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Thioredoxin Is a New Target for the Phytotoxicity of Small Lactone Mycotoxins, Patulin and Penicillic Acid on Maize Seedlings

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Abstract: The phytotoxic mechanisms of patulin (PAT) and penicillic acid (PA) have not been identified unambiguously. This study aims to clarify their effects on thioredoxins (Trxs). Aflatoxin B1 (AFB1), PAT and PA were isolated by solvent extraction and chromatographic techniques from the cultures of *Aspergillus flavus* Z2 (LC171449), *Penicillium vulpinum* CM1 and *Aspergillus ochraceus* EMCC516, respectively. The three mycotoxins showed phytotoxicity to the germination of maize seeds, which was manifested by inhibiting radicle and coleoptile emergence, in addition to their toxic effects on fresh weights and root and shoot lengths. The phytotoxicity of AFB1 and PAT was greater than that of PA. Due to the central roles exhibited by plant Trxs in cellular metabolic activities, they were tested as target proteins for PAT and PA using AFB1 as positive control. In vivo studies showed that the mycotoxins significantly reduced Trx activity measured in the roots and shoots of maize seedlings. PAT showed greater Trx-inhibiting activity than PA and AFB1. In vitro studies of the mycotoxins on Trx *y*1 (from *Arabidopsis thaliana*) and thioredoxin reductase (Tr, from rat liver) activities confirm the results of in vivo studies. The inactivation of Trx with PAT and PA was reduced in the presence of glutathione (GSH). Data obtained suggest that lactone mycotoxins are more highly reactive with simple low-molecular-weight thiols (like GSH) than with complex ones (like Trx).

Keywords: patulin (PAT); penicillic acid (PA); aflatoxin B1 (AFB1); maize; thioredoxin (Trx); thioredoxin reductase (Tr); glutathione (GSH)

Citation: Ismaiel, A.A.; Papenbrock, J. Thioredoxin Is a New Target for the Phytotoxicity of Small Lactone Mycotoxins, Patulin and Penicillic Acid on Maize Seedlings. *Agriculture* **2023**, *13*, 950. https://doi.org/10.3390/agriculture13050950

Academic Editors: Jing Qiu, Chunlin Fan and Dajin Yang

Received: 23 March 2023
Revised: 16 April 2023
Accepted: 24 April 2023
Published: 26 April 2023

1. Introduction

Mycotoxins, secondary metabolites of low molecular weight produced by filamentous fungi, can contaminate a wide range of agricultural crops [1]. The term mycotoxin was coined after an outbreak in England in the early 1960s that led to the death of more than 100,000 turkey poultry due to ingestion of aflatoxin-contaminated peanut meal [2]. Since this time, contamination of food and crops with mycotoxins has become a global issue. Furthermore, the hazardous nature and the possible mechanisms of mycotoxins' toxic effects need to be understood.

The majority of these mycotoxins are produced by a small number of fungal genera, such as *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium* and *Alternaria*. In the field, the phyllosphere of the growing plants is mainly infected by the fungal genera *Alternaria*, *Fusarium* and *Cladosporium*. In the harvesting and storage stages, *Aspergillus* and *Penicillium* are the predominant species [1]. The primary toxic effects of mycotoxins on humans and livestock result from the consumption of contaminated seeds or edible plant parts [3]. Mycotoxins are currently classified according to their chronic effects into mutagens, carcinogens or teratogens. For example, aflatoxins that are produced by *Aspergillus flavus* and *A. parasiticus* were listed as group 1 human carcinogens by the International Agency for Research on Cancer; ochratoxins that are produced mainly by *A. ochraceus* were classified as group

2B, a possible human carcinogen, and both patulin (PAT) and penicillic acid (PA) produced by many *Aspergillus* and *Penicillium* species were considered not classifiable as to their carcinogenicity to humans (Group 3) [4]. However, the two latter mycotoxins have been reported to be acutely toxic and cardiotoxic and were recognized as effective antibiotics [5].

In addition to the public health risks, mycotoxins have phytotoxic properties with a variety of biological activities, including morphological, physiological and metabolic effects. For example, aflatoxin was reported to inhibit the seed germination, seedling viability and growth of maize, wheat, sorghum, lettuce, mung, mustard, gram, cowpea and sesame [1]. The phytotoxic mechanism has been proposed on the basis of the inhibition of chlorophyll and carotenoid synthesis [6]. Suppression of protein and nucleic acid synthesis and amylase activity in these plants has been also reported [7,8]. Ochratoxin was found to induce plant cell death and growth inhibition of roots and shoots was also recorded at low concentrations. The phytotoxic activity of ochratoxin was reported on the basis of its interference with phenylalanine synthesis, membrane damage by induction of lipid peroxidation, disruption of calcium homeostasis, inhibition of cellular respiration and DNA damage [9]. The toxicity of PAT and PA on plants has been demonstrated by several authors. PAT was reported to inhibit plant elongation, causing as well decreases in number, weight and gain in biomass of seeds upon its application to these plants in aqueous solution [10,11]. PA also exhibited an inhibitory effect on the growth of young plants [12,13], in addition to its effect on overall metabolite turnover in maize [14]. The phytotoxic action of both PAT and PA was attributed to their inhibitory effect on the synthesis of protein, RNA or DNA, and their reactivity with sulfhydryl groups (-SH) was proved to cause genotoxic effects with induction of oxidative damage [15,16]. Inhibition of respiration by the two mycotoxins was also reported at subtoxic concentrations [12,17].

Thioredoxins (Trxs) are ubiquitous small proteins of 12–14 kDa that show protein disulfide reductase activity. They play essential roles in cellular metabolic activities by donating electrons to enzymes such as ribonucleotide reductase, methionine sulfoxide reductases and peroxiredoxins [18]. In addition, Trx-catalyzed disulfide bond reduction regulates an array of target proteins ranging from mammalian transcription factors to plant enzymes involved in photosynthesis [19]. Plant Trxs were discovered in the 1970s as a regulator of several enzymes localized in the chloroplasts and related to photosynthesis and malate synthesis. In plants, seven different Trx types were classified based on their primary structures and localization of their members: *f*, *m*, *x*, *z* and *y* in chloroplasts, *o* in mitochondria and *h* mainly localized in the cytosol [20]. They play essential roles in response to environmental stresses and cell growth and division [21]. Furthermore, they were reported to participate in several cellular processes, including enzyme regulation, oxidative stress response, transcription and translation [20]. In chloroplasts, the *f* and *m* types were the first plastidal Trxs characterized and named according to their ability to reduce plastidal fructose-1,6 bisphosphatase (FBPase) and malate dehydrogenase (MDH), respectively. These Trxs regulate the activity of enzymes in the chloroplast by thiol disulfide interchange mechanisms. After each reaction, they have to be reduced again via ferredoxin-Trx-reductase in the presence of ferredoxin [20]. Trx *y* reduces 2-cys peroxiredoxin A and B as well as peroxiredoxin Q, and through this action they participate in the antioxidant metabolism in chloroplasts [20]. Type *h* is believed to be cytosolic and conserved in monocots and dicots [18]. In cereal seeds, Trx *h* is abundant during development and germination. In germinating seeds, this Trx reduces storage proteins and α-amylase inhibitors [22]. Additionally, it reduces and activates a seed-specific serine protease, thiocalsin, thereby playing an essential role in the mobilization of storage proteins at an early stage of germination [23]. In developing seeds, Trx *h* was suggested to have a regulatory role in the passage of compounds to the endosperm as it was reported to be found in the maize pedicle [24] and wheat seeds [25].

