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Reactive Oxygen Species in Host-Plant Are Required for an Early Defense Response Against Attack of *Stagonospora nodorum* Berk. Necrotrophic Effectors SnTox †

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Abstract: Reactive oxygen species (ROS) play central role in plant immune responses. The most important virulence factors of the *Stagonospora nodorum* are multiple fungal necrotrophic effectors (NEs) (SnTox) that affect the redox-status and cause necrosis and/or chlorosis on wheat lines possessing dominant susceptibility genes (*Snn*). However, the effect of NEs on ROS generation in the early stages of infection has not been studied. In this study, our aim was to research the effect of *S. nodorum* effectors SnToxA, SnTox1, SnTox3 on development of disease symptoms, generation of hydrogen peroxide and enzymes activity of redox- metabolism at early stage of infection in various wheat genotypes infected with isolates of *S nodorum* - Sn4VD, SnB and Sn9MN, carrying a various set of NEs genes. Our result indicates that all three NEs of SnToxA, SnTox1, SnTox3 played an important role in inhibition of ROS at the initial stage of infection. The *Tsn1*-SnToxA and *Snn3*-SnTox3 inhibited ROS production in wheat by affecting on NADPH-oxidases, peroxidases, superoxide dismutase and catalase. The *Snn1*-SnTox1 inhibited the production of ROS in wheat by mainly affecting the peroxidase. NEs suppress of ROS production only in the presence of the susceptibility genes *Tsn1*, *Snn1*, *Snn3*.

Keywords: *Stagonospora nodorum*; *Triticum aestivum*; necrotrophic effectors; reactive oxygen species; peroxidase

1. Introduction

Plants have developed several levels of defense against microbial pathogens, which have been described in the «zig-zag» model of the plant immune system [1]. The first line of defense in plants is through the perception of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), which leads to development of basal immunity, known as PAMP-triggered immunity (PTI) [1]. However, the pathogen can suppress PTI using effectors which leads to the development of effector-triggered susceptibility (ETS). The second line of defense in plants is called effector-triggered immunity (ETI) and develops when an effector is recognized by products of effector-specific resistance genes [1]. The development of PTI and ETI induces similar responses in plants: both lines of defense can be separated in time and space, but both are closely related to the production of reactive oxygen species (ROS) [2]. PAMP-triggered ROS perform two functions. The apoplastic ROS are cytotoxic to kill pathogens, ROS act as signaling molecules to activate of plant

defenses [2]. The development of ETI leads to oxidative burst and formation of necrosis and restriction of the biotrophic pathogens growth [1].

Until recently, necrotrophic pathogens were considered universal and non-host specific. However, recent studies have revealed that about 20 necrotrophic fungal species in the Dothideomycete class produced effector proteins also known as host-selective toxins (HSTs) or necrotrophic effectors (NEs) that interact either directly or indirectly with dominant sensitivity/susceptibility gene products to induce disease [3]. When a specific NE is recognized by the corresponding host gene, a host response follows that allows necrotrophs to penetrate, grow and sporulate. Additional studies of host response to recognition of NE discovered hallmarks of an ETI response [4]. Thus, NEs use the host's ETI pathway to develop sensitivity, resulting in NE-triggered susceptibility (NETS) [3,4]. Classical pathogens producing HST are pathogens of the genera *Cochliobolus*, *Alternaria*, *Pyrenophora* and *Stagonospora* [3].

Pathogenic fungus *Stagonospora nodorum* Berk. (syn. *Septoria*, *Parastagonospora*; *teleo*, *Phaeosphaeria*) is the causative agent of Septoria nodorum blotch (SNB) of wheat. The most important factors of *Stagonospora nodorum* virulence include numerous fungal necrotrophic effectors (NEs) encoded by *SnTox* genes [5,6]. To date, three effector genes have been identified in the genome of *S. nodorum* (*SnToxA*, *SnTox1*, *SnTox3*) [5]. SnToxA causes necrosis on wheat varieties that contain *Tsn1* gene [7]. Sensitivity to SnTox1 is defined by the *Snn1* gene located on wheat chromosome 1BS [5,8]. Sensitivity to SnTox3 is conferred by *Snn3-B1* and *Snn3-D1* located on wheat chromosomes 5BS and 5DS, respectively [5].

