

Effect of heat-shock pre-treatment on tomato plants infected by powdery mildew fungus

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Abstract: The effect of plant heat-shock (HS) pre-treatment (40.5°C, 2 h) on *Pseudoidium neolycopersici* development in the susceptible and moderately resistant *Solanum* spp. genotypes was studied together with biochemical responses (endogenous concentrations of salicylic (SA), jasmonic (JA), abscisic acid (ABA), and peroxidase (POX) activity). In HS pre-treated *S. lycopersicum*, an acceleration of pathogen, chlorosis and necrosis development, strong SA, JA accumulation, and increased POX activity were detected. In *S. chmielewskii*, HS pre-treatment caused a slight suppression of pathogen development, increase in JA, ABA concentrations, and POX activity. HS accelerated and strengthened the development of symptoms and biochemical responses to the infection in the susceptible genotype in contrast to moderately resistant genotype with a robust defence response to an infection *per se*.

Keywords: abscisic acid; jasmonic acid; peroxidase; *Pseudoidium neolycopersici*; salicylic acid; *Solanum* spp.

Abbreviations: ABA – abscisic acid; dai – days after inoculation; FW – fresh weight; HR – hypersensitive reaction; HS – heat shock; IHP – infected heat-shock pre-treated; JA – jasmonic acid; POX – peroxidase; ROS – reactive oxygen species; SA – salicylic acid

Tomato powdery mildew, caused by *Pseudoidium* (= *Oidium*) *neolycopersici* (*P. neolycopersici*) (L. Kiss) L. Kiss is a disease predominantly devastating glass-house tomato crops worldwide (LEBEDA *et al.* 2014). A proper understanding of the processes taking part in *Solanum* spp. during *P. neolycopersici* infection may help in the development of strategies for crop protection against this pathogen. Until recently, the studies were concentrated on the morphological and molecular characterisations of this pathogen (WHIPPS *et al.* 1998; MIESLEROVÁ *et al.* 2002), the host range (LEBEDA & MIESLEROVÁ 1999; HUANG *et al.* 2000),

and on searching for resistance sources within the genus *Solanum* (LINDHOUT *et al.* 1994; MIESLEROVÁ *et al.* 2000; LEBEDA & MIESLEROVÁ 2002). In the last 15 years, an intensive research has been made to describe the biochemical processes occurring in the tomato during its infection by *P. neolycopersici* including a role of plant hormones (ACHUO *et al.* 2004, 2006; LI *et al.* 2012), reactive oxygen species (ROS) (MLÍČKOVÁ *et al.* 2004; TOMÁNKOVÁ *et al.* 2006), reactive nitrogen species (PITERKOVÁ *et al.* 2009, 2013) and a role of elicitors (oligandrin, BABA) in resistance responses (SATKOVÁ *et al.* 2017). How-

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ever, detailed descriptions of the defence signalling pathways have still been missing.

Salicylic acid (SA) has one of the key positions in defence signalling pathways expressed during the plant–pathogen interaction (VLOT *et al.* 2009; JIN *et al.* 2017). Besides SA, other plant stress hormones such as abscisic acid (ABA) (MAUCH-MANI & MAUCH 2005; CAO *et al.* 2011) and jasmonic acid (JA) (POZO *et al.* 2004; HALIM *et al.* 2006) may act in the defence signalling pathways in plant–pathogen interactions (SHIGENAGA & ARGUESO 2016). For example, exogenous application of JA effectively protected barley plants against infection by biotrophic powdery mildew fungus (*Blumeria = Erysiphe graminis* f.sp. *hordei*) (SCHWEIZER *et al.* 1993). As far as ABA is concerned, treatment of barley roots (WIESE *et al.* 2004) or phlox leaves (TALIEVA *et al.* 1999) with ABA resulted in enhanced resistance against powdery mildew infection. In contrast, ACHUO *et al.* (2006) demonstrated that ABA deficiency in tomato resulted in increased resistance to *O. (= P.) neolyopersici* and *Botrytis cinerea*. Exogenously applied ABA increased the susceptibility of ABA-deficient tomato to both pathogens (ACHUO *et al.* 2006).

An early plant cellular response, following successful pathogen recognition, is the production of reactive oxygen species (ROS) (HÜCKELHOVEN & KOGEL 2003; CAMEJO *et al.* 2016). One group of enzymes participating in ROS metabolism consists of the plant peroxidases (POXs) that catalyse oxidation-reduction reactions of various substrates using H₂O₂ (HIRAGA *et al.* 2001). POXs have several functions in plant defence, including direct toxicity against pathogens, production of phytoalexins, cellular growth and may contribute to disease resistance in different host–pathogen interactions (WOJTASZEK 1997; ALMAGRO *et al.* 2009). An increase of POX activity was detected in resistant genotypes of *Solanum* spp. in response to *O. (= P.) neolyopersici* infection (MLÍČKOVÁ *et al.* 2004). High POX activity correlated with intensive H₂O₂ production and with the first demonstration of hypersensitive reaction (HR) in resistant genotypes (MLÍČKOVÁ *et al.* 2004; TOMÁNKOVÁ *et al.* 2006).