Although PAT and PA have been reported as important tools in several biological studies, their cellular toxic mechanism still remains unclear. We have previously studied the phytotoxic action of PAT via its notable influence on glutathione (GSH) concentration [11] and glutathione-S-transferase (GST) and antioxidant enzyme activities of maize

seedlings [26]. Various enzymes were shown to be inhibited by PAT and PA, among them thiol enzymes, such as muscle lactic dehydrogenase, yeast alcohol dehydrogenase and muscle aldolase [27,28]. Due to evidence of the phytotoxicity of AFB1 [1], it was included in this study as a positive control for comparison with PAT and PA. Since Trx is a major thiol-disulfide oxidoreductase with both intracellular and extracellular functions and exists in either a reduced form (Trx-SH_2) or an oxidized form (Trx-S_2), the present study aimed at utilizing Trx of maize seedlings as a sensitive measure for assessing the phytotoxicity of the small lactone mycotoxins, PAT and PA, for the first time.

2. Materials and Methods

2.1. Chemicals

Aflatoxin B1 (AFB1), patulin (PAT), penicillic acid (PA), reduced glutathione (GSH), dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA) disodium salt, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), potassium phosphate, phenylhydrazine hydrochloride, NADPH, insulin from bovine pancreas, thioredoxin reductase (Tr, from rat liver) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals used were obtained from Roth (Karlsruhe, Germany) or Applichem (Darmstadt, Germany).

2.2. Fungal Strains for Production of the Mycotoxins

Three fungal strains from our lab stocks were used in this study. The first strain was *Aspergillus flavus* Z2, an AFB1-producing fungus that was previously isolated from local maize seeds (*Zea mays* L.) and deposited in the GenBank with accession number LC171449. The second strain, *Penicillium vulpinum* CM1, produced for PAT, was previously recovered from soil samples cultivated with maize [11]. The third strain, *Aspergillus ochraceus* EMCC516, produced for PA, was obtained from the Egyptian Microbial Culture Collection (Microbiological Resources Centre, MIRCEN, Cairo, Egypt). The fungal strains were sub-cultured on yeast–sucrose (YES) agar slants (yeast extract 20 g, sucrose 200 g, agar 20 g, distilled water 1 L), after which they were stored at 7 °C. After an interval of 3 to 5 months, sub-culturing of the fungal strains was repeated.

2.3. Preparation of Fungal Inoculum and Culture Conditions

Inoculum preparation and culture conditions were carried out according to our previous study [11]. Fungal inoculum of the three strains was separately prepared from actively growing slants (5 days old), which were then flooded with 0.1% Tween 20 diluted with sterile distilled water. After gently scrapping off the spores, a hemocytometer was used to adjust the spore concentration at 2×10^6 mL^{-1}. One mL of each individual fungal inoculum was aseptically transferred into an Erlenmeyer flask (500 mL volume) containing 100 mL YES broth adjusted at pH 6.0, followed by dark incubation at 30 °C for 10 days.

2.4. Extraction and Purification of Mycotoxins

Prior to extraction of mycotoxins from the culture flasks of the three fungal strains, their cultures were filtered via Whatman no. 1 filter papers. Thereafter, the filtrate was subjected to extraction procedure.

2.4.1. AFB1

The culture filtrate of *A. flavus* Z2 (LC171449) was defatted with *n*-hexane and then extracted with equal volume of chloroform [29]. This was carried out in a separating funnel by shaking for about 30 min. The chloroform layer (after 30 min standing) was filtered over anhydrous sodium sulfate and then concentrated under vacuum until dry using a rotary evaporator (IKA, RV10, Staufen, Germany). The dried crude extract was then dissolved in chloroform:hexane (1:1, *v*:*v*) and applied to column chromatography packed with C18 silica gel, after which AFB1 was eluted by benzene:ethanol (94:6, *v*:*v*) [30]. The fractions resulting from the column chromatography were evaporated until dry and

checked for AFB1 using thin layer chromatography (TLC) on a 0.25-mm F-254 silica thin layer plate (Merck, Darmstadt, Germany). The dried fractions were dissolved in methanol and loaded with the reference standard AFB1 on the TLC plate, which was then developed in a solvent system composed of toluene: ethyl acetate: formic acid (6:3:1, *v*:*v*:*v*). When the solvent system reached the end of the plate by 2 cm, the plate was allowed to dry and then examined under short- and long-wavelength (254 and 366 nm) ultraviolet light using Min UVIS, DUOUV source for TLC. Consequently, AFB1 appeared as blue fluorescence spots, which were scrapped off, eluted with methanol and the absorption measured at 363 nm using a Deuterium UV 21 D Milton Roy spectrophotometer [29]. A standard curve was made to quantify the concentration of AFB1.

2.4.2. PAT and PA

PAT and PA were extracted from a culture filtrate of *P. vulpinum* CM1 and *A. ochraceus* EMCC516 after acidification to pH 3.0 and defatting with *n*-hexane. Both mycotoxins were extracted by ethyl acetate in a separating funnel (under conditions described previously for extraction of AFB1). The crude extract was then dissolved in chloroform:hexane (1:1, *v*:*v*) and passed through column chromatography packed with C18 silica gel. Both mycotoxins were eluted with ether:*n*-hexane:formic acid (60:20:5, *v*:*v*:*v*) [30]. The fractions resulting from the column chromatography were evaporated, dissolved in chloroform and loaded onto TLC plates (as described earlier) in the presence of the PAT and PA standard. Toluene: ethyl acetate: formic acid (6:3:1, *v*:*v*:*v*) was used as a developing system. PAT spots appeared yellow after TLC treatment with 2.0% (*w*:*v*) phenylhydrazine hydrochloride solution followed by heating for 15 min at 130 °C. These spots were eluted with *n*-butanol and their color absorbance measured at 540 nm, and PAT was then quantified from a standard curve [31]. PA spots appeared as bright blue inflorescence under long-wavelength ultraviolet light, and were more visualized by exposing the TLC to ammonia vapor for 10 min followed by heating for 5 min [32]. The two mycotoxins were also quantified without chemical treatment by eluting their spots in methanol, and their concentrations were determined using the known molar absorption coefficients (PAT ε_{276} = 1436; PA ε_{224} = 1063).

2.5. Preparation of Mycotoxin Solutions

Mycotoxins (AFB1, PAT and PA) separated from chromatographic techniques were individually pooled and dissolved in chloroform to give a concentration of 20 mg mL^{-1}, which was stored at −20 °C. In the germination tests, the chloroform was evaporated, and each crystalline pure mycotoxin was dissolved in sterile water containing 0.1% DMSO.

