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## Time-course experiment of *Fusarium* infestation of wheat genotypes with the emphasis on the physiological response

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### ABSTRACT

Wheat (*Triticum aestivum* L.) is one of the most significant food cereal in the world. Under natural conditions, biotic and abiotic stress factors can seriously endanger the plant growth and development. Fusarium head blight (FHB), mainly caused by *F. graminearum* and *F. culmorum*, is a disease that has negative effects on economy, namely on the yield and the quality of the grain. In this research, the activities of guaiacol peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and polyphenol oxidase (PPO), as well as H<sub>2</sub>O<sub>2</sub> concentration and malondialdehyde (MDA) content, were determined in three wheat genotypes of various resistance to FHB (“Super Žitarka”-sensitive; “Lucija”- moderately resistant, and “Apache”- resistant) after the 2<sup>nd</sup> and the 4<sup>th</sup> day of infestation. The infected plants of “Lucija” showed higher POD activity compared to control after the 4<sup>th</sup> day, and increased POD activity compared to the 2<sup>nd</sup> day of infection. On the 2<sup>nd</sup> day, “Apache” responded to pathogen by the enhanced APX activity, and higher MDA level, compared to the 4<sup>th</sup> day of treatment when it started to decrease. Moreover, the treated plants of the same genotype showed the increased MDA level on the 2<sup>nd</sup> day, unlike the untreated plants. Contrarily, H<sub>2</sub>O<sub>2</sub> concentration in “Apache” showed the tendency to increase with the time of *Fusarium*-exposure. In comparison to the 2<sup>nd</sup> day of treatment, PPO activity decreased in “Super Žitarka” on the 4<sup>th</sup> day. In all genotypes at both sampling points, CAT activity did not change significantly under the pathogen attack. Overproduction of H<sub>2</sub>O<sub>2</sub> accompanied with the increased APX activity 2 days after infection may lead to the conclusion that FHB tolerance of “Apache” is ensured by the earlier activation of the defence mechanisms.

### Introduction

Fusarium head blight (FHB) caused by *Fusarium* species is an economically important wheat (*Triticum aestivum* L.) disease that has a negative impact on the yield and grain quality. Moderate to warm temperatures in conjunction with high humidity during the stage of anthesis and early kernel development increase the possibility of infection (Gilbert and Tekauz, 2000). Infected kernels become bleached, while contaminated grains

are often shrivelled with the presence of mycotoxins that have a harmful effect on human and animal health (Bai and Shaner, 2004).

In nature, plant copes with a numerous biotic and abiotic stresses. Biotic stresses include plant infestation by different pathogen elicitors or insects. Exposure of plants to *Fusarium* infection results in the activation of plant defence mechanisms (Nanda et al., 2010). These mechanisms can provide the efficient protection for the plant against reactive oxygen species (ROS) in the manner they remove them. ROS pool consists of H<sub>2</sub>O<sub>2</sub>, singlet oxygen (<sup>1</sup>O<sub>2</sub>), superoxide (O<sub>2</sub><sup>-</sup>) and hydroxyl (OH<sup>•</sup>) radicals

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and their generation in plant cell depends on the environmental conditions (Thannickal and Fanburg, 2000). Overaccumulation of ROS in plant leads to a disruption of the membranes' phospholipid bilayer, DNA damages and protein denaturation, thereby affecting the physiological processes in plants (Dat et al., 2000; García-Limones et al., 2002). Under the stress exposure, plant activates an enzymatic antioxidative system, which includes catalase (CAT), ascorbate peroxidase (APX) and non-specific peroxidase (POD), as well as nonenzymatic antioxidative detoxifying enzymes such as polyphenol oxidase (PPO). Major role of PPO is to eliminate ROS using flavonoids and phenolics as substrates (Boeckx et al., 2015; Mayer, 2006). There is a growing body of evidence suggesting that high induction of antioxidative enzymes in plants contributes to the pathogen resistance (Madadkhah et al., 2012; Racchi, 2013; Shahbazi et al., 2010; Sorahinobar et al., 2015).  $H_2O_2$  has the poorest reactivity and ability to diffuse easily across the membranes, which distinguishes it from other ROS particles. One of the roles of  $H_2O_2$  under pathogen attack is to reduce the growth and viability of pathogen in order to restrict its entrance into plant (Kuzniak and Urbanek, 2000).

