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Research article

Early response of wheat antioxidant system with special reference to Fusarium head blight stress

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ABSTRACT

Fusarium head blight (FHB) is a destructive fungal disease of wheat (*Triticum aestivum* L.) that causes significant grain yield losses and end-use quality reduction associated with contamination by the mycotoxin deoxynivalenol (DON). Three winter wheat varieties ('Vulkan', 'Kraljica' and 'Golubica') were screened for FHB resistance using artificial inoculation technique under field conditions. The aim of this study was to examine a relationship between FHB resistance and the effectiveness of enzyme antioxidant system of wheat varieties under different sampling times (3, 15, 24, 48, 96, 120 and 336 hai). In the time-course experiments FHB-resistant variety 'Vulkan' showed rapid induction of ascorbate peroxidase (APX) and polyphenol oxidase (PPO) activity in the early stages after infection (3 hai) and it seems that in 'Vulkan' FHB-resistance is associated with antioxidative enzymes activity. Moderately FHB resistant variety 'Kraljica' showed the higher guaiacol peroxidase (POD) activity and higher H_2O_2 content after 24 hai, increased malondialdehyde (MDA) content at the beginning of infection (3, 15 hai) while induction of catalase (CAT), APX and PPO was delayed. FHB-susceptible variety 'Golubica' involved antioxidant enzymes in defense response much later. Based on our results the activity of antioxidant enzymes (APX and PPO) was more pronounced in 'Vulkan' than in FHB-medium resistant variety 'Kraljica' and FHB-susceptible 'Golubica'. The differences in antioxidant response of wheat varieties under Fusarium infestation could be the result of genetic properties.

1. Introduction

In natural conditions, plants are exposed to both abiotic and biotic stress factors that can seriously endanger plant growth and production (Mittler et al., 2004). The most common stress factors are salt, drought, water excess, UV-B radiation, extreme temperatures, chemicals, ozone, oil nutrient deprivation and pathogen attack (Caverzan et al., 2012). Fusarium head blight (FHB) caused by fungi *Fusarium* spp., is one of the most important wheat (*Triticum aestivum* L.) disease that can reduce yields up to 30–50% during the epidemic years (McMullen et al., 1997). Aside from its destructive role in overall crop production, fungi also produce mycotoxins that can be, in high levels, toxic to humans and livestock. The infection process begins with brown spots on the glumes and rachis, where head may appear bleached. For these reasons, the best strategy for controlling FHB is breeding of new varieties with reduced susceptibility.

In plants, biotic stress stimulates the overproduction of reactive oxygen species (ROS), often called oxidative stress. These highly reactive particles such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$) and the hydroxyl radical ($OH^{\bullet}$) interact with cell molecules thereby causing the denaturation of proteins, DNA damage or degradation of membrane lipids. In order to reduce deleterious effect of ROS, plants have developed enzymatic and non-enzymatic antioxidant defense mechanisms that are involved in ROS detoxification. The most important enzymes include guaiacol peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD). Involvement of antioxidant enzymes in ROS removal is considered as a great indicator of genotype resistance under pathogen infestation (Madadkhah et al., 2012; Sorahinobar et al., 2015). SOD is a first line of defense against ROS directly involved in dismutation of superoxide anion to H_2O_2 which is converted by CAT and peroxidases to oxygen and water (Dat et al., 2000). Polyphenol oxidase (PPO) is a multi-copper oxidase, involved in non-enzymatic plant response to

Abbreviations: FHB, Fusarium head blight; hai, hours after inoculation.

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environmental stresses. Although, there are numerous different opinions about the role of PPOs under stress conditions (Fothergill and Rees, 2006), PPO is directly involved in ROS detoxification by oxidation of flavonoids and phenolic acids induced by pathogen attack (Madadkhah et al., 2012).

The aim of this research was to investigate the influence of *Fusarium* spp. in the ears of three wheat varieties ('Golubica', 'Kraljica' and 'Vulkan') after 3, 15, 24, 48, 96, 120 and 336 h after Fusarium inoculation by determining hydrogen peroxide concentration, lipid membrane peroxidation and activities of APX, POD, PPO and CAT in order to explore whether there is a possibility of using antioxidant enzyme system as an indicator of Fusarium resistance. We also wanted to verify if there are differences in response of wheat varieties to Fusarium attack. It is important to note that most of previous studies on antioxidant pathways were performed in controlled environment, but which cannot fully reveal real field conditions. In our research the results of antioxidant system measurement in the field are presented. To our knowledge, this is the first report on the early response of antioxidant enzymes after inoculation with Fusarium.

2. Materials and methods

2.1. Inoculum production

Inoculum was a mixture of two different *Fusarium* species (1:1). *F. culmorum* strain (IFA 104), DON chemotype and highly aggressive, was obtained from Institute of Biotechnology, IFA-Tulln, Austria. *F. graminearum* isolate was collected from the field of eastern Croatia obtained from a single spore technique.

To produce macroconidia of *F. culmorum*, a mixture of wheat and oat grains (3:1 by volume) was soaked in water overnight in 250 mL glass bottles. Water was decanted and seeds autoclaved. After seeding with the *Fusarium* strain, the seeds were kept for 2 weeks at 25 °C in the dark and thereafter incubated in the refrigerator for 3 weeks. Conidia were washed from the kernels and the concentration of the conidial suspension was set to 1×10^5 mL^{-1}. Inoculum with *F. graminearum* was prepared with the bubble breeding method using a liquid mung bean medium (Lemmens et al., 2004). Final concentration of the conidial suspension of *F. graminearum* inoculum was set to 1×10^5 mL^{-1}.

