Ph. D. Thesis

Ethylene-dependent effects of fusaric acid and fumonisin B1 on photosynthetic activity and reactive oxygen species metabolism in tomato (*Solanum lycopersicum* L.) leaves

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1. Introduction

Plants are exposed to various environmental stresses, especially biotic stresses such as pathogenic *Fusarium* species that can affect crop productivity and cause yield reduction and economic losses. These *Fusarium* species can infect several crops and produce noxious mycotoxins upon infection. *Fusarium* mycotoxins such as fusaric acid (FA) and fumonisin B1 (FB1) are secondary mycotoxins and have phytotoxic effects on maize, wheat, tobacco, tomato, corn, corn-based products, and animal feeds. Both FA and FB1 are cytotoxic, hepatotoxic, neurotoxic, and genotoxic in nature. Therefore, due to their adverse impacts on animals and plants, serious global attention is required to tackle with this problem and reduce their toxic effects on living organisms. FA and FB1 can induce elevated electrolytic leakage, modified membrane permeability, inhibited respiratory activity, reduced ATP levels, altered membrane potential, reduced stomatal conductance, disrupted mitochondrial activity, increased chloroplastic dysfunction, wilted cotyledons, increased lipid peroxidation, as well as induced chromatin condensation and programmed cell death (PCD) eventually. Mycotoxins can perturb photosynthesis due to the inhibition of electron transport chain and reduce stomatal conductance in plants.

Furthermore, the exposure of such noxious mycotoxins results in oxidative stress in the form of extensive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Mycotoxin-induced oxidative burst can induce PCD and reduce cell viability in plants. However, plants have evolved potent defensive mechanisms to detoxify the excessive ROS or RNS accumulation under mycotoxin exposure in the form of enzymatic and non-enzymatic antioxidants to maintain cellular homeostasis. Among phytohormones, ethylene (ET) has vital importance due to its involvement in germination, senescence, fruit ripening and stress responses under biotic stresses such as *Fusarium* infection. ET can bind with ET-receptors to regulate ET-mediated defence responses in plants under mycotoxins stress. Therefore, the ET-dependent effects of mycotoxins (FA and FB1) can be studied in detail by utilizing ET

signalling mutants to investigate its potential role in the regulation of ROS metabolism, photosynthesis, genetic and proteomic modifications. However, ET is still required to fill certain research gaps to understand its role in defence responses of plants against mycotoxins. In parallel, the regulation of PCD induction by ET under exposure of both mycotoxins FA and FB1 needs further explanation to explore the mechanisms in ROS production, ROS detoxification, and other associated changes in the function of photosynthetic apparatus at the subcellular level.

Therefore, this research work was conducted to investigate the phytotoxic effects of both *Fusarium* mycotoxins FA and FB1 on wild-type (WT) tomato (*Solanum lycopersicum* L. cv. Ailsa Craig) and ET-receptor mutant *Never ripe* (*Nr*) plants in a time- and concentration-dependent manner. Furthermore, the effects of mycotoxins on the photosynthetic activity and its associated photosynthetic pigments, proteins, oxidative stress, and ROS metabolism were also explored. Most importantly, the vital role of ET was also determined to analyse its involvement in the induction or regulation of mycotoxins-induced PCD in plants.

2. Aims

We aimed to examine the effects of FA- and FB1-provoked oxidative burst and the roles of key enzymatic and non-enzymatic antioxidants were studied in wild-type (WT) and ET-receptor mutant *Never ripe* (*Nr*) tomato plants while treating plants with sublethal (0.1 mM FA and 1 μM FB1) and cell death-mediating (1 mM FA and 10 μM FB1) concentrations of mycotoxins, for 24 and 72 h in the case of FA and 72 h for FB1. Therefore, our research objectives were:

1. To investigate the effects of FB1 and FA on the photosynthetic activity in WT and *Nr* mutant tomato plants.

2. To analyse and quantify the ROS production under FB1 and FA exposure in both tomato genotypes.

3. To reveal the role of ET in the induction and regulation of oxidative stress-induced PCD under mycotoxin exposure.

4. To explore the role of enzymatic and non-enzymatic antioxidants under FB1 and FA stress in WT and *Nr* plants.

5. To find out the expression of genes encoding the key antioxidant enzymes after FB1 and FA treatments in WT and *Nr* tomato plants.
6. To identify specific defence and photosynthetic proteins in both tomato genotypes subjected to FA and FB1 exposure.