In many host–pathogen interactions, plant resistance and/or susceptibility is influenced by high temperature (SCHWEIZER *et al.* 1995; KUBIENOVÁ *et al.* 2013; LEBEDA *et al.* 2014). Heat stress may modify the level of endogenous concentrations of the chemical compounds that subsequently influence plant resistance/susceptibility to the pathogen. Plant stress hormones and ROS may participate in heat stress plant response

(LARKINDALE & HUANG 2005; KOTAK *et al.* 2007; CLARKE *et al.* 2009). ABA (KUMAR *et al.* 2012), JA (SHARMA & LAXMI 2016), and SA (LOPEZ-DELGADO *et al.* 2004) are known to act as heat stress signalling compounds. Furthermore, a considerable overlap among cellular processes in response to heat and oxidative stress stimuli in plants was revealed, suggesting an intimate relationship between the heat shock (HS) and oxidative stress responses (REDDY *et al.* 2009). An oxidative burst (VALLÉLIAN-BINDSCHEDLER *et al.* 1998) and related changes in POX activity (ALMESELMANI *et al.* 2006) have been observed in heat treated plants. Moreover, the existence of a causal link between SA, oxidative stress, and synthesis of antioxidants after heat treatment may play a significant role in resistance to high temperature stress (DAT *et al.* 2000; CINGOZ & GUREL 2016).

The objective of this work is to contribute to still unknown mosaic of knowledge about the biochemical signalling pathways (ABA, JA, SA accumulation and POX activity) involved in rapid defence responses of both susceptible (*Solanum* (*S.*) *lycopersicum* cv. Amateur) and moderately resistant (*Solanum* (*S.*) *chmielewskii*) tomato plants to *P. neolyopersici* infection. *S. chmielewskii* was used because it is one of the best characterized *Solanum* genotypes regarding the biochemical aspects of resistance to *P. neolyopersici* infection, and its resistance response is characterised by very strong hypersensitivity (TOMÁNKOVÁ *et al.* 2006). Gradually, the attacked leaf is covered by black spots (HR), and by only slightly developed pathogen mycelia, followed by abscission of the leaf (MIESLEROVÁ *et al.* 2004).

Furthermore, we tried to reveal whether the short-term (2 h) exposure of the studied tomato plants to high temperature (40.5°C) prior to inoculation influenced the levels of resistance of the host plants to the pathogen, as well as the development of the pathogen. The gained insights can aid in improving crop performance under stress combinations.

MATERIAL AND METHODS

We used identical plant materials, growth conditions, *P. neolyopersici* isolate and its cultivation, HS pre-treatment, and inoculation procedure as in our previous paper (PROKOPOVÁ *et al.* 2010). Therefore, these sections are presented here briefly.

Plant material. Two different genotypes of tomato (*Solanum* spp.) were used in the experiments: a

susceptible genotype *S. lycopersicum* L. cv. Amateur, and moderately resistant genotype *S. chmielewskii* (Rick, Kesickii, Forbes & Holle) Spooner, Anderson, and Jansen (LA 2663).

Pathogen isolate and its cultivation. One isolate of *P. neolycopersici* (C-2), collected on tomatoes (*S. lycopersicum* cv. Lucy) grown in glasshouses of the State Phytosanitary Administration, Olomouc, Czech Republic (HUANG *et al.* 1998; LEBEDA & MIESLEROVÁ 1999, 2002) was used in all studies. The isolate is part of the collection of the Department of Botany, Palacký University in Olomouc, and is included in the Czech National Collection of Microorganisms (collection number UPOC-FUN-127). The isolate was maintained on 2–3-month-old tomato plants (*L. esculentum* cv. Amateur) held under plastic covers in a growth chamber at temperature 18–20°C and 12 h photoperiod (110 $\mu\text{mol photons per m}^2/\text{s}$).

Heat-shock pre-treatment. Experimental plants were exposed to 40.5°C for 2 h in a cultivation box (SANYO E&E Europe, Etten-Leur, the Netherlands). During this HS treatment, plants were illuminated with white light (45 $\mu\text{mol photons per m}^2/\text{s}$). In the case of infected plants, the HS pre-treatment was applied immediately before inoculation. Three plants per each variant were used.

Inoculation by *P. neolycopersici*. The adaxial side of the 3rd and 4th leaves (counted from the plant base up) of the selected plants (8–10 weeks old) was inoculated by surface contact (dusting/tapping) using leaves of tomato (*S. lycopersicum* cv. Amateur) that were covered (> 80%) by fresh sporulating mycelium of *P. neolycopersici* (8–10 days old) (LEBEDA & MIESLEROVÁ 2010). Plants representing a mock control of inoculation were treated by tapping with uninfected tomato leaves.

Physiological parameters were measured within 9 days after inoculation (dai), because this period is crucial for full expression of disease symptoms. Due to the defoliation of the infected leaves in *S. chmielewskii*, the measurements could only be done till 4 dai on plants without HS treatment (only one leaf left 7 dai – no possibility of statistics), and till 7 dai on HS pre-treated plants (only two leaves were left on the plants and could be used for the measurements).

Macroscopic and microscopic observations of pathogen and symptom developments. The macroscopic symptoms of infection were recorded at 2, 3, 4, 7, and 9 dai. The macroscopic photos were taken on an Olympus SZ 40 stereo zoom microscope with an Olympus DP 70 digital camera (both (Olympus, Tokyo, Japan).

The microscopic photos were made on an Olympus BX60 light microscope (Olympus, Japan), with an Olympus DP 70 digital camera at 2, 3, 4, 7, and 9 dai. The materials were prepared as described below. 48 h glacial acetic acid (acetic acid 99%; Lach-Ner, Neratovice, Czech Republic), mounting, and storage in glycerol (glycerolum 85%; Tamda, Olomouc, Czech Republic), staining with 1% cotton blue (methyl blue, Anilin blue; Sigma-Aldrich, Prague, Czech Republic) before observation (LEBEDA & REININK 1994; MIESLEROVÁ *et al.* 2004). The 4th leaf of the tested plants was photographed. At least 3 leaves per each variant (except defoliated ones) were used.