The aim of this research was to explore the effect of *Fusarium* spp. in three wheat genotypes with different FHB resistance ("Super Žitarka"-sensitive; "Lucija"- moderately resistant, and "Apache"- resistant) after the 2<sup>nd</sup> and the 4<sup>th</sup> day of infestation by determining the activity of the antioxidative enzymes (APX, CAT, and POD), lipid peroxidation content,  $H_2O_2$  level and PPO activity.

## Materials and methods

### *Field trials and pathogen inoculum preparation*

The experiment was carried out under field conditions. Wheat spikes of the wheat genotypes ("Super Žitarka", "Apache" and "Lucija") were treated with a mixture of *Fusarium graminearum* and *Fusarium culmorum* (stressed plants), while the second group of plants were left to natural infection (control). *Fusarium* treatment was performed by spraying the spikes at flowering (Zadok's scale 65) (Zadoks et al., 1974) using a tractor back sprinkler in the afternoon hours. The plants were irrigated few times during the day in order to maintain moisture on the ears.

After the 2<sup>nd</sup> and 4<sup>th</sup> day of *Fusarium* treatment, spikes were collected and stored at -80 °C prior to analyses. For the antioxidative enzymes activity, fresh tissue (200 mg) was ground into fine powder by using pestle and mortar, homogenized in

50 mmol/dm<sup>3</sup> potassium phosphate buffer (pH 7.0) by adding 0.1 mmol/dm<sup>3</sup> of ethylenediaminetetraacetic acid, 5 mmol/dm<sup>3</sup> of ascorbate acid and polyvinylpolypyrrolidone (PVP), centrifuged at 14 000 rcf for 30 min at 4 °C. From the obtained supernatants, the enzyme activity and the total protein content (Bradford, 1976) were measured. The activity of enzymes was expressed as units (U) per milligram of proteins [ $U\ mg^{-1}\ proteins$ ].

### *Activity of antioxidative enzymes*

The POD activity was assayed at 470 nm following the method by Siegel and Galson (1967) as peroxidation of hydrogen peroxide with guaiacol as an electron donor. CAT activity was determined by the decline in absorbance at 240 nm (Aebi, 1984). APX activity was analysed by tracking the decline in absorbance of ascorbate at 290 nm (Nakano and Asada, 1981). PPO activity was estimated at 430 nm as a rate of oxidation of pyrogallol by the increase in absorbance (Raymond et al., 1993). Enzymes' activity was presented as units (U) of enzyme activity per milligram of protein [ $\mu\text{mol}\ \text{min}/\text{mg}/\text{proteins}$ ].

### *MDA content and $H_2O_2$ concentration*

For the MDA and  $H_2O_2$  determination, the spikes (400 mg) were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12 000 rcf for 15 min at 4 °C. The MDA content, final product of the lipid peroxidation, was determined according to protocol by Verma and Dubey (2003).

5 % TBA in 20% TCA was added to 0.5 mL of supernatant, heated at 95 °C for 30 min and cooled in an ice bath. After centrifugation (14 000 rcf for 15 min at 4°C), absorbance of supernatant was measured at 532 nm and 600 nm. The data for non-specific absorption at 600 nm was subtracted. As a blank, the 0.5 % TBA in 20 % TCA solution was used. The MDA content was calculated using the molar extinction coefficient as 155 mmol/dm<sup>3</sup>/cm.

$H_2O_2$  concentration was determined using the protocol by Velikova et al. (2000). The 0.5 mL of extract was added to the mixture of 10 mmol/dm<sup>3</sup> potassium phosphate buffer (pH 7.0) and 1 mol/dm<sup>3</sup> KI and kept in darkness for 20 min. Absorbance of the mixture was read at 390 nm.  $H_2O_2$  content was defined using a calibration curve obtained with  $H_2O_2$  solutions ranging from 20 to 700  $\mu\text{mol}/\text{dm}^3$  and expressed as millimoles per gram of fresh weight [ $\text{mmol}/\text{g}_{\text{FW}}$ ].