2.6. Plant Material and Growth Conditions

Egyptian dry corn seeds (*Zea mays*) were washed with tap water, surface-disinfected in 70% (*v*:*v*) ethyl alcohol for 5 min, thoroughly washed with sterile water, and soaked for 8 h in sterile water. Thereafter, ten uniform seeds were positioned vertically in a middle line of small strips (23 × 11 cm) of paper towels that were rolled loosely into several scrolls according to the method described by Ismaiel and Papenbrock [26]. Thereafter, the edge of the paper strip was soaked in plastic tubes containing 10 mL of the mycotoxin solution. The seeds were allowed to germinate in the circumference scroll when the plastic tubes were dark-incubated at 25 °C for 7 days. The germination treatments included the individual treatments of seeds with different concentrations of PAT and PA, which varied from 25 to 100 μg mL^{-1}. AFB1, used as a positive control, was tested at the same concentrations. Seeds grown in 0.1% DMSO served as a negative control. The growth parameters were determined, which included the percent of seeds showing inhibition of radicle and coleoptile emergence, length of roots and shoots (cm) and their fresh weights (g) per seed.

2.7. Enzymatic Assay of Maize Trxs

Using a pre-chilled mortar and pestle, the fresh plant material (either from roots or shoots) of all mycotoxin-treated seedlings was finely ground in liquid nitrogen. The powder

obtained was kept at −80 °C until analysis. Total Trxs were extracted at 4 °C from 100 mg of the fine powder sample in 600 µL of 200 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. Centrifugation of the homogeneous mixture was performed at 15,000× *g* for 20 min at 4 °C, after which the combined supernatant was ready for immediate analysis in enzyme activity assays. The Trx activity was determined using a microplate reader (Synergy Mx, BioTek, Bad Friedrichshall, Germany) based on the methods of Arnér and Holmgren [33], with some modifications. In each well, the assay mixture contained 170 µL of 100 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, 0.13 mM insulin from bovine pancreas and 0.15 mM NADPH. The reaction was initiated by adding 30 µL of crude extract. Consumption of NADPH was determined from the decrease in absorbance at 340 nm at 25 °C for 5 min. Wells containing the assay mixture without the crude extract were used as control. Specific activities were calculated using the extinction coefficient of NADPH at 340 nm (6.2 mM^{-1} cm^{-1}). The Trx activity in plant samples was also confirmed by a second method in which the rate of insulin reduction was measured at 650 nm as turbidity formation from the insulin B chain precipitation. The assay mixture used in this method was prepared as mentioned above, with the addition of 0.33 mM DDT (instead of NADPH) to initiate the reaction. The increase in absorbance at 650 nm was determined at 25 °C for 120 min. The rate of precipitation is defined as the maximal increase ΔA650 min^{-1} in absorbance. The protein content of all plant samples was determined spectrophotometrically via the Bradford technique [34] using BSA as a standard.

2.8. Expression and Purification of Recombinant Trx y1 from Arabidopsis Thaliana

The Trx *y*1 from *A. thaliana* was heterologously expressed in *Escherichia coli* and purified via nickel affinity chromatography as previously described [35].

2.9. In Vitro Experiments Using Pure Proteins

2.9.1. In Vitro Effects of the Mycotoxins on Trx *y*1 Activity

The purified Trx *y*1 (1.0 µg) was individually incubated with AFB1, PAT and PA at different concentrations (1.0, 3.0, 6.0 and 9.0 µg) to give the following ratios of mycotoxin: Trx *y*1 preparations: 1:1, 3:1, 6:1 and 9:1. Incubation of these mycotoxins–Trx *y*1 preparations were carried out in an 100 µL assay mixture solution (its composition was mentioned above) for 1 h at 25 °C, after which the activity of Trx *y*1 was determined at 340 nm as described earlier.

2.9.2. In Vitro Effects of the Mycotoxins on Tr Activity

Commercial Tr from rat liver kept in −20 °C was adjusted at 1.0 µg and separately incubated with the mycotoxins at different concentrations of 1.0, 3.0, 6.0 and 9.0 µg to give ratios of mycotoxin: Tr preparations of 1:1, 3:1, 6:1 and 9:1. The mycotoxin: Tr preparations were employed in an 100 µL assay mixture solution that was composed of 100 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, 5 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and 0.15 mM NADPH. Incubation was carried out for 1 h at 25 °C and the TR activity was determined spectrophotometrically by the reduction of DTNB, which was monitored by the increase in absorbance at 412 nm. Specific activity was calculated from the extinction coefficient of TNB (13.6 mM^{-1} cm^{-1}) [33].

2.9.3. In Vitro Effects of GSH on the Reactivity of PAT and PA with Trx *y*1

The activity of Trx *y*1 following addition of varied concentrations of GSH to the mycotoxin–Trx *y*1 mixture (9:1) was examined. In a 100 µL assay mixture solution, GSH was separately added to the mixture at 9.0, 18.0 and 27.0 µg by simultaneous and consecutive manner. In the first application, all reaction components were added at the same time; i.e., GSH was immediately added to mycotoxin and Trx *y*1 and the Trx *y*1 activity was immediately measured following incubation for 1 h at 25 °C. In the second application, the mycotoxin and Trx *y*1 were allowed to be incubated for 1 h at 25 °C, after which GSH was

added to the mixture and then incubated for an additional 1 h, followed by determination of Trx *y*1 activity as previously described.

2.10. Statistical Analysis

Results obtained were presented as the mean ± standard deviation (SD) or standard error (SE) and statistical significance was analyzed by an ANOVA test (SPSS software version 22, IBM Corp., New York, NY, USA), followed by a least significant difference (LSD) test at a 0.05 level.

3. Results

3.1. Effects of PAT and PA on Maize Seed Germination

Figure 1A,B illustrates the phytotoxicity of PAT and PA to maize seeds. Several parameters of maize seedlings were determined under the influence of different concentrations (25–100 μg mL^{-1}) of PAT and PA, and compared to those treated with the same concentrations of AFB1 (Figure 1A,B). The radicle inhibition was already observed at the initial concentration of 25 μg mL^{-1} of the three toxins, recording inhibition percentages of 23.3%, 20.0% and 3.3% with AFB1, PAT and PA, respectively. At 75 μg mL^{-1}, inhibition percentages recorded were 26.6%, 23.3% and 16.7%, respectively. At 100 μg mL^{-1}, the inhibition percentages of both AFB1 and PAT were equal (30.0%), and greater than that of PA (20.0%) (Figure 2A). Similar results for the effects of the mycotoxins on coleoptile emergence were obtained. At low concentration, 25 μg mL^{-1}, the percentage of coleoptile inhibition by AFB1, PAT and PA was 23.3%, 23.3% and 10.0%. It was increased upon applying the high concentration of 100 μg mL^{-1} to 30.0%, 30.0% and 26.6% (Figure 2B).

