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BIOCHEMICAL CHANGES IN EARS OF WHEAT GENOTYPES SUBJECTED TO *FUSARIUM* SPP. ATTACK

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In wheat, *Fusarium* fungus promotes the appearance of destructive disease named as Fusarium head blight (FHB) that can cause grain yield reduction and mycotoxin accumulation. The focus of this research was to verify the influence of *Fusarium graminearum* and *F. culmorum* on wheat genotypes with different susceptibility to FHB: "Super Žitarka" (susceptible), "Lucija" (moderately resistant) and "Apache" (resistant). The experiment was performed under field conditions by artificial spore inoculation of ears at the flowering stage. The effectiveness of antioxidative enzymes, hydrogen peroxide (H_2O_2) content and malondialdehyde (MDA) content were observed at several sampling points after *Fusarium* inoculation (3, 15 and 24 hours). "Lucija" responded to pathogen by increase of guaiacol peroxidase (POD) activity, high H_2O_2 and MDA content in the early post-inoculation times (3 and 15 hours), compared to control. "Super Žitarka" displayed inhibition of catalase (CAT) activity throughout the whole time course of the experiment. Infected plants of "Apache" showed notable decline in MDA content over time. Moreover, in "Apache" increased H_2O_2 accumulation was observed immediately after *Fusarium* exposure (3 and 15 hours), compared to 24 hours. Rapid overproduction of H_2O_2 under *Fusarium* stress marked "Apache" as FHB-resistant.

Keywords: Enzyme antioxidative system – *Fusarium* spp. – H_2O_2 content – lipid peroxidation – wheat genotypes

INTRODUCTION

Plant exposure to pathogens leads to the numerous physiological and molecular changes within plant. Aside being harmful for the plant growth and development, pathogens can directly affect the production of agricultural and industrial important plants. One of the most widespread wheat disease is Fusarium head blight (FHB) caused by fungi *Fusarium* spp. [20]. The disease occurrence and incidence depend on environmental conditions such as humid and warm weather that promotes the spore dissemination. The most sensitive period for the plant infection is the time of flowering and early stages of seed development [9]. The first visible symptoms of FHB infection manifest as small colourless area at the base of the glume of the flower inside the inflorescence. In the later stages of infection, ears become bleached,

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redorange and even pink with the wrinkled, reduced-weight grains [8, 14, 16]. FHB speeding is also a health problem because infected grains may contain mycotoxins which consequently can be present in the human and animal nutrition [29].

Reactive oxygen species (ROS) are highly reactive particles that plants produce constitutively in the chloroplast, mitochondria and peroxisomes under normal physiological conditions. In such optimal conditions, their presence generally is not harmful for the cell [22]. The problem becomes greate when the plant finds itself under biotic and abiotic stress conditions resulting in increment of ROS concentration in the cell. Such extensive ROS levels impede the cellular metabolism, cause the DNA damages and disrupt the cell integrity [5]. Catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POD) are the most important antioxidative enzymes involved in the reduction of H_2O_2 to H_2O [4]. POD is a multiple-role enzyme involved mostly in the processes related with the cell wall modifications such as cross-linking of the structural proteins, lignification, suberization and cell elongation but also has a protective role in plant-pathogen interactions [2]. Polyphenol oxidase (PPO) as a part of non-enzymatic antioxidative mechanism is involved in a defence response provoked by pathogen [18]. Induced activity of the antioxidative system together with the non-enzymatic pathways appeared to be an important indicator for disease resistance [13, 25]. In contrast, ROS production can be beneficial for the plant immunity. Immediately after the pathogen attached to the plant surface, it triggers the rapid production of huge amounts of ROS, especially hydrogen peroxide (H_2O_2) in the specific reaction called "oxidative burst". Such an early event during incompatible plant-pathogen interaction occurs in order to prevent the pathogen entrance and spreading [15, 38, 39].