3. Materials & Methods

Tomato (*Solanum lycopersicum* L. cv. Ailsa Craig) seeds of wild-type (WT) and ET-receptor mutant *Never ripe* (*Nr*) plants were germinated under dark condition and thereafter, plants were grown in hydroponic culture in a greenhouse under controlled conditions such as 12 h light and dark periods, temperature of 24°C during the day and 22°C at night, radiation flux of 200 µmol photon m^{-2} s^{-1}, and the level of relative humidity was maintained between 55-60% for 4 weeks. Nutrient solution of pH 5.8 was provided every second day. All experiments were performed with 6-7 weeks aged intact plants at 5 developed leaves stage. Plants were treated with FA and FB1 of sublethal (0.1 mM FA and 1 µM FB1) and cell death-mediating (1 mM FA and 10 µM FB1) concentrations of mycotoxins, for 24 and 72 h in the case of FA and 72 h for FB1. ET production was determined using Hewlett-Packard 5890 Series II gas chromatograph (Palo Alto, CA, USA). Chlorophyll fluorescence as well as P700 (PSI) redox status were determined using a Dual-PAM-100 instrument (Heinz-Walz, Effeltrich, Germany). Stomatal conductance and net photosynthetic rate were measured using photosynthesis measuring system (LI-6400, LI-COR, Inc., Lincoln, NE). The photosynthetic pigments, lipid peroxidation, hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) production, enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione S-transferase (GST), guaiacol-dependent

peroxidase (POD), and non-enzymatic antioxidants such as glutathione (GSH) and ascorbate (ASA) were measured according to standard protocols using spectrophotometer (Kontron , Milano, Italy). Further, NADPH oxidase, nitric oxide (NO) production, and electrolytic leakage (EL) were also recorded using specific protocols. The expression patterns of key antioxidant genes were assessed using qRT-PCR (qTOWER Real-Time qPCR System, Analytic Jena, Jena, Germany). Moreover, the effect of mycotoxins on defence and photosynthetic proteins were also observed using western blotting technique following standard protocol. Sigma Plot 11.0 software (SPSS Science Software, Erkrath, Germany) was used to perform statistical analysis. In addition, analysis of variance (ANOVA) was executed to find out the differences in all treatments by Tukey's test, and the significant difference was recorded if $p \leq 0.05$.

4. Summary

Mycotoxins such as fusaric acid (FA) and fumonisin B1 (FB1) are secondary metabolites of several *Fusarium* species which cause the reduction of crop productivity and yield worldwide. In addition to plants, these mycotoxins also prevail in animal feeds and human foods leading to severe health risks. Further, these mycotoxins are commonly found in *Arabidopsis*, tomato, tobacco, and other cereal crops such as wheat, maize, etc. resulting in economic losses. Furthermore, plants exposed to these mycotoxins and *Fusarium* infections exhibit disease symptoms such as development of lesions and necrotic spots, increased plasma membrane permeability, lipid peroxidation, electrolytic leakage, chlorosis, and eventually plant programmed cell death (PCD). Moreover, these noxious mycotoxins can also influence photosynthetic activity and stomatal conductance in plants. Exposure to such mycotoxins can induce oxidative/nitrosative stress by the accumulation of reactive oxygen- (ROS) and nitrogen species (RNS) which can affect the antioxidant defence system in plants. Various phytohormones such as ethylene (ET) regulate defence responses via modulating ROS/RNS metabolism by activating the plant antioxidant and detoxification

responses. Although numerous studies have examined the plant-fungal pathogen interactions and the consequent induction oxidative/nitrosative stress and defence responses, but the fundamental role of ET either in PCD induction or defence regulation remains less-studied. Therefore, the aim of this research was to explore the effects of FA and FB1 on wild-type (WT) and ET receptor mutant *Never ripe* (*Nr*) tomato plants after treatments with sublethal (0.1 mM FA and 1 μM FB1) and cell death-mediating (1 mM FA and 10 μM FB1) concentrations for 24 and 72 h in the case of FA but only for 72h for FB1, because in this case no significant changes were observed after 24 h. In addition to this, the expression of the key antioxidant enzyme-encoding genes, activity and accumulation of specific proteins, cell viability, and lipid peroxidation were also determined in both tomato genotypes.