Microscopic evaluation of pathogen germination and growth. At various terms (1/3, 1, 2, and 3 dai) the randomly selected leaf discs (12 mm in diameter) were cut from infected 4th leaves (HS-treated and non-treated), then immersed for 48 h in glacial acetic acid, mounted in glycerol, and stained with 1% cotton blue prior to observation in an Olympus BX60 light microscope (LEBEDA & REININK 1994; MIESLEROVÁ *et al.* 2004). Fungal development was assessed as the number of germ tubes per conidia, expressed in percent (%) of conidia producing germ tubes per 100 conidia, counted for each leaf disc at 1/3, 1, 2, and 3 dai; and as the length of the germ tubes assessed at 1/3, 1, 2, and 3 dai (1st tube with lobbed appressorium, and 2nd and 3rd tubes with nipple shaped appressoria). Four leaf discs were used for each time interval. A minimum of 120 conidia were observed per each accession for each time interval. The length of the germ tubes was measured using an ocular micrometer.

SA, JA, and ABA quantification. The endogenous concentrations of ABA, JA, and SA were measured in the 4th leaf 4, 7, and 9 dai. The measured leaflet was harvested and immediately weighed, frozen in liquid nitrogen, and stored at –80°C. The procedure used for ABA, JA, and SA extraction and purification was as described by HLAVÁČKOVÁ *et al.* (2006) (with slight modifications). The 100 pmol [²H₄]SA (Cambridge Isotope Laboratories, Andover, USA), 50 pmol [²H₆]ABA (Isotope Laboratory, IEB AS CR, Prague, Czech Republic), and 50 pmol [¹⁸O]JA (supplied by Els Prinsen, University of Antwerp, Belgium) were added as internal tracers for the recovery studies and quantification. The purified samples were quantified by the LC-MS/MS system, as in BERGOUNOUX *et al.* (2009). Three leaves were used and three independent experiments were performed for each treatment.

Enzyme extraction procedures and peroxidase activities. The 4th leaves (counted from the base of

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stem, each leaf contained 5 leaflets) were collected from infected and control plants at 1/3, 1, 2, 3, 4, 7, and 9 dai. Three leaves (the 4th) from three different plants were collected per each variant and term. The leaves were homogenised at the 1 : 2 (w/v) ratio with 0.1 M Tris (Lach-Ner, Czech Republic)/HCl buffer, pH 7. Leaf homogenates were centrifuged at 16 000 g for 10 min at 4°C; the supernatants were used immediately for the determination of peroxidase activity. Peroxidase activity assessment was achieved by the spectrophotometric method, with the use of guaiacol modified according to ANGELINI *et al.* (1990). The reaction was started by the addition of 50 µl of 10 mM H₂O₂ to the reaction medium, containing 100 µl of 0.1 M potassium-phosphate (Lach-Ner, Czech Republic) buffer, pH 6, 40 µl of 50 mM guaiacol (Sigma-Aldrich, Czech Republic) and 100 µl of sample (plant extract). All measurements were performed at 30°C; changes in absorbance were followed at $\lambda = 436$ nm every 5 s for 1 min on a Synergy HT Microplate Reader (BioTek, USA). All measured values were subtracted from the blank values, where the extracts were replaced in the reaction mixture by the same volume of extraction buffer (0.1 M potassium-phosphate buffer pH 7; 1% PVP (polyvinylpyrrolidone); 2 mM DTT(1,4-dithiothreitol); 0.5 mM Pefabloc[®]; 2 mM EDTA (edathamil); all Sigma-Aldrich, Czech Republic). For each variant, three measurements for each of the three independent sets of samples were conducted (i.e. nine values were obtained).

Statistical analysis. Data for the length of the conidial germ tubes (1st, 2nd, and 3rd), POX activity, as well as the SA, JA, and ABA concentrations are presented as the mean \pm SD, and were subjected to one-way analysis of variance (ANOVA; $\alpha = 0.05$) plus (when applicable) followed by a comparison of means using the test of Bonferroni. The statistical tests were performed using NCSS 2000 statistical software (Statistical Solutions, Cork, Ireland).

RESULTS

Susceptible genotype – Effect of infection. *S. lycopersicum* cv. Amateur is not able to generate HR in response to powdery mildew *P. neolycopersici*, and this pathogen usually exhibited intensive mycelia development and sporulation on the leaf surface, leading to the creation of a dense white coat of the pathogen mycelia covering the adaxial side of infected leaves. We observed typical pathogen development in

our experiments (Figures 1A and 1B). After conidia germination and successive mycelia development on the leaf surfaces, this significant progress continued until 9 dai, when the majority of the leaf surface was covered with the dense sporulating mycelium of the pathogen (Figure 1A).

The pathogen development was also confirmed microscopically. We monitored number and length of germ tubes, i.e. outgrowths producing by germinating conidia. The first germ tube frequently contained appressoria ensuring the first contact with the host and forming of haustoria. After successful initial contact, other germ tubes were produced (2nd, 3rd, 4th), each of them emerging directly from the conidium. Gradual growth of the 1st, 2nd, and 3rd germ tubes was observed within the first days after inoculation (1/3–3 dai) (Figure 2), until a dense mycelial net was created (Figure 1B, from 7 dai) and the formation of conidiophores started. Non-germinated conidia and germinated conidia with the 1st germ tube, with or without lobed appressoria, were found on plant leaves at 1/3 dai. At 1 dai, conidia with two germ tubes were found, the first one with a lobed appressorium, and the second tube usually longer than the first one (with nipple shaped appressorium); whereas, at 2 and 3 dai, conidia with two or three germ tubes were observed (Figure 2). A progressive growth of the first, second, and third germ tubes of *P. neolycopersici* during the experiment is evident from Figure 3.