The spore suspensions were set to a concentration so that single bottle of one strain contained a sufficient amount of suspension (>900 mL) which could be diluted in 100 L of water right before inoculation (100 mL per m^2).

2.2. Field trial

The field trial was set up at the Agricultural Institute Osijek (45°32′N, 18°44′E). The soil type was eutric cambisol. The average annual precipitation in vegetation period in 2015/16 was 595 mm and the average annual temperature was 9.73 °C. The experimental plot area was 7.56 m^2, where one treatment (control and artificially inoculated) was replicated in two plots. Spray inoculations with *Fusarium* species were performed on varieties during flowering time (Zadok's scale 65) using tractor back-sprayer in the late afternoon and repeated two days later. To maintain moisture at ears we sprayed water with tractor back-sprayer on several occasions during the day. All three genotypes are Croatian winter wheat varieties ('Vulkan', 'Kraljica' and 'Golubica') developed at Agricultural Institute Osijek. Variety 'Vulkan' is a bread wheat with high yield, moderate quality and early heading, earlier characterized as FHB resistant (Spanic and Drezner, 2011), while 'Kraljica' has good quality and high yield with lodging resistance. 'Golubica' is a high quality variety with moderate yield, previously characterized as Fusarium susceptible (Spanic et al., 2013).

The percentage of bleached spikelets (disease intensity) per plot was estimated according to a linear scale (0–100%) on days 10, 14, 18, 22 and 26 after inoculation. With this data the area under disease progress curve (AUDPC) for FHB intensity was calculated for each entry. FHB intensity per plot was taken as a measure for general resistance. Type I resistance represented a percentage of diseased ears per plot. 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. Inoculated and control ears were sampled at 3, 15, 24, 48, 96, 120 and 336 h after inoculation (hai) and frozen at −80 °C until use. At 336 h, there was the appearance of the first FHB symptoms and these heads were taken for further analysis. After harvest, for the determination of percentage of Fusarium colonized kernels (FCK), 100 kernels of each variety were randomly selected and washed in 90% ethanol for 20 s. After alcohol evaporation, the seeds were placed on moist filter paper in Petri dishes and incubated at 25 °C at a relative air humidity of 80%. On the 6th day after incubation the percentage of Fusarium infected kernels was assessed.

2.3. Enzyme activity

For enzyme extraction, five replicates were taken from the control and infected plants. Each sample was consisted of five ears. Ears tissue was ground into fine powder with liquid nitrogen in the presence of polyvinylpyrrolidone (PVP) using pestle and mortar. About 0.2 g of ear powder was extracted with 1 mL of 50 mM potassium phosphate buffer, pH 7.0 with 5 mM ascorbic acid and 0.1 mM EDTA. After centrifugation for 15 min at 14 000g and 4 °C, re-extraction with 1 mL of same buffer was performed and the joint supernatant was taken for enzyme assays.

2.4. Measurements of enzyme activities

Determination of guaiacol peroxidase (POD; EC 1.11.1.7) activity was done according to Siegel and Galston (1967). Guaiacol peroxidase activity was determined by monitoring the increase in absorbance at 470 nm over 2 min. The enzymatic reaction was initiated by the addition of 25 μL of protein extract to 975 μL of reaction mixture. Reaction mixture consisted of 5 mM guaiacol and 5 mM hydrogen peroxide in 0.2 M phosphate buffer (pH 5.8). The enzyme activity was defined as 1 μM of guaiacol oxidized per minute per mg protein (Unit mg^{-1} (protein)). Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to Nakano and Asada (1981) by monitoring the decrease in the absorbance at 290 nm over 2 min. The enzymatic reaction was started by adding 10 μL of 12 mM H_2O_2 in 990 μL of the reaction mixture. The reaction mixture consisted of 955 μL 50 mM potassium phosphate buffer (pH 7.0) with 0.1 mM EDTA, 10 μL 25 mM ascorbic acid and 25 μL of protein extract. The enzyme activity was expressed as Unit mg^{-1} (protein). One Unit of APX is defined as 1 μM of oxidized ascorbate per minute per mg protein. Catalase (CAT, EC 1.11.1.6) activity was measured by the method of Aebi (1984). The reaction was started by adding 50 μL of protein extract to the reaction mixture (950 μL) consisted of 50 mM potassium phosphate buffer (pH 7.0) and 5 mM H_2O_2 decrease in absorbance was monitored over 1 min and catalase activity was expressed as units μmol of H_2O_2 decomposed per minute per mg protein (Unit mg^{-1} (protein)). Polyphenol oxidase (PPO, EC 1.14.18.1) activity was determined as a rate of oxidation of pyrogallol to o-quinones at 40 °C (Raymond et al., 1993). Reaction was induced by addition of 15 μL protein extract to reaction mixture consisted of 2 mL 100 mM potassium phosphate buffer (pH 7.0) and 0.2 mL 100 mM pyrogallol. The increase of absorbance was recorded at 430 nm. The PPO