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Heat Treatments at Varying Ambient Temperatures and Durations Differentially Affect Plant Defense to *Blumeria hordei* in a Resistant and a Susceptible *Hordeum vulgare* Line

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Abstract

Our previous research showed that a powdery mildew resistant barley line (MvHV07-17) maintains its resistance to *Blumeria hordei* (Bh) even if plants are exposed to a long-term high temperature of 35°C for 120 h before Bh inoculation, whereas such high temperature pretreatment further increases susceptibility to infection in the susceptible barley line MvHV118-17. In the present study, we extended this approach using short-term high-temperature water treatment (49°C for 30 s) to determine how it affects powdery mildew resistance in these barley lines. We found that this short-term heat shock (HS) impaired plant defense responses, as reflected by development of Bh colonies and visible necrotic spots on leaves of MvHV07-17, which does not develop visible symptoms upon Bh inoculation under optimal growth conditions. In contrast, both HS and long-term heat stress enhanced susceptibility to Bh in MvHV118-17 plants. These results were supported by the measurement of Bh biomass using a qPCR method. Furthermore, microscopic examinations showed that HS elevated the rate of successful Bh penetration events and the spread of cell death in the surrounding mesophyll area and allowed for colony formation and sporulation in resistant barley, whereas early and effective plant defense responses, such as papilla formation and single-cell epidermal hypersensitive response, were significantly reduced. Furthermore, we found that the accumulation of hydrogen peroxide in both resistant and susceptible barley was correlated with susceptibility induced by HS and long-term heat-stress. This study may contribute to a better understanding of plant defense responses to Bh in barley exposed to heat.

Keywords: barley, barley powdery mildew, heat stress, hydrogen peroxide, hypersensitive response, penetration resistance, plant defense responses

As one of the most destructive environmental factors, high temperature induces heat stress in plants and fundamentally influences plant defense responses. Heat stress can affect plants in different ways, such as damaging cell membranes, disrupting genome integrity, disordering the conformation of proteins, altering cytoskeleton structure, and inducing the generation of reactive oxygen species (ROS), often leading to programmed cell death (Han et al. 2021; Parrotta et al. 2016; Zhou et al. 2019). These changes in heat-stressed plants usually negatively affect plant resistance mechanisms (Desaint et al. 2021). It is not surprising that plants react variously to different types of heat treatments. Heat shock (HS; short-term exposure to high temperatures), for example, submerging plants in 49°C water for 30 s 1 day before barley powdery mildew (*Blumeria hordei*, Bh) inoculation, resulted in the susceptibility of near-isogenic lines of barley (*H. vulgare* L. 'Ingrid') containing different resistance genes (*mlo5*, *Mlg*, *Mla12*) (Barna et al. 2014). The same HS further increased susceptibility to Bh in genetically susceptible barley (cultivar Ingrid *Mlo*) (Barna et al. 2014). We found that pre-exposures to long-term heat stress (35°C for 1 to 5 days) also has a negative effect on barley powdery mildew resistance in cultivar Ingrid near-isogenic barley lines manifested as increased powdery mildew growth (Kolozsváriné Nagy et al. 2022). However, we have found a resistant barley breeding line (MvHV07-17) that retains its ability to defend itself against barley powdery mildew even following a 5-day pre-exposure to high temperatures at 35°C (Schwarczinger et al. 2021). It can be concluded that the duration and the intensity of heat stress fundamentally influence plant defense responses (Jagadish et al. 2021). Extreme weather events may arise nowadays due to global climate change. According to the EUROSTAT report for 2019, Spain is the country with the largest barley cultivation area in the EU-28 (Bento et al. 2021). However, the Mediterranean region, and in particular the Iberian Peninsula, has been identified as prominent climate change hot spot (Alessandri et al. 2014). According to current meteorological data, the strongest heatwave so far was observed in June 2023, when parts of Spain warmed to above 40°C. Moreover, in some parts of Sicily, temperatures reached 47.4°C this summer. Therefore, it can be assumed that barley may be exposed to the same extreme environmental conditions in the field as in the experiments of this study. Therefore, it is important to know how plant–pathogen interactions are altered under such conditions.