In our previous study, we tested the response of several wheat genotypes to FHB and noted that FHB-resistant genotype had quicker response to infection [34]. For this reason the objective of this study was to complete our previously published results by examining the response of three wheat genotypes "Super Žitarka" (FHB-sensitive), "Lucija" (FHB-moderately resistant) and "Apache" (FHB-resistant) to *Fusarium* infection, in order to verify whether there are any differences in their physiological response to infection. Furthermore, we want to explore which defence mechanisms contribute to FHB resistance.

MATERIALS AND METHODS

Inoculum production

Macroconidial inoculum was used for FHB disease screening in the Osijek field experiment in 2016. The inoculum was a combination of two isolates (50 : 50) consisted of *F. graminearum,* isolated from wheat loved in Croatia and *F. culmorum* was obtained from the Department of Biotechnology, IFA-Tulln, Austria. Inoculum was produced and quantified according to Lemmens et al. [17] and Snijders et al. [30], with minor modifications. The final concentration was set up to 1×10^5 ml^{-1}.

Plant material and treatment

In the first part of field experiments, ears of wheat genotypes were treated with the mixture of spore suspension while second part of experiment were control plants exposed to natural infection. Spray inoculations were carried out in the stage of flowering (Zadok's scale 65) [40]. All experiments (both control and infected) were repeated twice. For physiological and biochemical assays, plant tissue was collected after at 3, 15 and 24 hours and stored at –80 °C prior to analyses. The percentage of bleached spikelets (disease intensity) per plot was estimated according to a linear scale (0–100%) at 10, 14, 18, 22 and 26 days after inoculation. With this data, the area under disease progress curve (AUDPC) for FHB incidence was calculated for each entry [28]. FHB incidence per plot was considered as a measure for general resistance (GR). Disease incidence was used as an indicator for Type I+II resistance. The percentage of diseased heads was calculated after assessing a random sample of 30 heads on 10, 14, 18, 22 and 26 days after inoculation. AUDPC for FHB incidence was calculated. After ripening, a sample of 50 ears per subplot was harvested manually and weighed. After threshing grains were weighed and the percentage of kernels infected with Fusarium (% FCK) was assessed. For the determination of % FCK, 100 kernels of each genotype were randomly selected and incubated at 25 °C at a relative air humidity of 80%. After the 6th day of incubation, the percentage of *Fusarium* infected grains was calculated.

Enzyme extraction

Five ears of treated and non-treated (biological repetitions) plots were collected at each time point and pulled. Ears were ground by addition of polyvinylpolypyrrolidone (PVP), using pestle and mortar. Tissue (200 mg) was homogenized in 1 ml ice cold 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM ascorbate acid and centrifuged at 14,000 g for 15 min at 4 °C. Subsequently, re-extraction of tissue was done again with addition 1 mL of same buffer and obtained supernatants were used for protein concentration determination and assays of antioxidative enzyme activities. The protein concentration was measured using bovine serum albumin as a standard [7]. The activity of enzymes was expressed as units (U) per milligram of proteins [U mg^{-1} proteins].

Assays of antioxidant enzyme activities

POD [Enzyme Commission number (EC) 1.11.1.7] activity was measured at 470 nm as an increase in absorbance as a result of guaiacol (Sigma) oxidation [27]. The reaction mixture contained 0.2 mM potassium phosphate buffer (pH 5.8), 5 mM guaiacol and 5 mM H_2O_2. The reaction started by addition 25 µL of extract to 975 µL of reac-

tion mixture. APX (EC 1.11.1.1) was obtained by tracking the decline in absorbance of ascorbate at 290 nm [21]. The reaction started by addition of 10 µL 12 mM H_2O_2 to 990 µL reaction solution contained 50 mM potassium phosphate buffer (pH 7.0), 10 µL 25 mM ascorbate acid, 25 µL and 0.1 mM EDTA. CAT activity (EC 1.11.1.6) was estimated as decline in absorbance at 240 nm [1]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0) and 5 mM H_2O_2. Enzymatic decomposition of H_2O_2 occurred after addition of 50 µL of extract to 950 µL of reaction mixture. The activity of PPO (EC 1.14.18.1) was determined as an increase of absorbance at 430 nm as a result of breakdown of pyrogallol to o-quinones at 40 °C [26]. Reaction started by adding 15 µL of extract to reaction mixture (2 mL) consisted of 100 mM potassium phosphate buffer (pH 7.0) and 0.2 mL 100 mM pyrogallol.