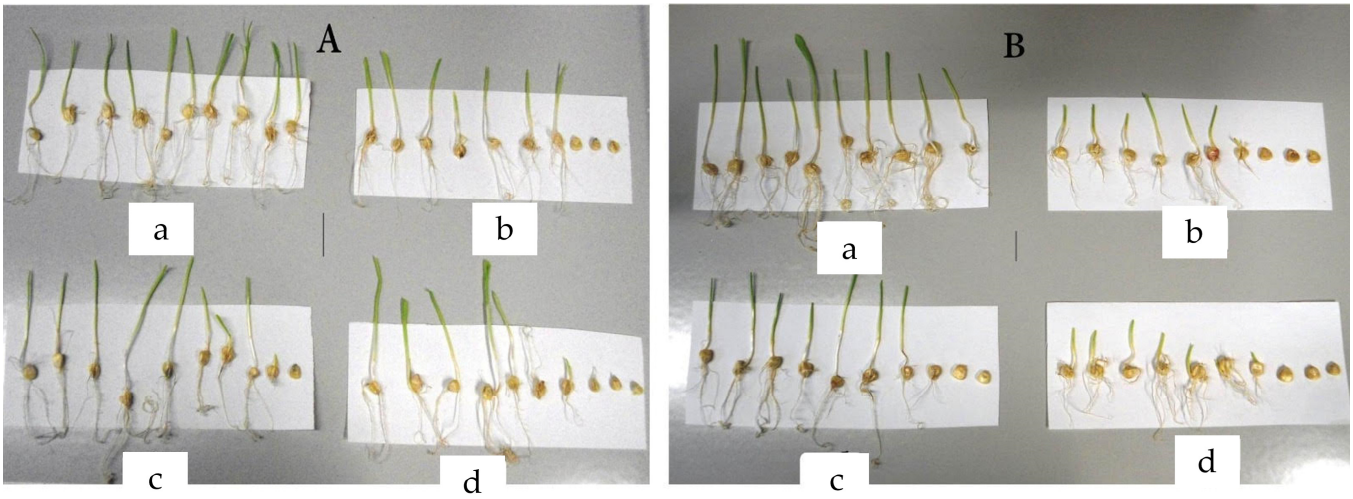

Figure 1. Photographs of 7-day-old maize seedlings following treatment with the mycotoxins solutions at 50 μg mL^{-1} (**A**) and 100 μg mL^{-1} (**B**). Maize seedlings of the control treatment (0.1% DMSO solution) (a); maize seedlings treated with AFB1 (b); maize seedlings treated with PA (c); and maize seedlings treated with PAT (d). In photograph A, the scale bar equals 7.4 cm. In photograph B, the scale bar equals 5.6 cm.

The effects of mycotoxins on fresh weights and lengths of roots and shoots were studied (Figure 3A–C). A significant reduction ($p \leq 0.05$) in the average values of these parameters was obtained at all applied concentrations of mycotoxins when compared with the control (without mycotoxin treatment). Upon individual application of AFB1, PAT and PA at 100 μg mL^{-1}, the reduction in the average values of fresh weights was significant, and was approximately 48.2%, 46.7% and 44.6% of the control. At this concentration of mycotoxins, the average root length was significantly reduced by 1.9, 1.8 and 1.4 times, respectively, compared to the control. A comparative significant decrease in the average

shoot length was obtained upon application of the respective mycotoxins, recording 1.8, 1.9 and 1.3 times compared to the control.

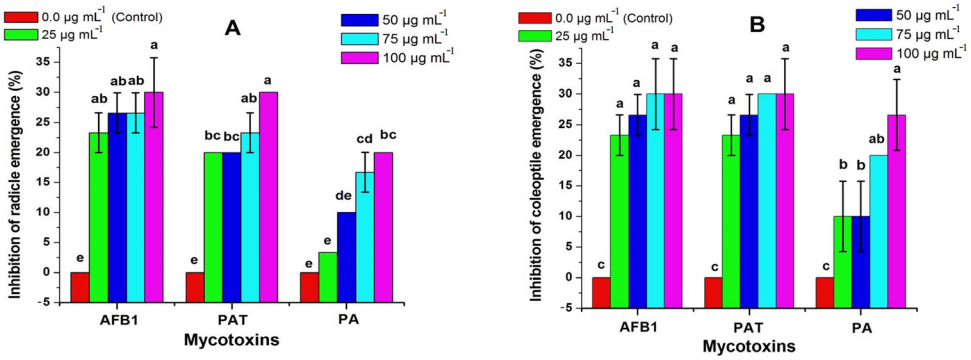

Figure 2. Effects of different concentrations of mycotoxins on radicle emergence (**A**) and coleoptile emergence (**B**) on 7-day-old maize seedlings. The values of radicle and coleoptile emergence were expressed as an inhibition percentage (%). DMSO solution (0.1%) was used as a control. Mean value is given for triplicates (each replicate included 10 samples) from three independent experiments ± SE. The different letters for inhibition percentages either for radicle or coleoptile at different mycotoxin concentrations are statistically different (LSD test, $p \leq 0.05$).

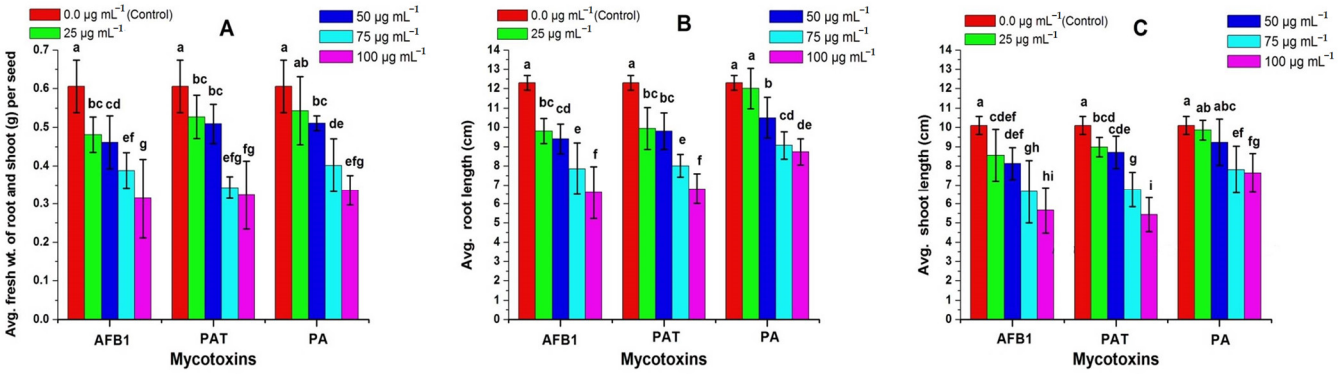

Figure 3. Effects of different concentrations of mycotoxins on fresh weights (**A**), length of root (measurements are for the main root) (**B**) and length of shoot (**C**) of 7-day-old maize seedlings. DMSO solution (0.1%) was used as a control. Mean value is for triplicate (each replicate included 10 samples) from three independent experiments ± SD. The different letters for fresh weights, root lengths or shoot lengths at different mycotoxin concentrations are statistically different (LSD test, $p \leq 0.05$).