Bh is an obligate biotrophic Ascomycete fungus, the causative agent of the devastating powdery mildew disease of barley. Early Bh recognition and the timely activation of barley defense responses during powdery mildew infection is necessary to successfully prevent the pathogen. Barley plants have evolved different defense mechanisms to overcome Bh infection (Kuska et al. 2018). The formation of plant epidermal cell wall appositions at sites of attempted penetration (papilla) is an early and effective method of plant defense to Bh, which may arrest powdery mildew penetration and prevent the emergence of the haustorium through which the fungus extracts nutrients and water from the host (Kim et al. 2014; Kusch and Panstruga 2017). If the papilla is unable to block the penetration of the invading powdery mildew, a second layer of plant defense

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(hypersensitive response, HR) is activated in resistant plants, limiting the growth and spread of the pathogen (Bai et al. 2012). The different defense pathways in barley can be linked to specific powdery mildew resistance genes. Papilla formation is characteristic of barley plants carrying *mlo* resistance genes, the single-cell HR is typical in barley plants carrying *Mlg* resistance genes, whereas spreading HR with visible localized necrotic symptoms is usually found in plants with *Mla* resistance genes (Hückelhoven and Kogel 1998). It has been found that in resistant barley, papilla formation initially attempts to block the penetration of Bh 14 to 24 h after inoculation, followed by epidermal single-cell HR expressed at 18 to 30 h post penetration, whereas multicellular HR is triggered in epidermal and surrounding mesophyll cells 24 to 72 h after inoculation (Hückelhoven et al. 1999; Schultheiss et al. 2003). The activation of both defense mechanisms (papilla and HR) correlates with the accumulation of ROS, especially hydrogen peroxide (H_2O_2) (Hückelhoven and Kogel 2003).

In the present study, we simultaneously tested how HS (49°C for 30 s) and long-term heat stress (35°C for 5 days) influence the defense responses of a powdery mildew resistant barley line (MvHV07-17) compared with a susceptible line (MvHV118-17). The effects of heat stress pretreatments on the level of visible powdery mildew symptoms were investigated along with microscopic examinations to determine cellular reactions of the selected lines to Bh, such as the frequency of fungal penetration, papilla formation, and cell death, as well as the development of hyphae, conidiophores, and conidia. Furthermore, powdery mildew biomass and H_2O_2 accumulation were also assessed at different time points following heat stress and powdery mildew inoculation in the selected resistant and susceptible barley lines.

According to our previous results, long-term heat stress (35°C for 5 days) has no significant effect on powdery mildew (Bh) resistance in the resistant barley line MvHV07-17; however, it significantly enhanced susceptibility to Bh in the susceptible line MvHV118-17 (Schwarczinger et al. 2021). In contrast, this study demonstrated that short-term HS significantly reduced Bh resistance in both resistant and susceptible barley lines (MvHV07-17 and MvHV118-17).

Materials and Methods

Plant material, heat treatment, and powdery mildew inoculation

The following barley (*Hordeum vulgare*) lines were used in our experiments: MvHV07-17, MvHV118-17, and Mv Initium. Plants were developed in pots containing potting soil in controlled environmental chambers at 20°C with a 16 h light/8 h dark photoperiod. Barley plants were exposed to long-term heat stress in controlled environmental chambers with a 35°C 16 h light/25°C 8 h dark photoperiod for 120 h (the heat treatment lasted from day 7 to day 12 of plant age) (Schwarczinger et al. 2021). Short-term HS was applied on 12-day-old plants by immersing the leaves into a hot water bath (49°C for 30 s) prepared in a cooker (Barna et al. 2014). Water temperature was continuously monitored by a thermometer. Pots were inverted, and the whole areal parts of plants were submerged. After heat treatments, barley plants were held at 20°C until inoculation. Non-heat-treated control plants were grown at 20°C and a 16/8 h day/night cycle. Bh race A6 was maintained on a susceptible barley cultivar Mv Initium. Freshly produced conidia were dusted onto the leaves of MvHV07-17 and MvHV118-17 2 h after heat treatments using a settling tower. Subsequently, inoculated plants were incubated in a growth chamber at 20°C as described above. Seven days after inoculation (DAI), powdery mildew symptoms were evaluated visually.