Level of lipid peroxidation and H_2O_2 concentration

For lipid peroxidation and H_2O_2 content measurements ears tissue was collected in the same way as for the determination of enzyme activity. 400 mg of fine powder was homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA), centrifuged at 12,000 g for 15 min at 4 °C. Malondialdehyde (MDA) content, a final product of decomposition of biomembranes was measured [37]. 1 mL of 0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA was added to 0.5 mL of supernatant, heated at 95 °C for 30 min, cooled immediately in an ice bath and centrifuged at 14,000 g for 15 min at 4 °C. The absorbance of supernatant was measured at 532 nm for non-specific turbidity by subtracting the absorbance at 600 nm. As a blank 0.5% TBA in 20% TCA solution was used. Level of lipid peroxidation was expressed as MDA in nanomols per gram of fresh weight [nmol g^{-1}_{FW}], using an extinction coefficient of 155 mM^{-1} cm^{-1}. For the determination of H_2O_2 content, 0.5 mL of supernatant was added in mixture of 1M KI and 10 mM potassium phosphate buffer (pH 7.0) and left in dark conditions [36]. After 20 min, the absorbance was recorded at 390 nm and H_2O_2 content was determined using calibration curve and expressed as nanomoles per gram of fresh weight [nmol g^{-1}_{FW}].

Statistical analysis

All results (enzymatic activity, MDA and H_2O_2 content) were expressed as means of five (technical repetitions) with the corresponding standard errors (± SE). The data were analysed by ANOVA. Differences between means were compared by the post-hoc Duncan's test at the 0.05 probability level. Data were analysed by STATISTICA 13.1 (Stat Soft Inc., USA) software package.

RESULTS

The AUDPC for general resistance (Fusarium severity) in Apache (1.25) was lower in comparison to Lucija (50.8) and Super Žitarka (87.5) (Table 1). The Fusarium colonised kernels showed a similar trend in inoculated plants, where Super Žitarka showed 25% of colonized kernels in comparison to Apache (3%) (Table 1). For Lucija and Super Žitarka, there was a significant increase in symptoms at 22 days (days after inoculation) (Table 2). The *Fusarium* incidence increased from 13.1 to 45.0% for Lucija and from 16.7 to 58.0% for Super Žitarka from 22 to 26 days.

In treated plants of "Lucija", POD activity increased after 3 hours compared to control (Table 3b). In "Lucija", POD showed higher activity immediately after the inoculation (3 h) in comparison to 15 and 24 hours while in "Super Žitarka" POD activity was inhibited at 15 hours in regard to 3 and 24 hours (infected plants) (Tables 3a, 3b).

In all sampling points (3, 15 and 24 hours) infected plants of "Super Žitarka" showed decreased CAT activity, compared to untreated plants, while at 24 hours "Apache" had the same response to *Fusarium* stress (Tables 3a, 3c).

Table 1
AUDPC for general resistance (A), Type I+II resistance (initial infection) to FHB (B) and *Fusarium* colonized kernels for three wheat varieties (C)

Genotype	(A)	(B)	(C) (%)	Resistance/susceptibility*
"Apache"	1.25	20.81	3	R
"Lucija"	50.75	137.45	18	MR
"Super Žitarka"	87.50	164.14	25	S

*R – resistant; MR – moderate resistant; S – susceptible.

Table 2
Disease symptoms and diseased ears for general resistance for Type I+II after 10, 14, 18, 22 and 26 days (d)

Genotype	Disease symptoms (%)				
	10d	14d	18d	22d	26d
"Super Žitarka"	0	0	5	12.5	40
"Lucija"	0	0	1.5	5	27.5
"Apache"	0	0	0	0.5	0.5
	Diseased ears (%)				
	10d	14d	18d	22d	26d
"Super Žitarka"	0	8.33	11.665	16.66	58.33
"Lucija"	3.33	3.33	9.995	13.33	44.995
"Apache"	0	0	3.33	3.33	4.995