Effectors SnToxA, SnTox1, SnTox3 are considered the main ones in the pathogen *S. nodorum* and are quite widespread among strains and isolates [8]. Effectors SnToxA, SnTox1, SnTox3 cause necrosis and chlorosis in susceptible wheat genotypes, it follows from this that they have an impact on the redox-metabolism of the host plant. Unfortunately, the whole signal transduction pathway from recognition of the effector by the receptor to the necrosis development is unknown [4,6]. However, the role of NEs SnToxA, SnTox1, SnTox3 in the suppression of PTI and the development of ETS is expected [6]. It is believed that suppression of primary ROS burst during PTI by effector proteins as virulence factors is a common adaptation of many virulent pathogens [2]. Much data has been accumulated on the effectors of various pathogens that suppress ROS burst during PTI [2]. However, there is no such data on NEs SnToxA, SnTox1, SnTox3.

In this study, our aim was to research the effect of *S. nodorum* effectors SnToxA, SnTox1, SnTox3 on the development of disease symptoms, generation of hydrogen peroxide, expression of oxidoreductase genes and the activity of their protein products at an early stage of infection in various cultivars of soft spring wheat infected with isolates of *S nodorum* - Sn4VD, SnB and Sn9MN, carrying a various set of NEs genes. Here, we evaluated the roles of each compatible interaction *Tsn1*-SnToxA, *Snn3*-SnTox3, *Snn1*-SnTox1 in suppressing ROS production at the initial stage of infection.

2. Material and Methods

2.1. Research Objects and Seedling Resistance

The objects of the study were three cultivars of soft spring wheat (*Triticum aestivum* L.) contrasting in resistance to *S. nodorum* Berk.: Omskaya 35 (Om35), Kazakhstanskaya 10 (Kaz10) and Zhnitsa. Plants were hydroponically grown on 10% solution of Hoagland–Arnon nutrient medium in a KS-200 SPU growth chamber (Russia) at 20/24 °C (night/day) at the irradiance 146 W/m^2 FAR (Osram lamps L 36W/77) and the 16-h photoperiod for seven days. The pathogen objects were three isolates of the fungus *S. nodorum*: SnB, Sn4VD and Sn9MN.

The evaluation of seedling resistance of cultivars was carried out by the lawns method, as previously described [9]. The development of SNB symptoms on wheat leaves was registered on the sixth day after infection with *S. nodorum* isolates using an SP-800UZ Image Stabilization camera (Olympus, Indonesia); the damage area was measured using the ImageJ computer program (rsbweb.nih.gov/ij/download. html) and expressed as a percentage of the total leaf area. In addition, the degree of lesion was also evaluated according to the International scale based on the percentage

of the damage area of plant organs: RR (0–5%)—varieties with very high and high resistance; R (up to 10–15%)—resistant varieties; M (up to 25%)—slightly susceptible varieties; S (up to 40–65%)—susceptible varieties; SS (over 65%)—varieties with very high and high susceptibility. Detriment was also scored on a qualitative scale rating the type of lesion from 0 to 5 as described by Liu et al. (2004) [10].

2.2. Isolation of DNA, RNA and Performing the Polymerase Chain Reaction (PCR) and Biochemical Parameters

DNA was isolated from wheat seedlings and 7-day fungus culture by the phenol-detergent method [11]. Identification of NEs genes SnToxA, SnTox1 and SnTox3 in *S. nodorum* isolates Sn4VD, SnB and Sn9MN was performed by PCR with gene-specific primers *SnToxA* (JX997419), *SnTox1* (JX997402) [4] and *SnTox3* (FJ823644) [12]. Primers for the housekeeping gene tubulin (S56922) [11] were used as an internal control for the presence of fungal DNA. PCR with the cDNA template was performed in a TP4-PCR-01-Tertsik type PCR machine (DNK-Tekhnologia, Russia).

The dominant allele of the *Tsn1* gene was identified in wheat cultivars by PCR with primers for the *Xfcp623* microsatellite marker on the internal region of the *Tsn1* gene [7]. The dominant allele of the *Snn1* gene was identified with primers for the intragenic marker *Snn1* (KP085710) [4]. The allelic state of the *Snn3-B1* locus was determined by PCR with primers for the *Xcfd20* and *Xgwm234* microsatellite markers [11]. In all cases, PCR products were resolved in 7% PAAG stained with ethidium bromide using the Gene Ruler DNA Ladder (Fermentas). The gels were photographed using a documenting system of GelDoc XR (Bio-Rad).