Only slightly higher endogenous concentrations of SA and ABA and lower of JA compared to healthy plants were observed 7–9 dai (Figure 4). A significant increase in the POX activity was detected 3 dai and the more pronounced second one 9 dai (Figure 5).

Susceptible genotype – Effect of heat-shock pre-treatment on healthy and infected leaves. HS pre-treatment of healthy non-inoculated plants evoked only slight changes in the measured parameters. We did not detect any macroscopic changes in HS pre-treated leaves (Figure 1A). HS pre-treatment *per se* did not cause any hormone accumulation (Figure 4). We observed a pronounced increase in POX activity at 1, 7, and 9 days after HS pre-treatment (Figure 5).

In infected HS pre-treated (IHP) leaves, contrary to infected leaves, chloroses appeared (from 7–9 dai) (Figure 1A) and developed progressively to necrosis in the later stages of infections. However, microscopically, the intensive sporulation was confirmed on IHP leaves (Figure 1B). It is evident from Figure 2 that the number of conidia producing three germ tubes increased at 2 dai after heat treatment compared with

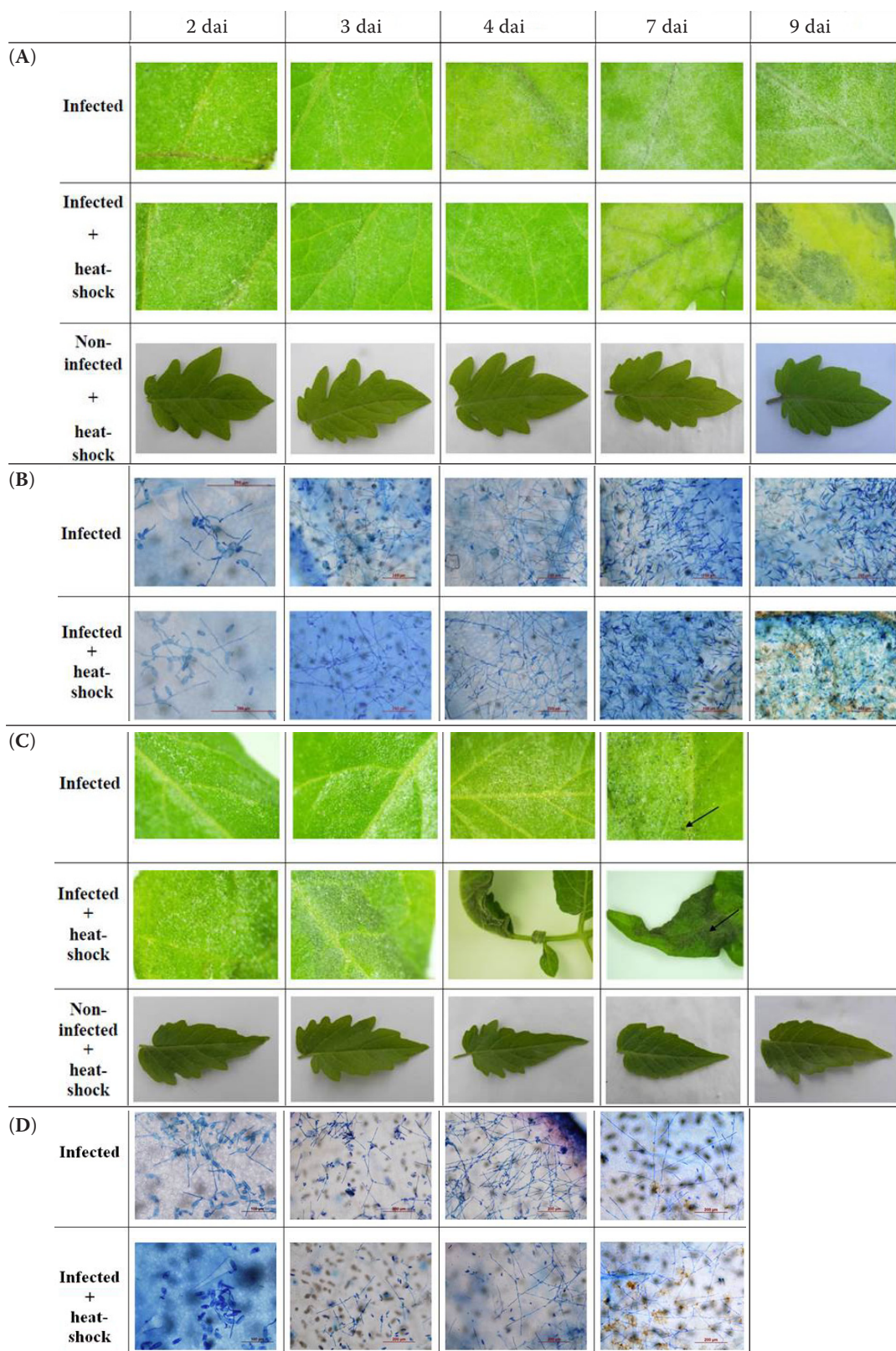


Figure 1. Macroscopic and microscopic observations of pathogen and symptom development on infected, infected heat-shock (HS) pre-treated, and non-infected HS pre-treated leaves of susceptible *S. lycopersicum* cv. Amateur (A, B) and moderately resistant *S. chmielewskii* (C, D) genotype of tomato plants. Due to the defoliation of the infected leaves in *S. chmielewskii*, photos at 9 dai are missing. Arrows show black (necrotic) spots. One representative sample is shown