Table 3a

Effects of *Fusarium* spp. on activity of antioxidative enzymes, MDA content and H_2O_2 concentration in genotype "Super Žitarka" at 3, 15 and 24 hours in control (0) and treated (T) plants

"Super Žitarka"	3h		15h		24h	
	0	T	0	T	0	T
POD	5.73 ± 0.43a	5.52 ± 0.15a	4.92 ± 0.49bc	4.62 ± 0.44b	5.23 ± 0.51ac	5.66 ± 0.27a
APX	0.69 ± 0.14a	0.54 ± 0.04a	0.55 ± 0.13a	0.56 ± 0.05a	0.62 ± 0.07ab	0.62 ± 0.06ab
CAT	0.022 ± 0.002c	0.017 ± 0.001ab	0.019 ± 0.002bc	0.016 ± 0.002a	0.022 ± 0.003c	0.018 ± 0.002ab
PPO	0.09 ± 0.01a	0.07 ± 0.01c	0.08 ± 0.01ac	_0.07 ± 0.01bc	0.08 ± 0.01abc	0.09 ± 0.01a
H_2O_2	173.56 ± 6.87a	177.20 ± 15.95abc	191.62 ± 10.22ac	197.95 ± 11.08a	195.48 ± 9.45a	187.93 ± 7.06abc
MDA	2.89 ± 1.07a	3.35 ± 0.34ab	4.15 ± 0.34c	3.61 ± 0.29abc	4.04 ± 0.15bc	3.37 ± 0.35ab

Remarks: Values are means ± SE (n≥5). Different lower-case letters indicate significantly different values (P<0.05) within each wheat genotype separately.

Table 3b

Effects of *Fusarium* spp. on activity of antioxidative enzymes, MDA content and H_2O_2 concentration in genotype "Lucija" at 3, 15 and 24 hours in control (0) and treated (T) plants

"Lucija"	3h		15h		24h	
	0	T	0	T	0	T
POD	5.23 ± 0.31a	6.83 ± 1.04b	4.91 ± 0.34a	5.67 ± 0.46a	5.18 ± 0.61a	5.68 ± 0.79a
APX	0.43 ± 0.08a	0.49 ± 0.08a	0.42 ± 0.07a	0.56 ± 0.13a	0.50 ± 0.08a	0.51 ± 0.13a
CAT	0.013 ± 0.002a	0.015 ± 0.003a	0.015 ± 0.003a	0.017 ± 0.004a	0.017 ± 0.005a	0.014 ± 0.002a
PPO	0.12 ± 0.01b	0.11 ± 0.01ab	0.11 ± 0.01ab	0.10 ± 0.01a	0.12 ± 0.01b	0.10 ± 0.01a
H_2O_2	147.96 ± 13.55a	178.84 ± 21.32b	142.14 ± 7.14a	195.10 ± 19.51b	146.14 ± 4.47a	219.01 ± 12.77c
MDA	3.43 ± 0.31a	7.46 ± 0.81ef	2.56 ± 0.12b	8.35 ± 0.58f	3.19 ± 0.38a	4.39 ± 0.20c

Remarks: Values are means ± SE (n≥5). Different lower-case letters indicate significantly different values (P<0.05) within each wheat genotype separately.

Table 3c

Effects of *Fusarium* spp. on activity of antioxidative enzymes, MDA content and H_2O_2 concentration in genotype "Apache" at 3, 15 and 24 hours in control (0) and treated (T) plants

"Apache"	3h		15h		24h	
	0	T	0	T	0	T
POD	4.32 ± 0.49b	4.88 ± 0.67ab	4.87 ± 0.25ab	5.09 ± 0.55a	5.14 ± 0.44a	4.87 ± 0.36ab
APX	0.61 ± 0.03a	0.67 ± 0.08a	0.63 ± 0.09a	0.72 ± 0.12a	0.68 ± 0.02a	0.66 ± 0.04a
CAT	0.027 ± 0.004a	0.023 ± 0.003ab	0.025 ± 0.003ab	0.022 ± 0.003ab	0.026 ± 0.028a	0.021 ± 0.003b
PPO	0.14 ± 0.03c	0.10 ± 0.01b	0.10 ± 0.01ab	0.08 ± 0.01a	0.08 ± 0.01ab	0.08 ± 0.003ab
H_2O_2	152.12 ± 2.39ab	158.67 ± 5.34bc	150.54 ± 4.91a	160.30 ± 8.18cd	167.36 ± 4.03d	134.15 ± 6.49e
MDA	3.56 ± 0.38bc	4.81 ± 0.37d	3.27 ± 0.25ab	3.82 ± 0.33c	3.28 ± 0.43ab	3.01 ± 0.32a