Total RNA was isolated from control and infected with *S. nodorum* isolates (Sn4VD, SnB and Sn9MN) wheat leaves of three varieties Om35, Kaz10, and Zhnitsa, fixed in liquid nitrogen, with Trizol reagent according to manufacturer's (Sigma, Germany) recommendations. Analysis of genes expression for NEs (*SnToxA*, *SnTox1* and *SnTox3*) in different isolates of *S. nodorum* during inoculation of wheat plants and expression of genes for wheat oxidoreductases (*TaRbohF*, *TaSod*, *TaPrx*) was performed by quantitative real-time polymerase chain reaction (RT-PCR) with an iCycler iQ5 Real-Time PCR Detection System (Bio-Rad, USA) and SYBR Green I intercalating dye (Sintol, Russia). Real-time PCR was performed using primers for genes encoding NADPH oxidase (*TaRbohF*, AY561153), superoxide dismutase (SOD) (*TaSod*, JX398977.1), anionic peroxidase (*TaPrx*, TC151917) [11], SnToxA (JX997419), SnTox1 (JX997402) [4], and SnTox3 (FJ823644) [12]. To normalize the expression results of the studied genes, primers were used to the tubulin gene of the fungus *S. nodorum* (S56922) [11] and to the gene of constitutively expressed protein that is a wheat ribonuclease inhibitor (RNase L inhibitor-like) *RLI* (AY059462) [11]. Changes in the expression of the gene of interest were estimated by the level of normalized gene expression calculated with the iCycler iQ5 Real-Time Detection System Software (Bio-Rad, USA).

The content of hydrogen peroxide (H_2O_2) and the enzymes activities of peroxidase (POX) and catalase in wheat leaves of three varieties Om35, Kaz10, and Zhnitsa were measured 24 h after inoculation with *S. nodorum* isolates as previously described [9].

2.4. Statistical Analysis

All experiments were carried out three times with three biological and three analytical replications (n = 9 in total), except for the measurements of infected area, which were performed in not less than 30 biological replications (n = 90 altogether). The figures and table report mean values and their confidence intervals calculated from their standard errors. Statistical analysis consisted of Student's t-test at a significance level of $p \leq 0.05$.

3. Results

3.1. The Role of Compatible Interactions in Causing Disease

The *Tsn1*-SnToxA, *Snn3*-SnTox3 and *Snn1*-SnTox1 interactions were investigated in this study. Alleles of susceptibility genes *Snn1*, *Tsn1* and locus *Snn3-B1* were identified by PCR in three varieties of soft spring wheat (Figure 1). Thus, Om 35 (*tsn1*/*Snn1*/*snn3*) was sensitive to NE of SnTox1. Variety Kaz10 (*tsn1*/*Snn1*/*Snn3*) was sensitive to NE of SnTox1 and SnTox3. The Zhnitsa (*Tsn1*/*Snn1*/*Snn3*) was sensitive to all three effectors (Figure 1).

Three *S. nodorum* isolates Sn4VD, SnB and Sn9MN were tested for presence of the three NEs genes *SnToxA*, *SnTox3* and *SnTox1* by PCR (Figure 2a). The isolates Sn4VD and Sn9MN contained three NEs genes *SnToxA*, *SnTox3* and *SnTox1* in their genome (Figure 2a). The isolate SnB contained only two NEs genes *SnToxA* and *SnTox3* in the genome (Figure 2a).

Investigation of the transcriptional activity of NEs genes in three *S. nodorum* isolates SnB, Sn4VD, and Sn9MN during inoculation of the susceptible genotype of the Zhnitsa (*Tsn1*/*Snn1*/*Snn3*) revealed differences in expression (Figure 2b). In isolate Sn4VD, expression of all three NEs genes *in planta* was not detected (Figure 2b). The SnB isolate expressed only two NEs genes, *SnToxA* and *SnTox3* (Figure 2b). In isolate Sn9MN, expression of three NEs genes *SnTox1*, *SnToxA*, and *SnTox3* was found (Figure 2b).