3.2. Effects of PAT and PA on Trx Activities of Maize Seedlings

The levels of Trx activities in the root and shoot tissue of maize were determined as affected by AFB1, PAT and PA (Figure 4A–C). Upon treatment with the three mycotoxins, Trx activity was significantly reduced ($p \leq 0.05$) in roots and shoots when compared with control treatments, an effect that was dose-dependent. The reduction in Trx activity was more pronounced and significant ($p \leq 0.05$) in roots than in shoots following individual application of mycotoxins. At the lower concentration, 25 µg mL^{-1}, total Trx activity was reduced by 15.5%–23.4% in root tissue and by 3.2%–6.8% in shoot tissue. Using the highest concentration, 100 µg mL^{-1}, the decline rate in the enzymatic activity was remarkably increased. In root tissue, total Trx activity was reduced by 35.5%, 46.3% and 41.3% after respective treatment with AFB1, PAT and PA. In shoot tissue, the enzymatic activity was reduced by 20.3%, 24.5% and 20.2% upon treatment with the respective mycotoxins.

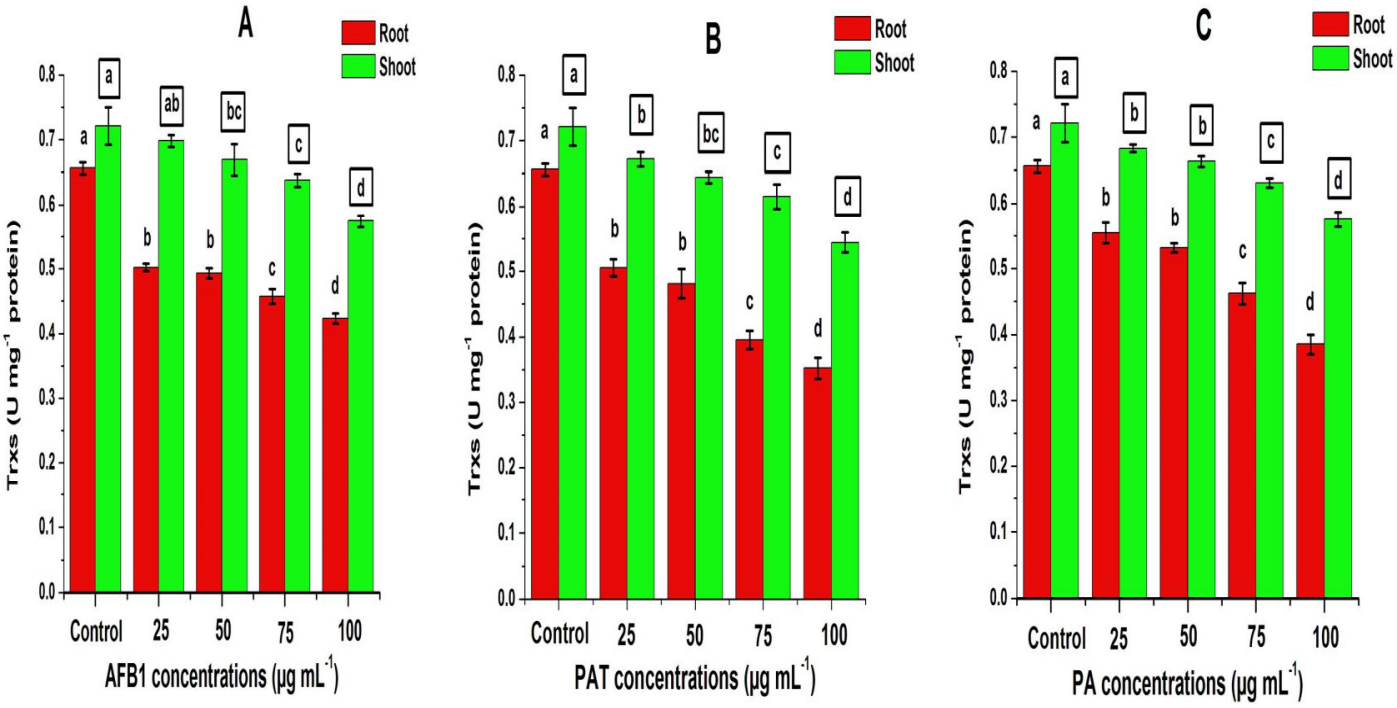

Figure 4. Trx activity in roots and shoots of 7-day-old maize seedlings treated with different concentrations of AFB1 (**A**), PAT (**B**) and PA (**C**). DMSO solution (0.1%) was used as a control. Data are presented as the mean ± SD of triplicate measurements from two independent experiments. Different letters either for roots (without squares) or shoots (in small squares) at different concentrations are statistically different (LSD test, $p \leq 0.05$).

3.3. Reactivity of PAT and PA with Trx y1 In Vitro

The in vitro effects of varying concentrations of PAT, PA and AFB1 on Trx *y*1 (from *Arabidopsis thaliana*) activity are presented in Table 1. Thioredoxin was incubated at a constant concentration (1 µg) with different concentrations of mycotoxins to give varying ratios. Individual pre-incubation of Trx with lower concentration and PAT or PA at 1 µg (giving a ratio of 1:1) showed that the inhibition percentage of Trx activity was 35.5% and 31.1%, respectively. A greater reduction in the percentage of Trx activity was significantly obtained upon increasing the concentration of both mycotoxins. The higher ratio (9:1) gave inhibition percentages of Trx activity of 92.9% and 49.1%, respectively. AFB1 had no recorded effect on the Trx activity at all concentrations tested. Data also indicated that PAT showed a greater inhibitory effect on Trx activity than PA at all varying ratios, and significant differences ($p \leq 0.05$) in the inhibition percentages of Trx activity were remarkable (Table 1).

Table 1. Influence of varying concentrations of mycotoxins on the activity of Trx *y*1 from *Arabidopsis thaliana*.

Mycotoxin: Trx *y*1 Ratio	Inhibition (%) of Trx *y*1 Activity		
	PAT	PA	AFB1
0:0 (control)	0.0 ^{e}	0.0 ^{d}	0.0
1:1	35.5 ± 4.33 ^{d,*}	31.1 ± 2.63 ^{c}	0.0
3:1	43.1 ± 4.17 ^{c,*}	37.8 ± 2.11 ^{b}	0.0
6:1	64.5 ± 0.58 ^{b,*}	41.9 ± 1.54 ^{b}	0.0
9:1	92.9 ± 0.89 ^{a,*}	49.1 ± 3.58 ^{a}	0.0

Trx *y* activity was assayed following incubation of the mycotoxins separately with Trx *y*1 at different ratios in a 100 mM assay mixture solution (pH 7.0; its composition was described in Materials and Methods) at 25 °C for 1 h. The initial mycotoxin concentrations were 1.0, 3.0, 6.0 and 9.0 µg. The initial Trx y concentration was constant (1.0 µg) for all treatments. Mean value is for triplicate measurements from two independent experiments ± SD; $^{a–e}$ different superscript letters indicate that the means in the same column are statistically different (LSD test, $p \leq 0.05$). * indicates that the means in the same row are significantly different from those obtained by PA and AFB1 (LSD test, $p \leq 0.05$).