Remarks: Values are means ± SE (n≥5). Different lower-case letters indicate significantly different values ($P<0.05$) within each wheat genotype separately.

For APX activity no significant interactions were noticed in infected plants (all P-values > 0.05) (Tables 3a–c).

Considering PPO activity, Duncan test showed a significant interaction between genotype and experimental period under control conditions (P = 0.001725) and *Fusarium* stress (P = 0.0035). "Super Žitarka" exhibited after 3 hours decreased PPO activity, compared to untreated plants (Table 3a). The same genotype showed decreased PPO activity under treatment both after 3 and 15 hours, compared to 24 hours.

Considering MDA content, in "Apache" and "Lucija", the time had a significant influence on treatment (P < 0.0001). *Fusarium* treated plants of "Lucija" showed increased MDA content at each point of collection (3, 15 and 24 hours) in regard to respective control plants while "Apache" showed increase in MDA content in comparison to untreated plants at 3 and 15 hours (Tables 3b, 3c). "Lucija" under pathogen infection exhibited the highest MDA content after 15 hours, compared to 24 hours, and increased MDA content after 3 hours, compared to treatment for 24 hours. Infected plants of "Apache" revealed a decline in MDA content at 15 and 24 hours compared to treatment for 3 hours, but higher MDA content at 15 hours than at 24 hours after treatment.

Regarding of H_2O_2 content Duncan test showed significant interaction between genotype and time of exposure under control conditions (P = 0.003651) and *Fusarium* treatment (P = 0.000044). Treated plants of "Lucija" showed significantly higher H_2O_2 concentration than control at all time points (3, 15 and 24 hours) of treatment (Table 3b). Furthermore, at 24 hours, H_2O_2 content increased in comparison to 3 or 15 hours. Stressed-plants of "Apache" showed higher H_2O_2 content in treated plants, at 15 hours compared to control, and decreased H_2O_2 concentration under infection compared to control at 24 hours (Table 3c).

DISCUSSION

The lower *Fusarium* symptoms in "Apache" compared to "Lucija" and "Super Žitarka" confirmed the variation in resistance, which ranked them as resistant ("Apache"), moderately resistant ("Lucija") and susceptible ("Super Žitarka") to FHB [12, 33].

Increased expression of POD activity under pathogen attack generates the toxic ambience for the fungi growth through rapid oxidative burst reaction [3, 24]. "Lucija" showed remarkable POD activity immediately after the inoculation (at 3 hours), in comparison to control, referring that in this genotype POD perhaps had an important role in early defence response to *Fusarium*. Diseased plants of "Lucija" also showed remarkable H_2O_2 accumulation at all time-points, compared to control. Moreover, in "Apache" increased H_2O_2 accumulation was observed immediately after *Fusarium* exposure (at 3 and 15 hours), compared to that observed after 24 hours. Such a response might be connected to the formation of the natural barrier against *Fusarium* penetration at the beginning of the spore infestation. A similar result was found in

FHB-partially resistant wheat genotype "Gaskozhen", which responded to *Fusarium graminearum* and *F. culmorum* by enhanced production of H_2O_2 and high induction of POD [13]. On the other hand, induced POD activity detected in "Lucija" after *Fusarium* attack could be the result of the "oxidative burst". Studies aimed at the intensity of oxidative burst in plants exposed to fungi elicitors demonstrated that the host response to the elicitor showed a delayed reaction (after 8–12 hours) in the manner of ROS generation, but the visible symptoms were already notable after 2–4 hours [11, 38]. Moreover, high H_2O_2 levels appear to inactivate CAT and APX activities referring them as less effective in H_2O_2 removal. Based on this observation we conclude that in "Lucija" *Fusarium* promotes an oxidative burst, which is a crucial event in compatible host-pathogen interaction. A correlation between ROS generation in host cells infected by necrotrophic fungi and pathogen survival was also observed [13]. In the same study, after experimental induction of oxidative burst by glucose/glucose oxidase treatment in wheat leaves, the increase of H_2O_2 and O_2^- concentration caused cell death, providing thus nutrition for the pathogen.