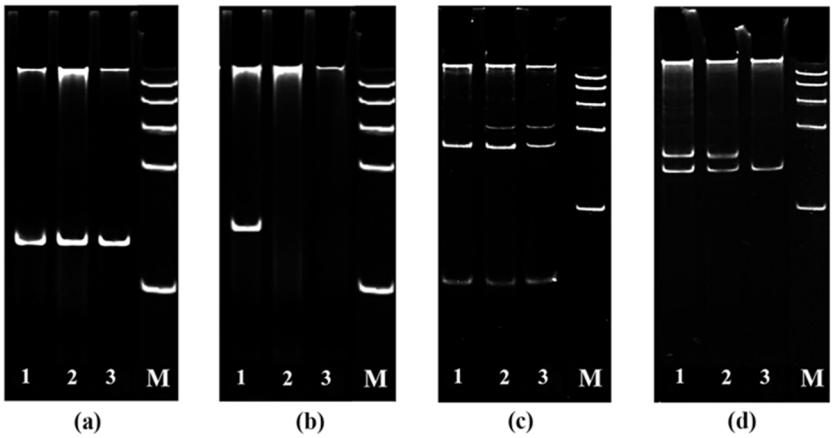

Figure 1. Identification of alleles of susceptibility genes *Snn1* (**a**), *Tsn1* (**b**) and *Snn3-B1* locus (**c**,**d**) using primers for SSR markers *Xcfd20* (**c**) and *Xgwm234* (**d**) in different wheat varieties by PCR. 1—Zhnitsa; 2—Kazakhstanskaya 10; 3—Omskaya 35; M—DNA molecular weight ladder 100–1000 bp.

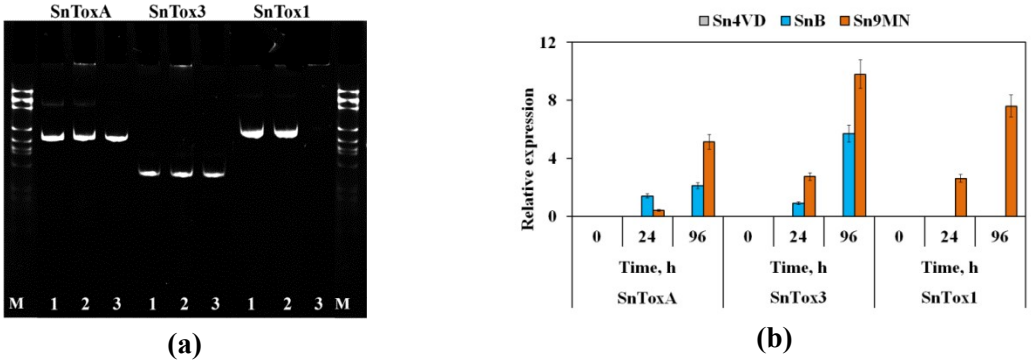

Figure 2. Identification of the *SnToxA*, *SnTox3* and *SnTox1* genes by PCR in tree isolates of *S. nodorum*: 1—Sn4VD; 2—Sn9MN; 3—SnB; M—DNA molecular weight ladder 100–1000 bp (**a**). Transcriptional activity of *SnToxA*, *SnTox1*, and *SnTox3* effector genes (*in planta*) during inoculation of the susceptible genotype of the Zhnitsa (*Tsn1*/*Snn1*/*Snn3*) with water (0) and three isolates of *S. nodorum* SnB, Sn9MN, Sn4VD (**b**).