3.4. Effects of PAT and PA on Tr Activity In Vitro

The in vitro effects of varying concentrations of PAT, PA and AFB1 on the activity of Tr (from rat liver) are presented (Table 2). The Tr activity was gradually affected at all tried concentrations of PAT and PA, and the greater the increase in concentration of both toxins, the greater the increase in the inhibition percentages of Tr activity. In contrast, Tr activity was not affected by AFB1 at all concentrations tested (Table 2). Upon incubation of PAT and PA with Tr at a low ratio of 1:1, the inhibition percentage of Tr activity was low, recording 9.8% and 8.3%, respectively. The inhibition percentage of enzyme activity was high, recording 30.5% and 25.1%, upon incubation of the two respective mycotoxins with Tr at a high ratio of 9:1. At this ratio, the inhibition percentage obtained by PAT and PA was approximately 3.0 times greater than that obtained at a ratio of 1:1. Comparing the results of the effects of PAT and PA on Tr activity statistically, it was found that significant differences in the inhibition percentages of enzyme activity were attained at ratios of 3:1 and 9:1.

Table 2. Influence of varying concentrations of mycotoxins on the activity of Tr from rat liver.

Mycotoxin: TR Ratio	Inhibition (%) of Tr Activity		
	PAT	PA	AFB1
0:0	0.0 ^{e}	0.0 ^{e}	0.0
1:1	9.8 ± 1.27 ^{d}	8.3 ± 1.73 ^{d}	0.0
3:1	17.9 ± 2.04 ^{c,*}	14.6 ± 1.79 ^{c}	0.0
6:1	23.8 ± 2.65 ^{b}	20.8 ± 0.85 ^{b}	0.0
9:1	30.5 ± 1.97 ^{a,*}	25.1 ± 2.53 ^{a}	0.0

Tr activity was assayed following incubation of the mycotoxins separately with Tr at different ratios in a 100 mM assay mixture solution (pH 7.0; its composition was described in Materials and Methods) at 25 °C for 1 h. The initial mycotoxin concentrations were 1.0, 3.0, 6.0 and 9.0 µg. The initial Tr concentration was constant (1.0 µg) for all treatments. Mean value is for triplicate measurements from two independent experiments ± SD; $^{a–e}$ different superscript letters indicate that the means in the same column are statistically different (LSD test, $p \leq 0.05$). * indicates that the means in the same row are significantly different from those obtained by PA and AFB1 (LSD test, $p \leq 0.05$).

3.5. Effects of GSH Addition to Mycotoxin–Trx y1 Mixture

GSH was added to the PAT–Trx and PA–Trx mixtures in different concentrations to give varying ratios of GSH: mycotoxin: Trx. Results of inhibition of Trx activity were obtained following simultaneous and consecutive addition of GSH to the mycotoxin–Trx *y*1 mixture (Table 3). For all treatments of GSH addition to the mycotoxin–Trx *y*1 mixture, either simultaneously or consecutively, the greater the increase in GSH concentration, the less the reduction in percentage of Trx activity. In particular, simultaneous application of GSH to the mycotoxin–Trx *y*1 mixture showed a greater inhibitory effect on Trx activity than consecutive application, with significant differences ($p \leq 0.05$) at all tried treatments

of GSH. At a low concentration of GSH (9 µg), attaining a ratio of GSH: PAT: Trx of 9:9:1, simultaneous and consecutive applications significantly reduced Trx activity to 58.7% and 26.7%, which represented 1.6 times their controls (without GSH). At the same ratio of GSH: PA: Trx, the reduction percentage of Trx activity from the two applications were 38.5% and 23.3%, which represented 1.3 times their controls. Upon increasing the ratio of GSH: mycotoxin: Trx to 27:9:1, PAT reduced Trx activity to 49.4% (the reduction level was 1.9 times that of the control) and 21.7% (the reduction level was 2.0 times that of the control) in simultaneous and consecutive applications, respectively. Meanwhile, PA reduced Trx activity to 22.8% and 18.8% (the reduction level was 2.2 and 1.6 times that of their controls, respectively).

Table 3. Effects of PAT and PA on the activity of Trx *y*1 from *Arabidopsis thaliana* after simultaneous and consecutive addition of GSH to the mycotoxin–Trx *y*1 mixture at varying concentrations.

GSH:Mycotoxin:Trx *y*1	Inhibition (%) of Trx *y*1 Activity			
	PAT		PA	
	Simult. *	Consec.	Simult. *	Consec.
0:9:1 (control)	92.2 ± 1.46 [a]	42.8 ± 1.39 [a]	49.5 ± 1.52 [a]	30.6 ± 0.50 [a]
9:9:1	58.7 ± 2.28 [b]	26.7 ± 0.70 [b]	38.5 ± 1.29 [b]	23.3 ± 0.49 [b]
18:9:1	54.9 ± 1.69 [c]	24.2 ± 0.94 [c]	27.6 ± 1.75 [c]	20.7 ± 0.69 [c]
27:9:1	49.4 ± 1.39 [d]	21.7 ± 0.76 [d]	22.8 ± 3.66 [d]	18.8 ± 0.75 [d]

Trx *y*1 activity was assayed following incubation of the mycotoxin–Trx *y*1 mixture (9:1) separately with GSH at different ratios in a 100 mM assay mixture solution (pH 7.0) at 25 °C for 1 h. GSH concentrations of 9.0, 18.0 and 27.0 µg were added to mycotoxin–Trx *y*1 mixture simultaneously and consecutively, as described in Section 2. Mean value is for triplicate measurements from two independent experiments ± SD; [a–d] different superscript letters indicate that the means in the same column are statistically different (LSD test, $p \leq 0.05$). * indicates that the means of simultaneous application are significantly different from those of consecutive application (LSD test, $p \leq 0.05$) either in PAT or PA treatments.

4. Discussion

PAT and PA are small lactone mycotoxins produced in various food and agricultural commodities [36]. In addition to their multiple toxic effects on several biological systems, they share some properties, such as structural similarities; both of them are polar acidic secondary fungal metabolites [5]. On the biological level, they were recognized early as effective antibiotics and they are highly reactive with SH-containing proteins and GSH [1,27,28]. In this study, three fungal strains, viz., *A. flavus* Z2 (LC171449), *P. vulpinum* CM1 and *A. ochraceus* EMCC516, were cultured on YES medium, and by means of solvent extraction and chromatographic techniques, they were found to produce AFB1, PAT and PA at a level of 41.6, 1042.0 and 714.9 µg 100 mL^{-1}, respectively. In the literature, it is well known that the three respective fungal species are major sources for the production of these mycotoxins [1,5,11,26,29].