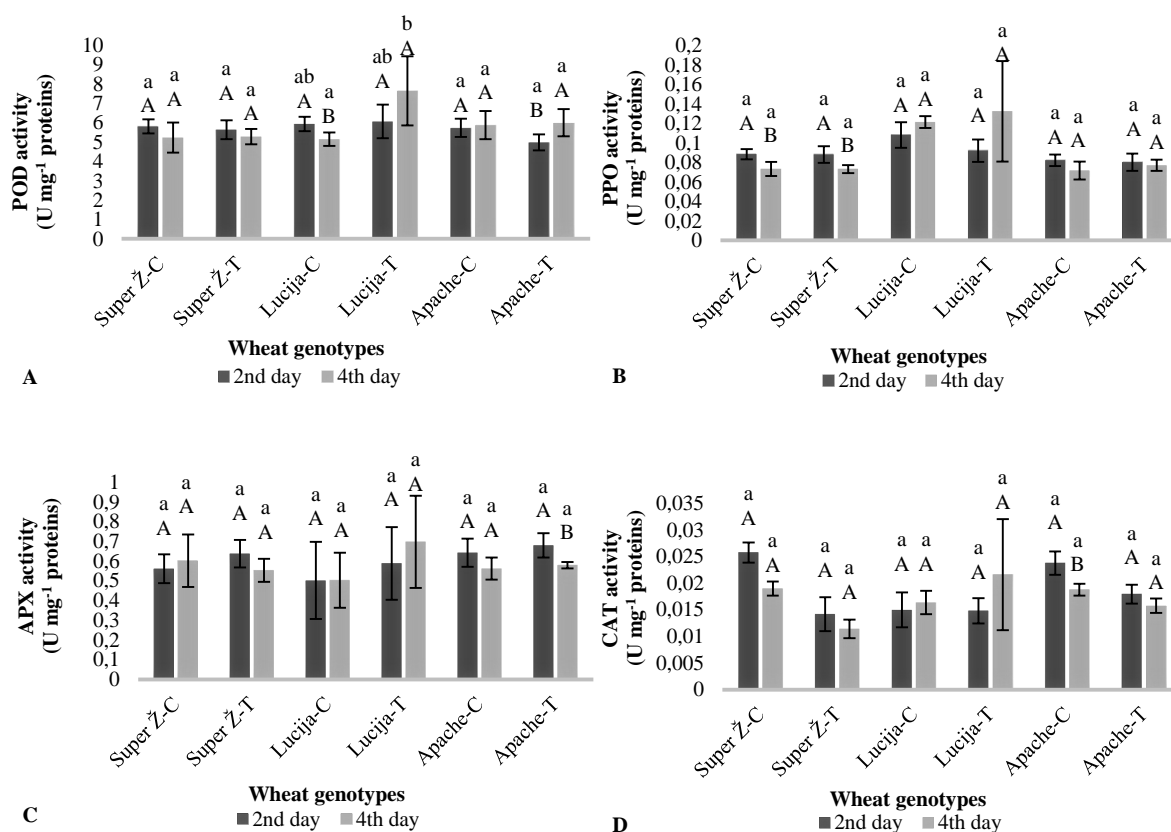
All results were expressed as means of five replicates with the corresponding standard errors. Differences between means were evaluated using the nonparametric Kruskal-Wallis test using Statistica 13 software.