The following main findings were obtained during this research work:

1. Both mycotoxins (FA and FB1) induced stress in WT as well as in *Nr* plants after 72 h in 1 mM FA and 10 μM FB1 concentrations, respectively. PCD induction was observed in WT and *Nr* tomato leaves based on the changes in cell viability, lipid peroxidation, oxidative stress, and ROS metabolism.
2. FA and FB1 mycotoxins induced significant ET emission in tomato plants but no significant difference was found between WT and *Nr* tomato genotypes. ET production was dependent on mycotoxins' dose and exposure time.
3. FA severely affected the photosynthetic activity, contents of photosynthetic pigments, stomatal conductance, net photosynthetic rate, electrolytic leakage, and expression of the key antioxidants in both tomato genotypes irrespective of ET signalling, however, FA induced oxidative stress also in ET-dependent (H_2O_2) and ET-independent ($O_2^{\cdot -}$) manner.
4. FA in an ET-dependent manner regulated the activation of enzymatic and non-enzymatic antioxidants to decrease ROS accumulation under FA exposure.

5. FB1 exposure reduced photosynthetic activity in both tomato genotypes by decreasing photosynthetic parameters, stomatal conductance, and net photosynthetic rate while enhanced oxidative stress (H_2O_2 accumulation) in *Nr* plants and decreased NO production in WT plants.
6. FB1-treated *Nr* plants also showed higher SOD and GST activities. FB1 treatment also resulted in ET-dependent EL increase in WT plants.
7. Our findings conclusively demonstrate that FA mycotoxin in an ET-dependent manner regulated the induction of defence responses of plants by the activation of antioxidants while FB1 treatments resulted in ET-dependent induction of PCD by increasing EL and oxidative stress (H_2O_2 accumulation).

5. Összegzés

A mikotoxinok, mint például a fuzársav (FA) és a fumonizin B1 (FB1), melyek számos *Fusarium* faj másodlagos metabolitjai, világszerte csökkentik a gazdaságilag fontos növények produktivitását és a terméshozamát. Ezenkívül ezek a mikotoxinok általában megtalálhatók az Arabidopsisban, a paradicsomban, a dohányban és más gabonanövényekben, mint például a búzában és a kukoricában, ami gazdasági veszteségeket okoz. A mikotoxinok és a *Fusarium* fertőzésekneka növények esetében okozott károk mellett, részben ezeknek köszönhetően, felhalmozódhatnak az állati takarmányokban és az emberi élelmiszerekben is, ami további súlyos egészségügyi kockázatokat jelent. A mikotoxinoknak kitett növények speciális betegségtüneteket mutathatnak, mint például foltszerű elváltozások a leveleken (pl. nekrotikus foltok kialakulásának formájában), amely mögött a megnövekedett plazmamembrán-permeabilitás, a lipidperoxidáció, az ionkieresztés, a klorózis és végül a növényi programozott sejthalál (PCD) állhat. Ezenkívül a mikotoxinok a növények fotoszintetikus aktivitását és a sztómák nyitottságát is befolyásolhatják. Az ilyen mikotoxinoknak való kitettség oxidatív/nitrozatív stresszt válthat ki a reaktív oxigén- (ROS) és nitrogénfajták (RNS) felhalmozódása révén, amelyek hatással

lehetnek a növények antioxidáns védekező rendszerére is. Különféle fitohormonok, például az etilén (ET) szabályozzák ezeket a védekezési válaszokat a ROS/RNS metabolizmus modulálásával, aktiválva a növényi antioxidáns és méregtelenítő válaszlépéseket. Bár számos tanulmány vizsgálta a növény-gomba kórokozók kölcsönhatásait és az ebből adódó oxidatív/nitrozatív stressz- és védekezési reakciókat, az ET alapvető szerepe mind a PCD-indukcióban, mind pedig a védekezés szabályozásában továbbra is kevésbé ismert. Ezért kutatásunk célja az volt, hogy feltárja az FA és FB1 toxinok vad típusú (WT) és ET receptor mutáns, *Never ripe* (*Nr*) paradicsomnövényekre gyakorolt hatását szubletális (0,1 mM FA és 1 µM FB1) és sejthalált indukáló (1 mM FA és 10 µM FB1) koncentrációk esetében. Az FA esetében már 24 óra után szignifikáns elváltozások voltak megfigyehetőek, de a teljes hatás megjelenéséig 72 órára volt szükség, az FB1 esetében azonban csak 72 óra után vizsgáltuk meg alaposabban a növényeket, mivel ebben az esetben 24 óra elteltével nem volt szignifikáns változás a toxin kezelés hatására a vizsgált élettani és molekuláris folyamatokban. A fenotipikus vizsgálatok mellett mindkét paradicsom genotípusban meghatároztuk a fotoszintetikus aktivitást leíró paraméterek változását, a kulcsfontosságú antioxidáns enzimeket kódoló gének expresszióját és aktivitását, bizonyos (védekezéshez és fotoszintézishez köthető) fehérjék aktivitását és felhalmozódását, a sejtek életképességét, valamint a lipidperoxidáció mértékét is.