In this study, the phytotoxic effects of PAT and PA were demonstrated on maize seedlings, as compared with the difuranocoumarin mycotoxin, AFB1. Regarding the effects of the three mycotoxins (25–75 µg mL^{-1}) on radicle and coleoptile emergence in maize, mycotoxins can be arranged according to their inhibitory activity in the following order: AFB1 > PAT > PA. However, at the high dose 100 µg mL^{-1}, comparable inhibition percentages were obtained by the three mycotoxins, where the radicle inhibition percentage by AFB1 and PAT was 30.0% and that of PA was 20.0%. At the same dose, the coleoptile inhibition percentage was 30.0% by AFB1 and PAT and 26.6% by PA. In the literature, the phytotoxicity of mycotoxins was reported to be variable among plant varieties. In this regard, Keromnes and Thouvenot [12] stated that the phytotoxicity of AFB1 was greater than PA. They further showed that the necessary doses of the two respective mycotoxins that cause 50.0% inhibition of maize seed germination (Limagrain LG 9 variety) were 25 and 500 µg mL^{-1}. In our previous study [11] investigating the phytotoxicity of PAT to germination of maize seeds (cv. Montello), a radicle inhibition was evident at 20 and 25 µg mL^{-1}, recording percentages of 2.5 and 5.0%, respectively, whereas coleoptile

inhibition was found at 5 to 25 μg mL^{-1}, recording 2.5–10.0%. Crisan [37] reported that 100 μg aflatoxin mL^{-1} causes inhibition of hypocotyl and root elongation in Cruciferae species. Yamaji et al. [38] found that PA at 62.5 and 125 μg mL^{-1} did not cause phytotoxicity in *Picea glehnii* seedlings, whereas concentrations of 250 or 500 μg PA mL^{-1} caused slight browning of *P. glehnii* roots without root destruction. Moreover, these authors found that at 250 or 500 μg mL^{-1}, PA collapsed the root structure of lettuce and inhibited the growth of lettuce seedlings.

The average values of fresh weights and root and shoot lengths of maize seedlings were affected by the three mycotoxins. Significant reduction percentages (44.6%–48.0%) in fresh weights were obtained upon treatment of maize seeds with mycotoxins, and AFB1, followed by PAT, was more effective than PA. The lengths of roots and shoots were also significantly reduced at all tried concentrations of mycotoxins, and the toxic effect was in the following order: AFB1 > PAT > PA. Interestingly, at a high dose (100 μg mL^{-1}), shoot length was more affected by PAT than AFB1. Significant reductions in the average values of fresh weights and lengths of roots and shoots due to PAT treatments were observed as preliminary signs of phytotoxicity [11]. Reductions in radicle length, the germination index and, hence, the seedling viability of barley and sorghum were found with AFB1 at 1000 and 5000 μg mL^{-1} [7]. In *Nicotiana tabacum*, the inhibitory action of AFB1 on the development and mass of both root and leaf was significant and dose-dependent upon increasing the concentration above 0.5 AFB1 μg·mL^{-1} [39]. In germinating seeds, aflatoxin was reported to inhibit chlorophyll *a* and *b*, in addition to its ability to inhibit macromolecule synthesis in cell (protein, DNA and RNA) and seedling amylase activity [7,8]. In maize seedlings, the percentage inhibition of root growth was found to be directly proportional to the concentrations of PAT and PA [11,12]. Both PAT and PA were reported to have genotoxic effects in addition to their activity with respect to lessening the respiration of seedlings [1,12,17].

Thioredoxins are small thiol:disulfide oxidoreductases characterized by consensus amino acid sequence WC(G/P)PC in the redox active site [19,20]. In their reduced state, Trxs reduce disulfide bridges in numerous target proteins. Subsequently, the oxidized Trxs are reduced by the flavoenzyme Tr, and thereby participate in central cellular processes such as synthesis of deoxyribonucleotides, sulfur metabolism, regulation of gene expression and oxidative stress defenses [20,21]. Compared to animals, plants have a great variety of Trx types that are specific to one or two subcellular compartments. Based on their primary structures, biochemical properties and subcellular localizations, Trxs can be classified into different groups. The *A. thaliana* genome encodes for about 20 Trx isoforms that are localized in cytosol, the chloroplast or mitochondria, in addition to a dozen proteins closely related to Trxs [18,20]. Based on the amino acid sequence, Trxs in *A. thaliana* can be classified into seven subfamilies (*h*, *f*, *m*, *z*, *x*, *y*, and *o*) [20]. In this study, the in vivo results for the effects of PAT and PA on the Trx activities of maize seedlings using AFB1 as a positive control showed that the three mycotoxins have significant inhibitory influence on the Trx activity of roots and shoots. Either in roots or shoots of maize, PAT was more effective on Trx activity than PA and AFB1. Upon seedling treatment with PAT, the reduction percentage was 46.3% in root tissue and 24.5% in shoot tissue. Mycotoxins were reported to induce increases in production of ROS that can disrupt the redox status of cells, resulting in oxidative stress, which plays a major role in the toxic effects of major mycotoxins [40]. In plant cells, damage to proteins, carbohydrates, lipids and nucleic acids is a consequence of oxidative stress [41]. In order to cope with high rates of ROS production and hence prevent ROS injury, plant cells exhibit a remarkable antioxidative defense system that includes low-molecular compounds such as ascorbic acid and GSH (non-enzymatic machinery) and various enzymes (enzymatic machinery) such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione-S-transferase (GST), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). Among these enzymes, Trxs were reported to participate in plant antioxidant defense mechanisms, and this suggests that Trxs are key components in protecting the plant from oxidative damage [21]. The

decline in Trx activities either in roots or shoots due to PAT and PA treatments may be interpreted on the basis of both mycotoxins' active toxicity. As discussed above, PAT and PA are highly reactive with SH-containing enzymes and GSH [1,27,28], and this may indicate the high specificity of these enzymes to PAT and PA. Moreover, it was reported that PAT induces an elevation in ROS production that causes an increase in lipid peroxidation [42]. As a result, the decline in Trx activity could be interpreted on the basis of the increased H_2O_2 concentration, which mediates the inactivation of Trxs by PAT via oxidation of their thiol groups. A relation between the activities of APX and GR and sulfur was reported, since a deficiency in sulfur significantly suppresses their activities, and the decline recorded in enzymatic activity is related to depletion of GSH [43]. Recently, we reported on the decrease in GST activity in roots and shoots of maize following the treatment of seedlings with PAT and the decrease in enzymatic activity was similarly interpreted [26]. Along the same lines, a decline in the antioxidant enzyme activities of SOD and CAT due to PAT treatment was reported [44]. Another interpretation of the decrease of Trx activity by PAT and PA suggests that both mycotoxins impaired the biosynthesis of intermediate metabolic compound(s), thereby causing inhibition in the synthesis of proteins, DNA and RNA [15,16].

Unlike for PAT and PA, it is well known that AFB1 has no affinity for –SH groups. Therefore, the inhibitory effect of AFB1 on the Trx activity of maize seedlings could be taking place in an indirect matter. There is evidence reported in the literature that AFB1 has an inhibitory action on the biosynthesis of chlorophyll *a*, chlorophyll *b* and protochlorophyllide and on the activity of amylase in plants [7,8]. At least 23 biological processes in chloroplasts are controlled by Trxs, such as the Calvin cycle, the photosynthetic electron-transport chain, starch metabolism, nitrogen metabolism, lipid biosynthesis, protein folding and translation [45]. Thus, Trxs act as key redox sensors in plants, where they obtain electrons from ferredoxin, which is directly reduced by photosystem I [46]. Our results further showed that Trx activity was higher in shoots than in roots. Along the same lines, the activity of both APX and GR was considerably more pronounced in shoots than in roots of maize seedlings [26]. It is evident that upon treatment of maize seeds with mycotoxins, they translocate via plant organs and the expression of Trxs in shoots is higher than in roots. In plants, the non-photosynthetic plastids (such as amyloplasts) are found in seeds and roots, which are important for storage and mobilization of starch needed for plant development. Though amyloplasts do not perform photosynthesis, they contain the ferredoxin thioredoxin system [47]. Additionally, in amyloplasts (unlike in chloroplasts), the ferredoxin seems to be reduced by metabolically generated NADPH (not by light) via ferredoxin NADP reductase, which was reported to be induced in root plastids during nitrate assimilation [48]. Trx *f* and *m* were recognized in the roots and flowers of pea (non-photosynthetic parts) [49].