Thus, incompatible interactions or resistance were observed when cultivars were inoculated with the Sn4VD isolate, because the isolate did not produce NEs (Table 1). The incompatible interaction was observed in the combination cultivar/isolate Om35/SnB (*tsn1*/*snn3*/*Snn1*—*ToxA*/*Tox3*/*tox1*). The minimal damage areas were observed in all incompatible interactions in variants of Om35/Sn4VD, Kaz10/Sn4VD, Zh/Sn4VD, Om35/SnB (Table 1). It can be explained by hypersensitivity reactions or by the influence of other genes or effectors with minor effects [5]. The *Snn1*-SnTox1 interaction and the effect of SnTox1 on the development of the disease were observed in the combination Om35/Sn9MN (the combination of genotypes is presented in Table 1). The *Snn1*-SnTox1 interaction led to the formation of necrosis, occupying almost 25% of the leaf area and did not cause the formation of chlorosis (Table 1). The *Snn3*-SnTox3 interaction and the effect of SnTox3 on disease progression were observed in combination Kaz10/SnB (Table 1). The *Snn3*-SnTox3 interaction led to the formation of necrosis and significant chlorosis, together occupying more than 50% of the leaf area (Table 1). The *Snn3*-SnTox3 and *Snn1*-SnTox1 interactions were observed in combination Kaz10/Sn9MN (Table 1). However, we did not find the expected significant increase in the total lesion, but there was an increase in necrotic spots compared to the Kaz10/SnB variant (*Snn3*-SnTox3) (Table 1). This reaction could be explained by the fact that *Snn1*-SnTox1 interaction is epistatic to *Snn3*-SnTox3 [5]. The *Tsn1*-SnToxA and *Snn3*-SnTox3 interactions were observed in combination Zh/SnB (Table 1). These interactions led to a significant increase in the damage areas compared to the Kaz10/SnB variant (*Snn3*-SnTox3), chlorosis and necrosis occupied more than 70% of the leaf area (Table 1). The *Tsn1*-SnToxA, *Snn3*-SnTox3, *Snn1*-SnTox1 interactions were observed in combination Zh/Sn9MN (Table 1). In this variant, an increase in the affected areas was found in comparison with the Zh/SnB variant (*Snn3*-SnTox3 and *Tsn1*-SnToxA), mainly due to an increase in the formation of necrosis (Table 1). Thus, according to our results, NE SnTox1 caused the development of necrosis, NE SnTox3 stimulated the development of both necrosis and chlorosis, NE SnToxA induced the development of extensive chlorosis in susceptible wheat plants, which is consistent with literature data [5,8,12].

Table 1. Reaction to damage of wheat varieties to infected with *S. nodorum* SnB, Sn9MN and Sn4VD isolates.

Variety	Reaction to Damage	Isolate *S. nodorum*		
		Sn4VD (*toxa*/*tox3*/*tox1*)	**SnB (*ToxA*/*Tox3*/*tox1*)**	**Sn9MN (*ToxA*/*Tox3*/*Tox1*)**
Omskaya 35 (*tsn1*/*snn3*/*Snn1*)	Necrosis, %	0.05 ± 0.002	5 ± 0.7	23 ± 2
	Chlorosis, %	0	3 ± 0.5	0
	Damage zone, %	0.05 ± 0.001	8 ± 1	23 ± 2
	Damage score	1	2	3
	Resistance group *	RR	R	M
Kazahstanskaya 10 (*tsn1*/*Snn3*/*Snn1*)	Necrosis, %	0.05 ± 0.002	16 ± 2	31 ± 3
	Chlorosis, %	0	35 ± 3	25 ± 2
	Damage zone, %	0.05 ± 0.001	51 ± 5	56 ± 4
	Damage score	1	4	4
	Resistance group *	RR	S	S
Zhnitsa (*Tsn1*/*Snn3*/*Snn1*)	Necrosis, %	1 ± 0.1	18 ± 2	27 ± 2
	Chlorosis, %	2 ± 0.2	55 ± 4	57 ± 5
	Damage zone, %	3 ± 0.3	73 ± 6	84 ± 6
	Damage score	1	5	5
	Resistance group *	RR	SS	SS

Note:*RR (0–5%)—varieties with very high and high resistance; R (up to 10–15%)—resistant varieties; M (up to 25%)—slightly susceptible varieties; S (up to 40–65%)—susceptible varieties; SS (up to 90–100%)—varieties with very high and high susceptibility.

3.2. The Role of Compatible Interactions in Suppression of ROS Production

It is known that one of the earliest responses to the invasion of pathogen is the local generation of ROS—oxidative "burst", which plays an important role in the development of systemic resistance and is controlled by enzymes of the pro-/antioxidant system [13]. NADPH oxidases, also known as Respiratory Burst Oxidase Homologs (RBOHs), are responsible for production of ROS in plants during pathogen infection [14]. In addition, the apoplastic peroxidases play an important role in production of ROS in PTI [14].