Considering the MDA content, partially FHB-resistant "Lucija" revealed increased MDA values, compared to the untreated group, through the whole time course of the experiment, which is likely connected with the remarkable increase of H_2O_2 concentration and indicates that rapid oxidative explosion caused the destruction of membranes leading to cell death. Although "Lucija" showed great POD activity under infection (especially at 3 hours after treatment), its expression cannot be linked with *Fusarium* resistance due to extensive MDA content. Increased MDA content and remarkably higher H_2O_2 accumulation was also noticed in FHB-sensitive wheat cultivar "Falat" and the FHB-resistant wheat cultivar "Sumai3" subjected to *F. graminearum* [31].

Inoculation of "Super Žitarka" with Fusarium spores revealed inhibition of CAT activity after 3, 15 and 24 hours, of the treatment and of the PPO activity 3 hours, after the spore addition, compared to control group, suggesting that CAT and PPO activity levels may be associated both with sensitivity and disease symptoms. The link between disease appearance (caused by *Pseudomonas syringae* pv tomato) and PPO was described in transgenic tomato genotypes with both enhanced or reduced PPO activity [35]. PPO-supressed tomato genotypes had more pronounced disease symptoms, while PPO-enhanced genotypes showed small disease occurrence.

Agent of being involved in ROS generation under different environmental stress factors in order to improve the pro-antioxidative activity of plant, the role of PPO has also been connected with non-enzymatic plant defence response against a pathogen [6, 23]. In infected plants of "Apache" PPO and CAT activity decreased after 3 hours and 24 hours, compared to control group. Moreover, H_2O_2 content increased in "Apache" (at 15 hours), compared to the control, but at the last time point of treatment (24 hours) it decreased, suggesting possible changes in the regulatory pathways. Namely, in response to pathogen plant restricts its entrance by stomata closure regulated by salicylic acid (SA), which may evoke the inhibition of CAT and APX activity, increasing thus H_2O_2 accumulation [10]. Inhibition of CAT activity under exposure of FHB-resistant wheat genotype "Sumai3" to *F. graminearum* crude extract was

also noticed by Soranhinobar et al. [32]. However, in spite of enzymes inhibition, "Apache" was able to prevent somehow the membrane damages under infection, which is reflected by the decline of MDA content. The observation leads to the conclusion that in the FHB-resistant "Apache" the antioxidative system is not involved in the defence response. However a different observation was reported in our previous work where we pointed out the importance of early response of antioxidative system in developing FHB-resistance [34]. Such differences in the resistance reaction of FHB-resistant, wheat genotypes could be ascribed to different biological properties of the *Fusarium* races. It was shown that aggressiveness of *F. graminearum* isolates showed a high level of genetic variations even in samples that were growing at same field location [19].

CONCLUSIONS

Our study revealed various physiological responses of wheat genotypes with different genetic backgrounds to *Fusarium*. In spite of rapid induction of POD (at 3 hours) and increased H_2O_2 accumulation over time, compared to control, moderately FHB-resistant genotype "Lucija" cannot be characterized as FHB-resistant due to the high MDA content. Exposure of FHB-resistant genotype to *Fusarium* sp. showed higher H_2O_2 accumulation at the beginning of treatment (at 3 and 15 hours), compared to a 24-hour treatment. These findings show that a resistant genotype reacts more promptly to FHB disease. FHB-susceptible genotype "Super Žitarka" under infection showed decline in CAT activity, compared to control (at in all XXXX points), suggesting its inefficiency in responding to biotic stress caused by *Fusarium*.

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