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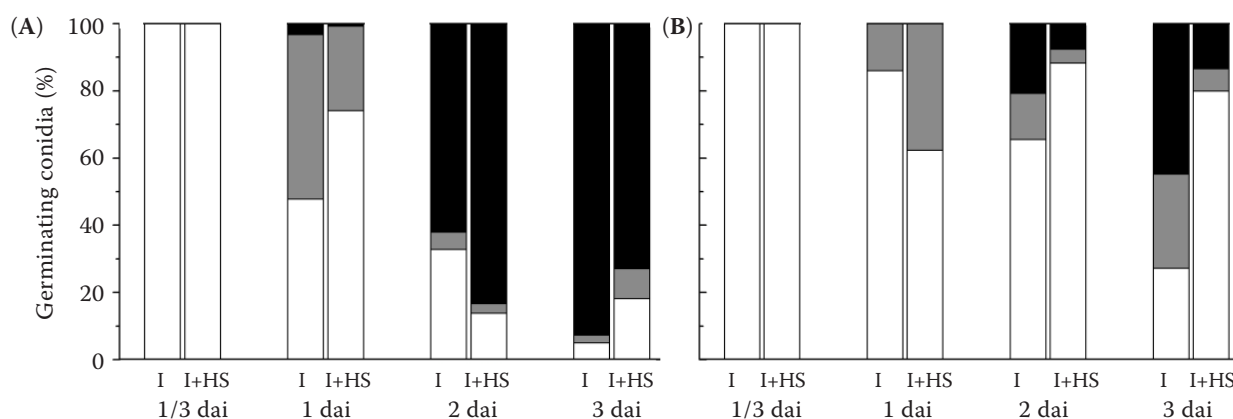


Figure 2. Representation of germinating conidia of *P. neolycopersici* with □ 1st, ■ 2nd, and ■ 3rd germ tubes on (A) *S. lycopersicum* cv. Amateur, as well as (B) *S. chmielewskii* 1/3, 1, 2, and 3 dai on leaves of infected non-treated (I) and infected heat shock pre-treated (I + HS) plants. Assessment of a number of germ tubes – see Materials and Methods

the infected non-HS pre-treated plants. The length of conidial germ tubes after heat treatment slightly extended 2 and 3 dai in comparison with infected leaves without HS (Figure 3). Thus, comparing the infected and IHP leaves in the early stages after inoculation (up to 2 dai), HS pre-treatment slightly accelerated pathogen development, with attention mainly paid to the speed of germ tube growth (Figures 2 and 3).

The combined stress of HS and infection evoked the strong accumulation of SA and JA. After 9 dai, the endogenous level of SA in IHP leaves was about 5 times higher than that in infected leaves (Figure 4). SA gradually accumulated within 4–9 dai, which could be related to chloroses development observed on IHP leaves at that time (Figure 1A). Even the endogenous concentration of JA in IHP leaves rose

about 3 times above the levels in infected leaves at 9 dai (Figure 4). No accumulation of endogenous ABA was observed in IHP leaves when compared to the infected ones (Figure 4).

The effect of HS pre-treatment was also obvious in the case of POX activity. The rise in POX activity was significant 1, 4, and 9 dai in IHP leaves, in comparison with healthy non-treated leaves or infected ones (Figure 5). POX activity was significantly higher even compared to HS pre-treated healthy leaves 4 and 9 dai (Figure 5), again indicating the action of both stresses in this response.

Moderately resistant genotype – Effect of infection. The early development of powdery mildew on infected leaves of *S. chmielewskii* was similar as in the case of highly susceptible *S. lycopersicum*

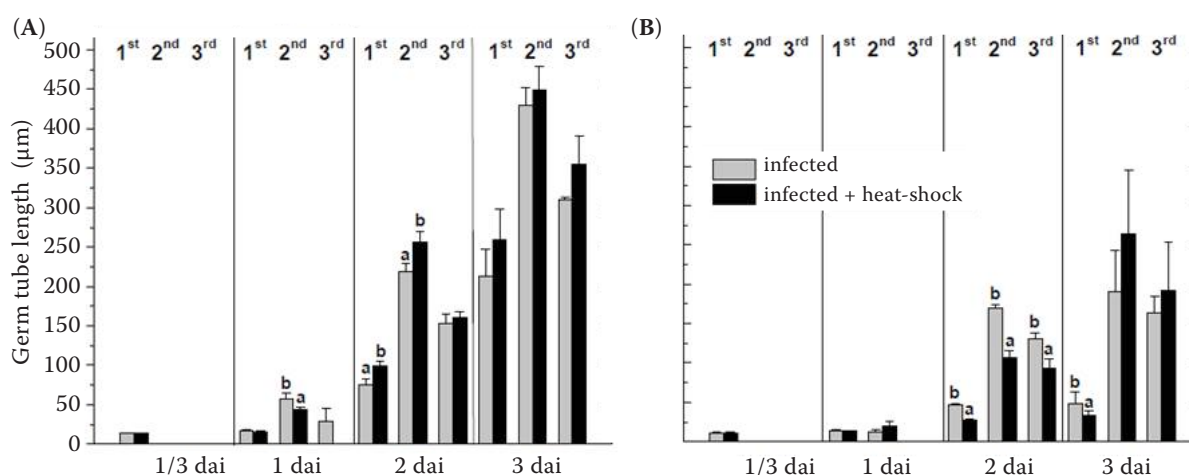


Figure 3. Length of the 1st, 2nd, and 3rd germ tubes arising from conidia of *P. neolycopersici* on (A) *S. lycopersicum* cv. Amateur, as well as (B) *S. chmielewskii* on leaves of non-treated (infected) and heat shock pre-treated (infected + heat-shock) plants within 1/3, 1, 2, and 3 dai. Statistically significant differences are indicated by different letters. Assessment of length of germ tubes – see Materials and Methods