Earlier, we found that the resistance of the *T. aestivum* to the pathogen *S. nodorum* was determined by the intensive generation of ROS, mainly H_2O_2, due to an increase in peroxidase activity and a decrease or absence of an increase in catalase activity in the initial stage of infection [9]. It is known that peroxidases are involved both in the generation processes and in the processes of ROS utilization in the apoplast, and catalase activate the decomposition reaction of H_2O_2 molecules [13]. In this study, the twofold and threefold increase in the H_2O_2 content was observed in variants with incompatible interactions (Om35/Sn4VD, Kaz10/Sn4VD, Zh/Sn4VD, Om35/SnB) (Figure 3a). The increase in H_2O_2 production in wheat to inoculation with the avirulent isolate Sn4VD, occurred mainly due to high activity of peroxidase (Figure 3c) and high transcript level of gene encoding anionic peroxidase (*TaPrx*) (Figure 3f) and also due to low activity of catalase (Figure 3e) at the early stage of infection (24 h). Regarding genes *TaRbohF* and *TaSod*, small increase in transcript level was observed at the early stage of infection to inoculation with the avirulent isolate Sn4VD (Figure 3b, d). When the resistant variety Om35 was inoculated with the SnB virulent isolate and an incompatible interaction developed, the large increase in transcript level of genes *TaRbohF* and *TaSod* was observed (Figure 3b,d). Thus, a typical reaction of resistance to *S. nodorum* was observed in all incompatible interactions, which led to the development of an oxidative burst and restriction of the pathogen growth.

All compatible interactions inhibited H_2O_2 production in susceptible wheat varieties at early stage of infection compared with incompatible interactions (Figure 3a). The *Snn1*-SnTox1 (Om35/Sn9MN) and *Snn3*-SnTox3 (Kaz10/SnB) interactions reduced H_2O_2 production by 2 times as compared with the incompatible interaction in variant Om35/SnB (Figure 3a). The presence of two or three compatible interactions in wheat plants led to an even greater decrease in H_2O_2 production (Figure 3a). All three interactions (*Tsn1*-SnToxA, *Snn3*-SnTox3, *Snn1*-SnTox1), individually or in combination, led to a decrease in peroxidase activity and decline of transcript level of genes *TaPrx*, *TaSod* in wheat at the early stage of infection (Figure 3c,d,f).

It should be noted that the *Snn1*-SnTox1 interaction did not lead to a decrease in the transcript level of gene *TaRbohF* and an increase in the activity of catalase compared to the incompatible interaction in variant Om35/SnB, unlike other interactions (*Tsn1*-SnToxA, *Snn3*-SnTox3) (Figure 3b,e). The transcript level of gene *TaRbohF* and activity of catalase in variants Om35/SnB and Om35/Sn9MN were found to be similar. Our results show that in the Kaz10/Sn9MN and Zh/Sn9MN variants, the *Snn1*-SnTox1 interaction did not affect the transcript level of gene *TaRbohF* (Figure 3b). We found similar results in changes in catalase activity (Figure 3e). Each of interaction *Snn3*-SnTox3 or *Tsn1*-SnToxA increased catalase activity by 1.5 times in the Kaz10/SnB and Zh/SnB variants, respectively (Figure 3e). In the Kaz10/Sn9MN and Zh/Sn9MN variants, the *Snn1*-SnTox1 interaction did not affect the catalase activity (Figure 3e).