A kutatás során a következő főbb megállapítások voltak levonhatóak:

1. Mindkét mikotoxin (FA és FB1) stresszt indukált a vad típusú, valamint az *Nr* növényekben 72 óra elteltével, 1 mM FA és 10 µM FB1 koncentrációban. A WT és Nr paradicsom növények leveleiben PCD indukálódott a sejtek életképességében, a lipid peroxidációban, az oxidatív stresszben, valamint a ROS metabolizmusban bekövetkezett változások alapján.
2. Az FA és FB1 is szignifikáns ET emissziót indukált a paradicsomnövényekben, de szignifikáns különbség nem volt kimutatható a különböző genotípusok és a különböző mikotoxinokkal végzett kezelések között sem. Az ET termelés

alapvetően a mikotoxinok dózisától és expozíciós idejétől függött.

3. Ezen eredmények alapján arra a következtetésre jutottunk, hogy az FA jelentősen befolyásolta a fotoszintetikus aktivitást, a fotoszintetikus pigmentek mennyiségét, a sztómakonduktanciát, a nettó fotoszintetikus rátát, az ionkieresztést és a legfontosabb antioxidánsok expresszióját mindkét paradicsom genotípusban, függetlenül az ET-jelátviteltől, azonban az FA az oxidatív stressz kialakulását ET-függő (H_2O_2) és ET-független ($O_2^{\cdot-}$) útvonalakon keresztül egyaránt indukálta.
4. Az FA ET-függő módon szabályozta az enzimatikus és nem-enzimatikus antioxidánsok aktiválását, hogy csökkentse a ROS felhalmozódását FA-expozíció alatt.
5. Az FB1 expozíció mindkét paradicsom genotípusban csökkentette a fotoszintetikus aktivitást (erre a fotoszintetikus aktivitást leíró paraméterek csökkenéséből, valamint a csökkenő sztómakonduktanciából és nettó fotoszintetikus sebességből lehetett következtetni), miközben fokozta az oxidatív stresszt (H_2O_2 felhalmozódást) az *Nr* növényekben és csökkentette az NO-termelést a WT növényekben.
6. Az FB1-kezelt *Nr* növények magasabb SOD és GST aktivitást mutattak. Az FB1 kezelés ET-függő EL növekedést is eredményezett a WT növényekben.
7. Eredményeink meggyőzően bizonyítják, hogy az FA mikotoxin ET-függő módon szabályozta a növények védekező reakcióinak indukálását az antioxidáns védekezési válaszok aktiválása révén, míg az FB1 kezelések ET-függő PCD indukciót eredményeztek az EL és az oxidatív stressz (H_2O_2 akkumuláció) növelésével.

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7. List of publications MTMT Identifier: 10076143

7.1. Publications used in this thesis.

Iqbal, N., Czékus, Z., Poór, P., Ördög, A., 2021. Plant defence mechanisms against mycotoxin Fumonisin B1. Chem.-Biol. Interact. 343, 109494. https://doi.org/10.1016/j.cbi.2021.109494. **IF: 5.192**

Iqbal, N., Czékus, Z., Ördög, A., Poór, P., 2021. Ethylene-dependent effects of fusaric acid on the photosynthetic activity of tomato plants. Photosynthetica 59(2), 337-348. DOI 10.32615/ps.2021.029. **IF:2.482**

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Fareeha Saeed, Muhammad Shahzad, Nida Nazir, and **Nadeem Iqbal.** Effects of Ca_2SiO_4 Foliar Fertilization on the Growth and Mineral nutrient content of Maize Grown under Salinity Stress presented in *6th International Conference on Environmentally Sustainable Development (ESDev-2016)* April 25-27, 2016.

Statement

As the corresponding author of the following journal publications, I verify that all the results presented in this thesis and scientific publications were not used before to obtain any Ph.D. degree and will not be used in future as well.

Iqbal, N., Czékus, Z., Poór, P., Ördög, A., 2021. Plant defence mechanisms against mycotoxin Fumonisin B1. Chem.-Biol. Interact.343, 109494. https://doi.org/10.1016/j.cbi.2021.109494.

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