Monitoring powdery mildew accumulation by real-time quantitative PCR (qPCR)

To monitor Bh biomass, heat-treated (or non-heat-treated control) and inoculated primary leaves were sampled 2 and 7 DAI. Leaf samples were homogenized in liquid nitrogen by using a QIAGEN TissueLyser II (QIAGEN, Valencia, CA, U.S.A.) for 30 s at 25 Hz. Fungal and plant DNA from homogenized plant tissue was isolated by using a Plant Genomic DNA Extraction Miniprep System Kit (Viogene Biotek Corp., New Taipei City, Taiwan) according to the manufacturer's instructions. The accumulation of Bh biomass in inoculated barley leaves was assayed by using a BioRad CFX96 real-time thermocycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.) running a standard program (95°C for 2 min, 40 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s). To determine fungal biomass, we assayed relative expression of the Bh *Glyceraldehyde-3-phosphate dehydrogenase* (*BhGAPDH*) gene, which has been described as a Bh gene optimal for qPCR assays during the pathogen infection cycle (Pennington et al. 2016) together with the barley reference gene *Ubiquitin* (*HvUbi*) as an internal control. The BhGAPDH primer pair (Pennington et al. 2016) and the HvUbi primers (Trujillo et al. 2006) have been described previously. The qPCR was performed by using 7.5 µl of 2× SYBR Green qPCR master mix (KAPA Biosystems, Wilmington, MA, U.S.A.) along with 0.75 µl of each primer (5 µM) and 2.5 µl of 20-fold diluted DNA template in a 15-µl total reaction volume. The relative fungal biomass was calculated by using the ΔΔ threshold cycle (Ct) method (Schmittgen and Livak 2008). All reactions were performed by using three independent biological experiments with three technical replicates.

Microscopy and 3,3′-diaminobenzidine histochemical staining

Fluorescence and light microscopy were used to monitor fungal development, plant defense responses (papilla formation, cell death) and H_2O_2 localization. Primary leaves of barley were fixed 2 and 7 DAI in 0.15% (wt/vol) trichloroacetic acid in ethanol/chloroform 4:1 (vol/vol) and stored in 50% (vol/vol) glycerol (Hückelhoven and Kogel 1998). To stain Bh structures for bright-field microscopy, cleared barley leaves were incubated in 10% (vol/vol) blue ink (Pelikan) in 25% (vol/vol) acetic acid for 1 min and then washed with water to remove the excess ink as described by Hückelhoven and Kogel (1998). Microscopy was carried out with a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Whole-cell autofluorescence was taken as an indicator of plant cell death (Koga et al. 1988), detected by fluorescence microscopy using a fluorescence filter set composed of a 450- to 490-nm excitation filter, a 495-nm dichroic mirror, and a 500- to 550-nm barrier filter. Photographs were taken with a Zeiss AxioCam ICc5 camera using Zeiss ZEN 2011 software. At least 100 plant/fungus interaction sites were scored in each primary leaf, and five inoculated barley leaves were examined per treatment. Only short epidermal cells of adaxial type A and type B (Koga et al. 1990), and only cells with one penetration attempt were evaluated. Furthermore, only host cells with one penetration attempt were recorded. In addition, we also recorded average lesion diameters at 7 DAI on those leaves where mesophyll cell death was found. Detection of H_2O_2 was performed by an endogenous peroxidase-dependent in situ histochemical staining using 3,3′-diaminobenzidine tetrahydrochloride (DAB) according to Thordal-Christensen et al. (1997). Detached leaves were vacuum-infiltrated with 1 mg ml^{-1} DAB and incubated at room temperature for 2 h before fixation. Clearance and storage of leaf segments, staining of fungal structures and microscopy were carried out as described above. To quantify H_2O_2 production macroscopic photos of DAB-stained leaves were processed using color deconvolution, a plugin from ImageJ software. The average staining intensity of leaves was expressed as a percentage of maximum pixel intensity found in the image.

Statistical analysis

The statistical evaluation of the frequency of Bh/barley interaction types, the relative amount of Bh biomass and quantification of H_2O_2 production (DAB staining) was carried out using the ANOVA method followed by Tukey post-hoc test by using Statistica 13 software (TIBCO Software, Palo Alto, CA, U.S.A.). Significant differences were determined at the 0.05 level of confidence.

Results

According to our previous research, a barley powdery mildew-resistant barley line (MvHV07-17) retains its resistance to Bh in response to pre-exposures to prolonged high temperatures (35°C for 1 to 5 days) manifested as both the lack of powdery mildew symptoms and only a marginal increase in powdery mildew biomass (Schwarczinger et al. 2021). To clarify if powdery mildew resistance of MvHV07-17 is also effective following HS, groups of plants were exposed either to long-term heat treatment (35°C for 5 days) or to HS (49°C for 30 s, 2 h before Bh A6 inoculation). Primary leaves of barley were evaluated visually for powdery mildew symptoms in heat-pretreated and control (held at 20°C) plants 7 DAI. Similar to our previous results (Schwarczinger et al. 2021), MvHV07-17 barley grown at 20°C and plants pretreated with long-term heat stress (35°C for 5 days) did not show any visible powdery mildew symptoms (Fig. 1). However, HS (49°C for 30 s) pretreatment triggered the appearance of chlorosis, local necrotic lesions (hypersensitive response; HR) and development of tiny Bh colonies on the MvHV07-17 leaves (Fig. 1). On the other hand, enhanced powdery mildew symptom severity was observed in the susceptible barley line (MvHV118-17) following exposures to both 35°C at 120 h and 49°C for 20 s compared with control plants (Fig. 1). Heat treatments alone did not cause any visible