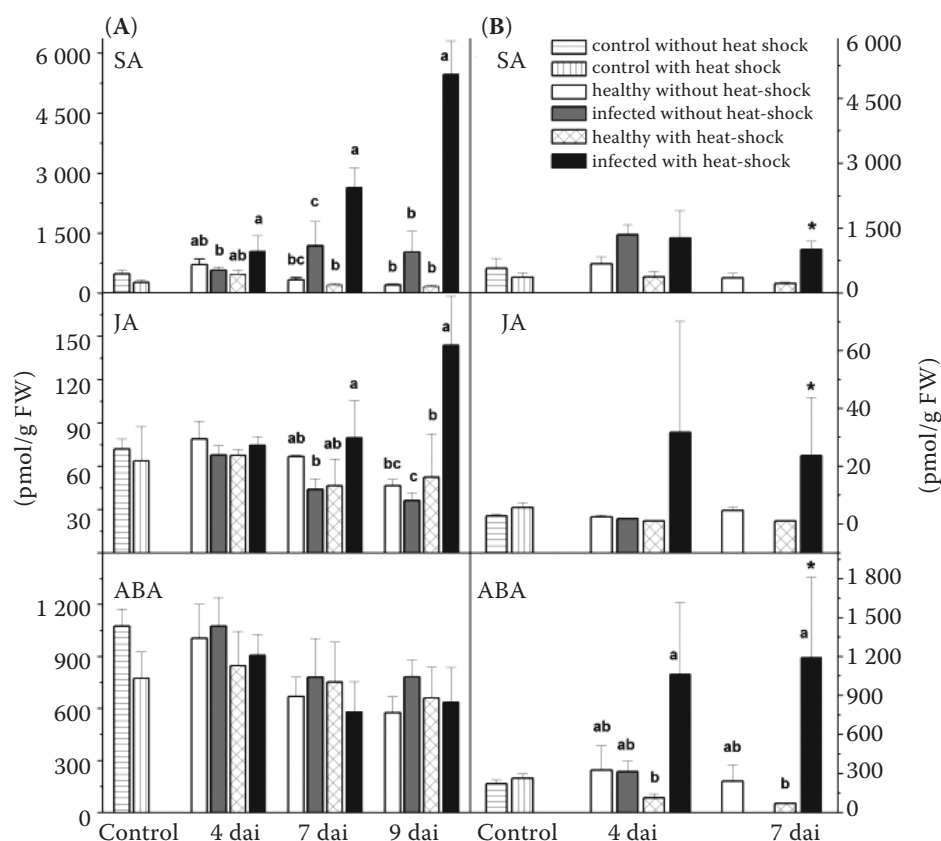


Figure 4. Endogenous concentration of salicylic (SA), jasmonic (JA), and abscisic acid (ABA) in (A) *S. lycopersicum* cv. Amateur, as well as (B) *S. chmielewskii* 4, 7, and 9 days after inoculation by *P. neolycopersici*. Columns “control” designate healthy non-treated plants that were measured 1 day before experiment running, and which belong to the series of plants pre-treated or non-pre-treated by heat shock. Means and SD are shown, $n = 3$ (* $n = 2$); statistically significant differences are indicated by different letters. Missing data for *S. chmielewskii* are caused by abscission of leaves. The figure is modified according to LEBEDA *et al.* (2014)

cv. Amateur, although the speed of germ tube development was slightly lower (Figure 2). Mycelium branching was recorded at 7 dai (Figure 1D). The length of pathogen germ tubes on *S. chmielewskii* was shorter, in comparison with the susceptible genotype, although the germ tubes gradually grew within 3 dai (Figure 3). The difference in resistance between these *Solanum* spp. genotypes was evident mainly in the later stages of pathogen development, when an intensive HR appeared in moderately resistant genotype (Figure 1C, arrows). HR pronouncedly suppressed the pathogen development and led to consecutive necrotisation, wilting, bending, and eventually to the abscising of the leaves.

The endogenous levels of SA, JA, and ABA in infected leaves of *S. chmielewskii* did not change significantly compared to the control plants within 4 dai (Figure 4). Most leaves (except one) for analysis defoliated very early after infection therefore there are no data for 7–9 dai in Figure 4. We did not detect any significant

changes in POX activity in the infected moderately resistant genotype within 4 dai (Figure 5).

Moderately resistant genotype – Effect of heat-shock pre-treatment on healthy and infected leaves. As in the case of the susceptible genotype, HS pre-treatment of healthy non-inoculated *S. chmielewskii* evoked slight changes in the measured parameters. No macroscopic changes in HS pre-treated leaves were observed (Figure 1C). The SA, JA, and ABA levels did not increase in the leaves of *S. chmielewskii* in response to HS pre-treatment, rather a decrease in endogenous ABA concentration was observed within 4–7 days (Figure 4). In the case of POX activity, an increase was observed 1–7 days after HS treatment, in comparison with the healthy non-treated plants (Figure 5).

Initially, IHP leaves expressed similar HR progress as did the infected leaves without HS, when the black spots and leaf wilting were observed (Figure 1C). Eventually, IHP leaves lost their turgor, wilted, and defoliated within 7 to 9 dai in a manner similar to