## Results and discussion

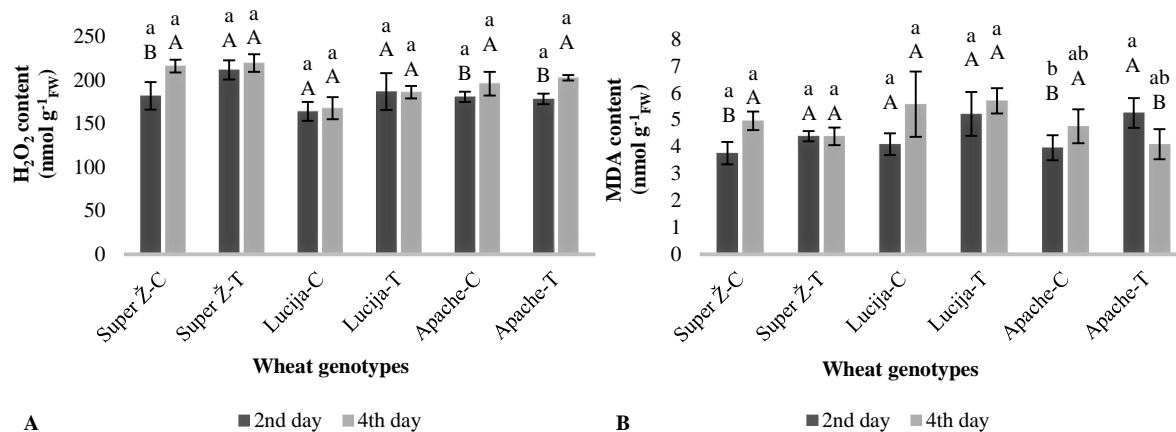
In plants, increased POD and PPO activity are mostly related to the straightening of the cell wall in order to restrict the pathogen entrance (Mohammadi and Karr, 2002; Morkunas and Gemerek, 2007). In stressed plants of “Apache”, POD activity increased over time (it was higher on the 4<sup>th</sup> day in comparison to the 2<sup>nd</sup> day of infection) (Fig. 1A). Such a delayed reaction could be the result of the stronger macroconidia colonization and penetration of the host plant cells. Similar result was observed in FHB-resistant wheat genotype “Vulkan”, in which *Fusarium* treated plant exhibited higher POD induction at a later point of collection (96 hai) than at 48 hai (Španić et al., 2017). The partially FHB-resistant genotype “Lucija” showed an increase in POD activity under *Fusarium* attack on the 4<sup>th</sup> day in regard to respective control group (Fig. 1A) suggesting that POD in “Lucija” is involved in overcoming stress injuries provoked by pathogen. The increase of POD activity after the 4<sup>th</sup> day of

infection was also described in tomato leaves exposed to *Botrytis cinerea* (Kuźniak and Skłodowska, 2005).

Apart from the involvement in ROS removal, the role of PPO is connected with non-enzymatic plant defence response against pathogen (Boeckx et al., 2015; Parveen et al., 2010; Thipyapong et al., 2004). In all tested genotypes, PPO activity did not show significant changes during time-course experiment (Fig. 1B). Exception was FHB-sensitive “Super Žitarka”, which responded to pathogen elicitor by an enhanced expression of PPO activity at an earlier time-point (2<sup>nd</sup> day) compared to later sampling time (4<sup>th</sup> day), which lead us to conclusion that “Super Žitarka” activates the non-enzymatic antioxidative system in response reaction earlier. On the other hand, FHB-sensitive wheat cultivar “Falat” showed induction of PPO activity after 5 days of *Fusarium* stress (Sorahinobar et al., 2015). The same was noticed in roots of *Fusarium*-susceptible melon genotypes on the 4<sup>th</sup> day of exposure to *F. oxysporum* f. sp. *melonis* (Madadkhah et al., 2012).



**Fig. 1.** Activity of POD (A), PPO (B) APX (C) and CAT (D) in ears of wheat genotypes (“Super Žitarka”, “Apache” and “Lucija”) infected with *Fusarium* spp. after 2<sup>nd</sup> and 4<sup>th</sup> day (C-control, T-treatment). Values are means of five replicates  $\pm$  SE. Different capital letters indicate significantly different values ( $p < 0.05$ ) among different time points and different genotypes under the same treatment, while different lower-case letters represent significantly different values ( $p < 0.05$ ) within each genotype separately at same sampling point.