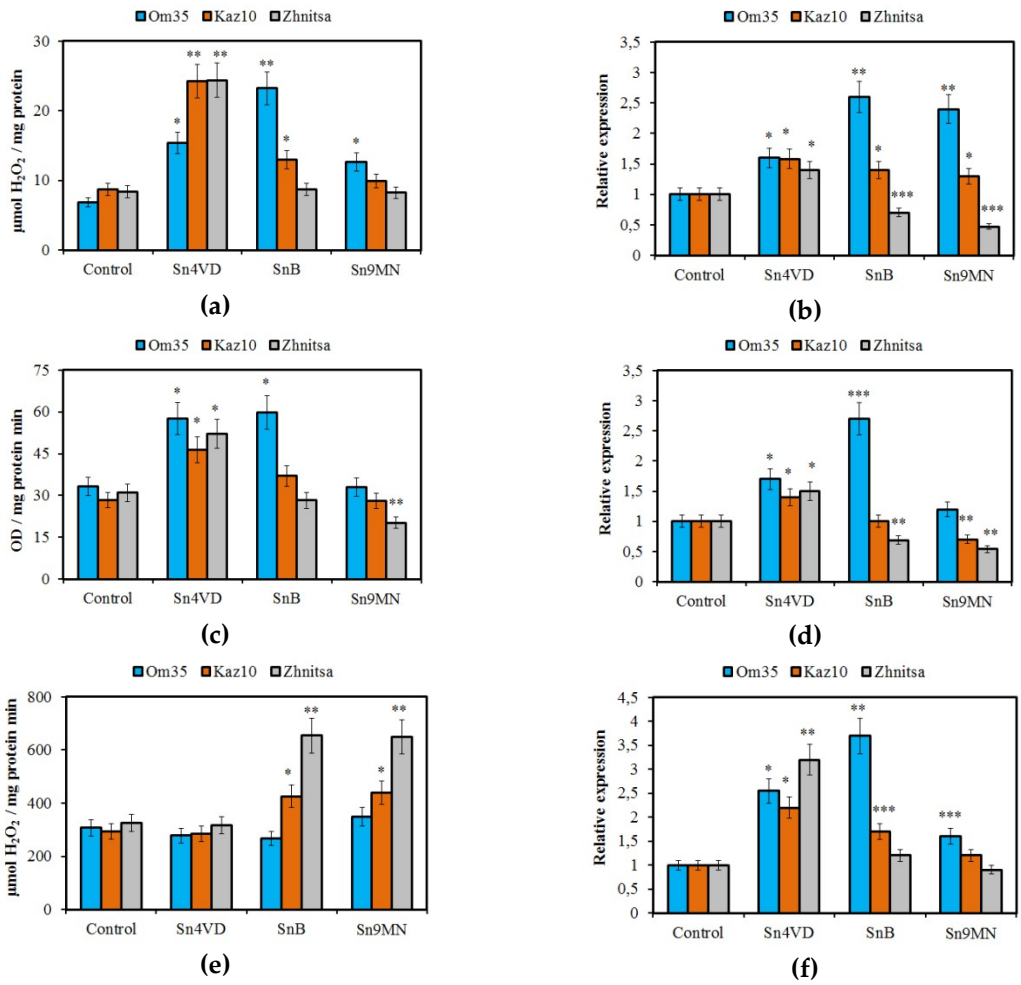

Figure 3. Hydrogen peroxide contents (**a**), enzyme activities of peroxidase (**c**) and catalase (**e**) as well as real-time polymerase chain reaction (PCR) analysis of genes encoding NADPH oxidase (*TaRbohF*) (**b**), superoxide dismutase (*TaSod*) (**d**) and anionic peroxidase (*TaPrx*) (**f**) in leaves of wheat varieties Om35, Kaz10 and Zhnitsa 24 h after inoculation with *S. nodorum* Sn4VD, SnB and Sn9MN isolates. The data are shown as means ± SE. Asterisks * show statistically significant differences from the control group, and different numbers of asterisks indicate differences between the variants significant at $p \leq 0.05$.

4. Discussion

Thereby, the suppression of the host defense response by the effectors of *S. nodorum* SnToxA, SnTox1, SnTox3 was carried out due to a decrease in the generation of H_2O_2 in the leaves of susceptible varieties at the initial stage of infection. The *Tsn1*-SnToxA and *Snn3*-SnTox3 interactions inhibited H_2O_2 production in wheat at the early stage of infection by affecting on four enzymes of redox metabolism: NADPH-oxidases, peroxidases, superoxide dismutase and catalase. The *Snn1*-SnTox1 interaction inhibited the production of H_2O_2 in wheat by mainly affecting the peroxidase activity and the transcript level of gene encoding anionic peroxidase (*TaPrx*).

5. Conclusions

This result indicates that all three NEs of *S. nodorum* SnToxA, SnTox1, SnTox3 played an important role in inhibition of ROS during PTI at the initial stage of infection, despite the fact that at the late stage of infection, all three NEs caused the formation of necrosis and chlorosis on leaves wheat of susceptible genotypes. Our results also showed that inhibition of ROS in PTI by NEs occurred only in the presence of the susceptibility genes *Tsn1*, *Snn1*, *Snn3*, which is also supported by other studies [6]. Therefore, our results suggest that effector–host sensitivity gene interactions

have the ability not only to hijack the host's own ETI pathway, but also suppress the host's own PTI pathway, resulting in NE-triggered susceptibility (NETS).

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

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