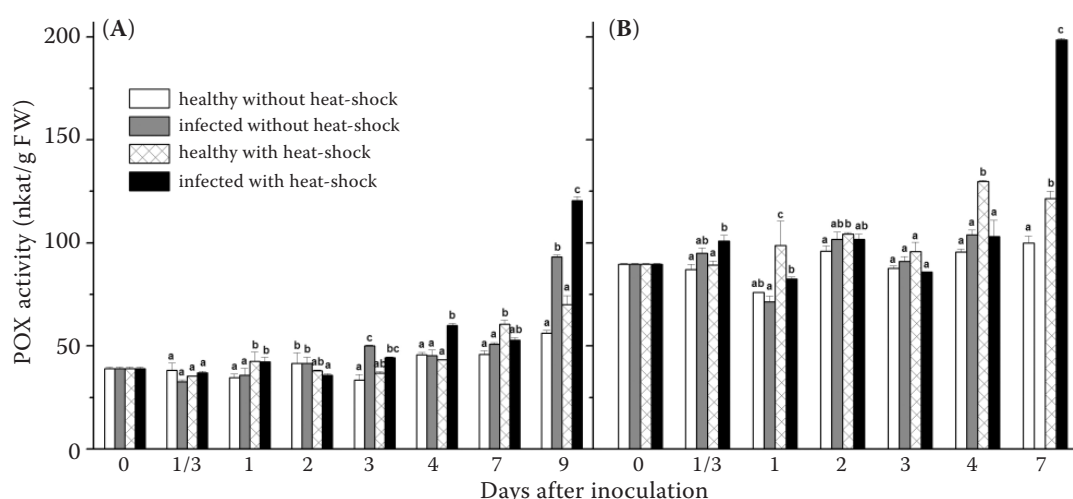


Figure 5. Guaiacol peroxidase (POX) activity in leaves of (A) *S. lycopersicum* cv. Amateur, as well as (B) *S. chmielewskii* 0, 1/3, 1, 2, 3, 4, 7, and 9 days after inoculation by *P. neolycopersici*. Means and SD are shown, $n = 3$; statistically significant differences are indicated by different letters. Missing data for *S. chmielewskii* are caused by abscission of leaves

infected leaves. However, compared to infected leaves without HS, slower germ tube development was detected 2–3 dai in IHP leaves (Figure 2). At 2 dai, the number of powdery mildew conidia producing three germ tubes was lower on *S. chmielewskii* after HS comparing to infected plants without HS (Figure 2) and the length of conidial germ tube was shorter (Figure 3), indicating that HR in this case was stronger in suppressing the pathogen's infection. Thus, in the early stage of infection, we could observe the slightly limited growth of tomato powdery mildew on the moderately resistant genotype after HS.

The SA level did not change significantly in IHP leaves 4 dai, only its slight accumulation at 7 dai was observed (Figure 4). However, a pronounced accumulation of JA and ABA in IHP leaves compared to the infected or HS treated plants was detected within 4 to 7 dai (Figure 4). ABA accumulation could be causally linked with loss of turgor, wilting, and defoliation observed in IHP leaves within 7 dai (Figure 1C). In IHP leaves, a significant increase in POX activity was detected at 1/3 dai (Figure 5). Following days the POX activity in IHP leaves was lower compared to HS pre-treated healthy plants until 7 dai, when the POX activity was doubled in IHP leaves compared to healthy non-treated plants and significantly higher compared to HS pre-treated healthy plants.

DISCUSSION

Susceptible genotype. In the susceptible tomato plants (*S. lycopersicum* cv. Amateur) infected by

powdery mildew *P. neolycopersici*, we observed a significant increase of POX activity 3 dai and mainly 9 dai (Figure 5) indicating the presence of oxidative stress in the attacked leaves. The oxidative stress in infected tomato leaves is in accordance with our previous results, where a slight accumulation of H_2O_2 (1–2 dai) was detected simultaneously with an increase in POX activity (1–5 dai) in the susceptible tomato genotype infected by *O. (= P.) neolycopersici* (TOMÁNKOVÁ *et al.* 2006). Moreover, a marked production of the superoxide anion (O_2^-) was detected at the first hours after inoculation and POX activity increased at 7 dai (MLÍČKOVÁ *et al.* 2004).

The simultaneous action of HS and the infection amplified and accelerated the infection symptoms (Figure 1A) and pathogen development (Figure 3). In this plant–pathogen interaction, the chlorosis and necrosis are usually detectable in the late timeframe, e.g. a few weeks after infection (LEBEDA & MIESLEROVÁ 2002). Rapid development of chlorosis and necrosis in IHP leaves were connected to a faster (4–9 dai) and more pronounced decrease of chlorophyll *a* and *b* pigment contents, stomatal closure, and inhibition of the rate of CO_2 assimilation, when compared to infected non-HS pre-treated leaves undergoing the same plant–pathogen interaction (PROKOPOVÁ *et al.* 2010). Heat-induced susceptibility has also been described in the barley infected by *Blumeria (= Erysiphe) graminis* f.sp. *hordei* (HAZEN & BUSHNELL 1983) or in coffee infected by the rust (SILVA *et al.* 1992). We anticipate that HS pre-treatment, followed by inoculation and infection process, amplified plant stress to such a great extent, that the leaves lost their

turgor and started to necrotise and die much earlier than in the case of infection without HS.

A significant increase in the endogenous levels of SA, JA (7 or 9 dai) and POX activity (mainly 4 and 9 dai) observed in the infected HS pre-treated susceptible tomato (Figures 4 and 5) suggests their important role in the signalling pathways leading to a susceptible response to the combined stresses. These signalling pathways can interact during the defence response and can be dependent on each other to a certain degree. Both antagonistic and also synergistic interactions have been observed between SA- and JA-dependent defences (ROBERT-SEILANIANZ *et al.* 2011; SHIGENAGA & ARGUESO 2016). Treatment of cucumber roots with phenolic acids led to membrane damages accompanied by lipid peroxidation (POLITYCKA 1997), which is a starting point of the JA biosynthetic pathway (WASTERNAK 2006). In line with this hypothesis, we detected an accumulation of free phenols and lipid peroxidation in tomato *S. lycopersicum* cv. Amateur infected by *O. (= P.) neolycopersici* 2–5 dai (TOMÁNKOVÁ *et al.* 2006). The combined stress of HS and infection probably amplified these processes, and the accumulation of JA was detected even at a later timeframe (mainly 9 dai) (Figure 4).