**Fig. 2.** H<sub>2</sub>O<sub>2</sub> (A) and MDA content (B) in ears of wheat genotypes “Super Žitarka”, “Apache” and “Lucija” infected with *Fusarium* spp. after 2<sup>nd</sup> and 4<sup>th</sup> day (C-control, T-treatment). Values are means of five replicates ±SE. Different capital letters indicate significantly different values ( $P < 0.05$ ) among different time points and different genotypes under the same treatment, while different lower-case letters represent significantly different values ( $P < 0.05$ ) within each genotype separately at same sampling point.

In “Apache”, at an early time point (2<sup>nd</sup> day) of infection, activity of APX was higher than on the 4<sup>th</sup> day (Fig. 1C) indicating that this genotype involved APX more in early response to *Fusarium*. The rapid APX activity under *Fusarium* treatment was also noticed in FHB-resistant wheat genotype “Vulkan” (Španić et al., 2017). Moreover, in “Apache”, H<sub>2</sub>O<sub>2</sub> concentration (Fig. 2A) was lower on the 2<sup>nd</sup> day of infection compared to the 4<sup>th</sup> day, which could be the result of an increased APX activity suggesting that APX had an important role in the early ROS decomposition. The same was found in pea (*Pisum sativum* L.) - *Sclerotinia sclerotiorum* interaction (Jain et al., 2013). The great importance of APX in ensuring the disease resistance was also noticed in *Avena sativa* subjected to *Puccinia coronata* stress (Figueiró et al., 2015).

Considering H<sub>2</sub>O<sub>2</sub> production under stressful environment, “Apache” exhibited overproduction of H<sub>2</sub>O<sub>2</sub> on the 4<sup>th</sup> day in comparison to the 2<sup>nd</sup> day (Fig. 2A). Higher H<sub>2</sub>O<sub>2</sub> accumulation was found 72 h after the infection of *Fusarium* tolerant-iso banana genotype with *Fusarium oxysporum* (Li et al., 2011). In our study, overproduction of H<sub>2</sub>O<sub>2</sub> in “Apache” under pathogen stress could be the result of its participation in plant defence mechanisms. Recent reports indicate that in response to elicitor, plant can increase H<sub>2</sub>O<sub>2</sub> level in order to induce the defence (Vranova et al., 2002) and resistant regulatory genes (Mittler et al., 2004).

MDA content, final product of lipid peroxidation, reached higher values in *Fusarium* exposed plants of “Apache” than control plants on the 2<sup>nd</sup> day (Fig. 2B).

Our results are similar to research by El-Zahaby et al. (1995) who noted increased MDA content in resistant variety of barely after exposure to powdery mildew fungi. Furthermore, in “Apache”, MDA content showed decline after the 4<sup>th</sup> day of infection in regard to the 2<sup>nd</sup> day (Fig. 2B). Higher induction of POD activity (Fig. 1A), accompanied with the low MDA values (Fig. 2B) on the 4<sup>th</sup> day of treatment lead us to conclusion that POD in “Apache” plays an important role in maintaining the integrity of the membranes providing thus FHB-resistance. This is in accordance with the research by Zhang et al. (2013) where peroxidase increased remarkably in resistant wheat variety compared to susceptible one.

## Conclusion

In “Apache”, *Fusarium* exposure caused the most notable changes in activity of the antioxidative enzymes. At an earlier time-point of infection (2<sup>nd</sup> day), the same genotype activates APX in response to *Fusarium*, while POD is a part of later defence strategies (4<sup>th</sup> day). Based on this, we can conclude that in “Apache”, APX and POD are important enzymes in providing the FHB-resistance. Unlike FHB-resistant “Apache”, treated plants of FHB-susceptible “Super Žitarka” sooner displayed the higher PPO activity (2<sup>nd</sup> day), which refers to the inclusion of non-enzymatic antioxidative as a reaction to *Fusarium* stress. In all tested genotypes, CAT did not show appreciable changes during *Fusarium* infection, which characterized it as a non-specific marker in screening the FHB disease.



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