T.; Proctor, R.H. The genetic basis for 3-ADON and 15-ADON trichothecene chemotypes in *Fusarium graminearum* sensu stricto. *Appl. Environ. Microbiol.* 2005, 71, 802–814. [CrossRef] [PubMed]

54. Arango, D.; Morohashi, K.; Yilmaz, A.; Kuramochi, K.; Parihar, A.; Brahimaj, B.; Grotewold, E.; Doseff, A.I. Molecular basis for the action of a dietary flavonoid revealed by the comprehensive identification of apigenin human targets. *Proc. Natl. Acad. Sci. USA* 2013, 110, E2153–E2162. [CrossRef] [PubMed]

55. Zhang, X.; Han, R.; Sun, X.; Li, G.; Yang, Q.; Li, Q.; Gai, W.; Zhang, M.; Chen, L.; Yang, G.; et al. The effect of the skeleton structure of flavanone and flavonoid on interaction with transferrin. *Bioorg. Med. Chem. Lett.* 2013, 23, 6677–6681. [CrossRef] [PubMed]

56. McCormick, S.P.; Stanley, A.M.; Stover, N.A.; Alexander, N.J. Trichothecenes: From Simple to Complex Mycotoxins. *Toxins* 2011, 3, 802–814. [CrossRef] [PubMed]

57. Tag, A.G.; Garifulina, 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]

58. Markovic, J.M.D.; Milenkovic, D.; Amie, D.; Popovic-Bijelic, A.; Mojovic, M.; Pasti, I.A.; Markovic, Z.S. Energy requirements of the reactions of kaempferol and selected radical species in different media: Towards the prediction of the possible radical scavenging mechanisms. *Struct. Chem.* 2014, 2, 1795–1804. [CrossRef]
60. Kulik, T.; Buśko, M.; Pszczółkowska, A.; Perkowski, J.; Okorski, A. Plant lignans inhibit growth and trichothecene biosynthesis in Fusarium graminearum. Lett. Appl. Microbiol. 2014, 59, 99–107. [CrossRef] [PubMed]

61. Lorenc-Kukula, K.; Wrobel-Kwiatkowska, M.; Starzycki, M.; Szopa, J. Engineering flax with increased flavonoid content and thus Fusarium resistance. Physiol. Mol. Plant Pathol. 2007, 70, 38–48. [CrossRef]

62. Gill, U.S.; Uppalapati, S.R.; Gallego-Giraldo, L.; Ishiga, Y.; Dixon, R.A.; Mysore, K.S. Metabolic flux towards the (iso)flavonoid pathway in lignin modified alfalfa lines induces resistance against Fusarium oxysporum f. sp. medicaginis. Plant Cell Environ. 2017. [CrossRef] [PubMed]

63. Kulik, T.; Abarenkov, K.; Buśko, M.; Bilska, K.; van Diepeningen, A.D.; Ostrowska-Kolodziejczak, A.; Krawczyk, K.; Brankovics, B.; Stenglein, S.; Sawicki, J.; et al. ToxGen: An improved reference database for the identification of type B-trichothecene genotypes in Fusarium. PeerJ 2017, 5, e2992. [CrossRef] [PubMed]

64. 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]

65. 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]

66. Re, R.; Pellergini, 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]

67. Perkowski, J.; Kiecana, I.; Kaczmarek, Z. Natural occurrence and distribution of Fusarium toxins in contaminated barley cultivars. Eur. J. Plant Pathol. 2003, 109, 331–339. [CrossRef]

68. 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]

69. 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/).