In vitro studies on the effects of PAT and PA on the activity of *A. thaliana* Trx *y*1 and rat liver Tr confirm the results of in vivo studies. At all different concentrations of PAT and PA, significant inhibition percentages of enzymatic activity of both enzymes were obtained and the inhibitory action of the two mycotoxins was dose-dependent. Data further showed that PAT had greater inhibitory activity on Trx *y*1 and Tr than PA. Trx *y*1 was reported to be localized in plastids and was suggested to have a very specific function in the photosynthesis of plants and other organisms [35,50]. In higher plants, Trx in the cytoplasm and mitochondria is reduced by NADPH-dependent Tr [20]. In chloroplasts, Trx isoforms and NADPH-dependent Tr act as redox regulatory factors involved in multiple plastid biogenesis and metabolic processes [51]. This enzyme contains a FAD-binding domain and a double Cys peptide motif in its catalytic center [51]. Tr (from rat liver) was employed in this study as a sensitive measurement for the in vitro effects of mycotoxins tested, since this enzyme is a key component of the Trx system (also called the Trx-Tr system, which is comprised of Trx, Tr and NADPH), which plays a major role in converting thiol and disulfide bonds in all cells from the three domains of life [51]. The inactivation of PAT and PA by thiols observed [1,11,27,28] supports our in vitro studies and has led to an

explanation of the mode of action of these mycotoxins via their binding with –SH residues in enzymes. Similar previous findings were obtained by Ashoor and Chu [27], who found that the two mycotoxins inhibited the yeast alcohol dehydrogenase and rabbit-muscle lactic dehydrogenase, which are also thiol enzymes. Moreover, these authors found that PAT had a higher affinity for dehydrogenases than PA. They reported that less PAT than PA was required to cause enzyme inhibition. These results support our findings concerning the higher reactivity of PAT than PA to Trx *y*1. The negative effect of AFB1 on the activity of Trx *y*1 and Tr was anticipated. As discussed earlier, in the in vivo studies, AFB1 could cause indirect influence on the Trx activity of the plant. This was interpreted on the basis of its interfering with the metabolic activities of the green pigments and amylase in plants. This indirect effect of AFB1 was not yet previously reported because AFB1 does not interact with –SH groups in enzymes, unlike PAT and PA.

The addition of GSH to PAT–Trx *y*1 or PA–Trx *y*1 either simultaneously or consecutively reduced the reactivity of both mycotoxins with Trx. Therefore, the inactivation of Trx with PAT and PA is reduced in the presence of GSH. The effect of GSH was dose-dependent. This study assumes that lactone mycotoxins are more highly reactive with simple low-molecular-weight thiols (like GSH) than with complex ones (like Trx). The rate of the inactivation process occurred more rapidly when GSH was more readily available in high concentration. This clearly explains why the inhibition in Trx activity was highly reduced upon increasing the GSH concentration to 27 µg (the ratio of GSH:mycotoxin:Trx at 27:9:1) in simultaneous or consecutive reactions. GSH, a tripeptide, is commonly the most abundant low-molecular mass thiol, with multiple functions in animal and plant cells [52,53]. It should be noted that in the control treatment (without addition of GSH, ratio of GSH:mycotoxin:Trx at 0:9:1), a greater reduction in the percentage of Trx inhibition activity was obtained after incubation for 2 h (consecutive reaction) than for 1 h (simultaneous reaction). Arafat et al. [54] stated that the inactivation rate of arginyl-tRNA synthetase (a thiol enzyme) is dependent on the concentration of PAT. These authors further found that slow inactivation of the enzyme with increasing time is induced by PAT. In our previous study regarding the in vitro effects of PAT on GSH, we found that a higher reduction in the percentage of free GSH inhibition was attained at lower PAT concentrations following incubation with GSH for 2 h rather than for 1 h [11].

5. Conclusions

Overall, this study presents new findings concerning the phytotoxicity of lactone mycotoxins (PAT and PA) through a demonstration of their inhibitory effects on Trx activity in a series of in vitro and in vivo experiments. The effects of both mycotoxins on germination of maize seeds and growth measurements of maize seedlings were assessed and compared with the effects induced by AFB1. Radicle and coleoptile emergence, fresh weights and lengths of roots and shoots of maize seedlings were significantly inhibited in a dose-dependent manner with increases in the concentrations of the three mycotoxins. AFB1, followed by PAT, was more effective on these growth parameters than PA. Meanwhile, comparable inhibition percentages were obtained by AFB1 and PAT upon treatment with the high dose of 100 µg mL^{-1}. Interestingly, at this dose, shoot length was more affected by PAT than AFB1. The remarkable effects of the three mycotoxins on the Trx activities of maize seedlings need to be highlighted: Significant reductions in Trx activity in roots and shoots were found and PAT was found to be more effective than AFB1 and PA. The in vitro studies conducted on Trx *y*1 and Tr showed that PAT had greater inhibitory activity than PA, whereas AFB1 had no activity. The addition of the thiol GSH to the reaction mixture of PAT–Trx *y*1 or PA–Trx *y*1 caused an apparent loss in enzyme activity. Interestingly, there is a greater reactivity of lactone mycotoxins with simple low-molecular-weight thiols (like GSH) than with complex ones (like Trx) and the higher the increase in GSH concentration, the higher was the inactivation rate of Trx. This study demonstrates, for the first time, one important mode of action of the phytotoxicity of small lactone mycotoxins on plants.

Author Contributions: Conceptualization, J.P.; methodology, A.A.I.; software, J.P.; validation, J.P.; formal analysis, J.P.; investigation, A.A.I.; resources, J.P.; data curation, A.A.I. and J.P.; writing—original draft preparation, A.A.I.; writing—review and editing, J.P. and A.A.I.; visualization, A.A.I. and J.P.; supervision, J.P.; project administration, J.P.; funding acquisition, A.A.I. and J.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Deutsche Forschungsgemeinschaft, grant number PA 764/19-1, AOBJ 618653.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data can be found according to the corresponding data source. Scholars requesting more specific data may email the corresponding author or the first author.

Acknowledgments: We thank Pamela von Trzebiatowski and Julia Volker for their excellent technical assistance. We also acknowledge Walid Abuelsoud, Institut für Botanik, Leibniz Universität Hannover, Germany, for his valuable advice concerning measurement of enzymatic activities.

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

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