The time correlation between the maximal SA and JA accumulation and increase in POX activity (Figures 4 and 5) suggested a causal link between these plant hormones and oxidative stress in the infected HS pre-treated susceptible tomato. Small molecules such as SA and/or some of the ROS direct cells to initiate cell death programs (HEATH 2000; HUYSMANS *et al.* 2017), which is in accordance with the extensive lesions observed in the same timeframe as the SA accumulation and POX activity increase (Figures 1A, 4, 5). In addition, an increase in POX activity (Figure 5) could be directly related to the membrane lipid peroxidation (TOMÁNKOVÁ *et al.* 2006) followed by JA biosynthesis. A connection between jasmonates and POX activity was firmed up by WALTERS *et al.* (2002), who demonstrated on barley seedlings infected by powdery mildew (*Blumeria graminis* f.sp. *hordei*) a significant increases in the activities of the plant defence-related enzymes, including POX, after the treatment of leaves with methyl jasmonate. A significant increase in POX activity was detected also in healthy HS pre-treated susceptible tomato 1 and 7 dai (Figure 5) suggesting that HS *per se* participated in the POX activation. ALMESELMANI *et al.* (2006) demonstrated a protective role of antioxidant enzymes (including POX)

under high temperature stress on wheat plants. The involvement of both SA and the antioxidant system in the induction of thermotolerance has been proposed (DAT *et al.* 2000; CINGOZ & GUREL 2016).

Thus, HS pre-treatment strengthened the effect of *P. neolycopersici* infection on the susceptible tomato genotype *S. lycopersicum* cv. Amateur by accelerating and amplifying the development of symptoms. The studied biochemical processes, activated by HS pre-treatment of infected plants, were closely interconnected and have a protective role, but with an insufficient capacity to defend the attacked susceptible tomato plants against the powdery mildew infection.

Moderately resistant genotype. Although in this experiment we did not detect any significant increase in POX activity 1–4 dai (Figure 5), our previous reports have shown that HR in *S. chmielewskii* was associated with an increase of POX activity observed during the first 4–12 h of infection, but primarily 5–7 dai (MLÍČKOVÁ *et al.* 2004); with H₂O₂ production 1–5 dai (TOMÁNKOVÁ *et al.* 2006). An increased POX activity and H₂O₂ production in leaf extracts of resistant *S. chmielewskii* correlated with the percentage of dead cells (MLÍČKOVÁ *et al.* 2004; TOMÁNKOVÁ *et al.* 2006). HS *per se* caused a gradual POX activation between 1–7 days in non-infected plants (Figure 5). This is in accordance with previous reports, where antioxidative enzymes (ALMESELMANI *et al.* 2006; REDDY *et al.* 2009) and H₂O₂ (ROS) (DAT *et al.* 1998; LARKINDALE & HUANG 2005; KOTAK *et al.* 2007) participated in the conferring of plant thermotolerance and thermo-protection. However, in the later phases after HS, the combined stress of HS and infection was needed for a more pronounced POX activity increase (Figure 5). Thus, it seems that analogous to the susceptible genotype, both stresses (pathogen and HS) have a cumulative effect on the increase in POX activity.

Infected leaves of *S. chmielewskii* pre-treated by heat showed macroscopically similar HR progress when compared to infected leaves (Figure 1C). More extensive turgor loss and wilting observed later in the IHP leaves might be caused (after the effects of infection) by the combined effect of mechanical damage during the inoculation procedure and HS. The microscopic observation revealed slower development of the pathogen after HS (Figures 2 and 3). A reduction of the infection and an induction of resistance have already been reported after heat-treatment of the leaves of a susceptible barley cultivar infected by powdery mildew (SCHWEIZER *et al.* 1995; VALLÉLIAN-BINDSCHEDLER *et al.* 1998). In contrast to similar

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accumulations of SA in both infected and infected heat-pre-treated leaves, the signalling pathways including ABA and JA only seemed to be activated by the combined stresses of HS and infection (Figure 4). A correlation between the activation of jasmonate signalling and enhanced resistance to pathogens has already been demonstrated in *Arabidopsis* (ELLIS *et al.* 2002). In barley, exogenous application of JA protected plants against powdery mildew *Blumeria* (= *Erysiphe*) *graminis* f.sp. *hordei* (SCHWEIZER *et al.* 1993). A pronounced accumulation of ABA could be causally linked with the observed turgor loss, wilting (Figure 1C), defoliation, senescence, as well as with the stomatal closing detected in our previous work in infected heat-pre-treated leaves within 3–7 dai (PROKOPOVÁ *et al.* 2010). ASSELBERGH *et al.* (2008) reviewed mechanisms of ABA action as a positive and negative regulator of disease resistance and as a factor interfering with both plant abiotic and biotic stress responses. The correlation between ABA accumulation and the reduction of pathogen infection was observed by ACHUO *et al.* (2006) on tomato, where drought stress resulted in a twofold increase in endogenous ABA, as well as in a subsequent significant suppression of *O. (= P.) neolyopersici*. An essential role of ABA in plant resistance and defence responses to pathogens and ABA-mediated induction of JA biosynthesis were presented in *Arabidopsis* plants (ADIE *et al.* 2007).

Our results suggest that stimulus by *P. neolyopersici* infection *per se* was strong enough to evoke a robust *S. chmielewskii* defence response in terms of HR occurrence, inhibition of pathogen development, and the activation of the antioxidant system of plants. HS before inoculation did not strengthen the response significantly; rather, it evoked additional defence signalling pathways, including the accumulation of plant stress hormones that participated in the robust plant stress response.

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