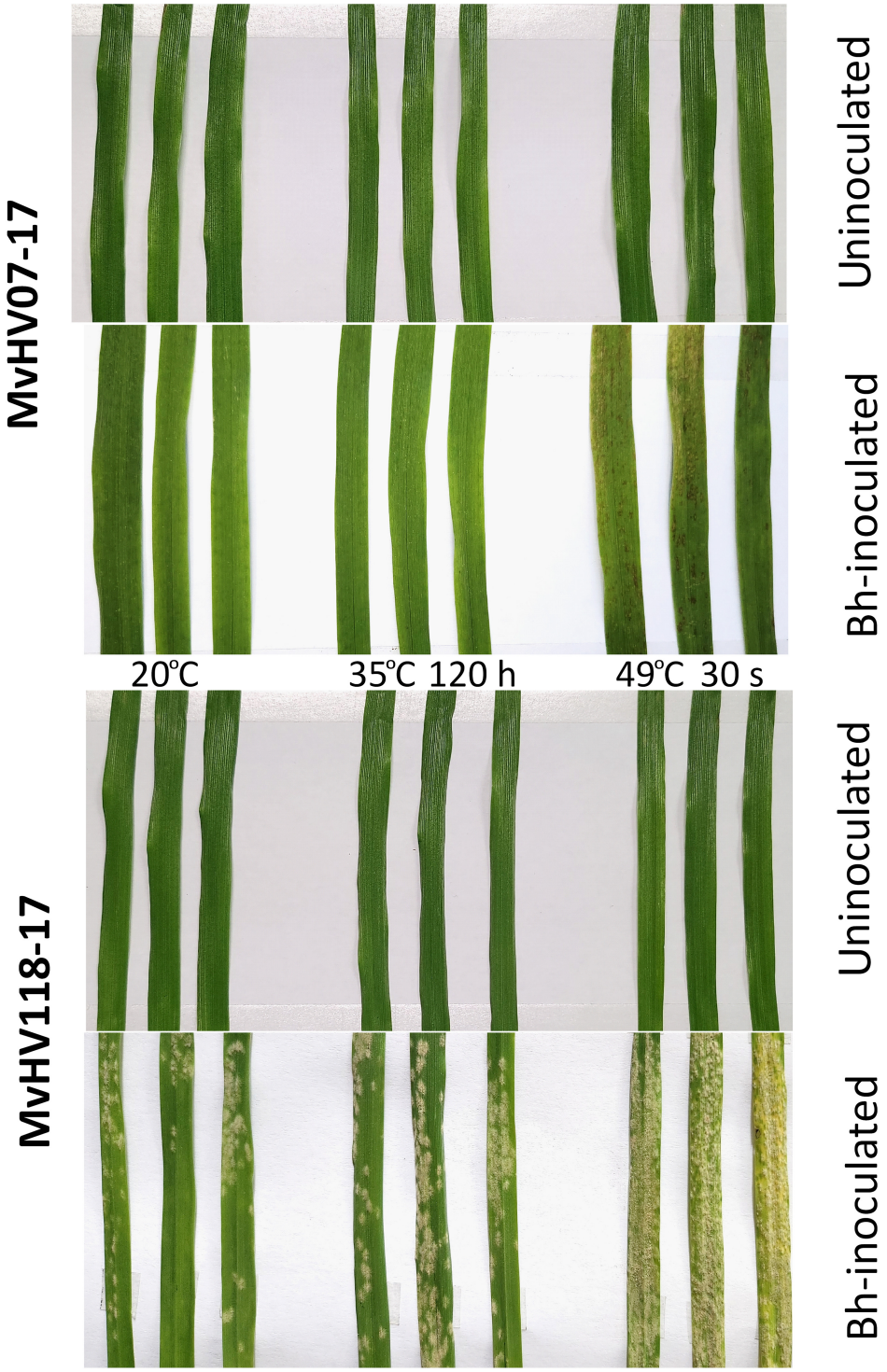

Fig. 1. Macroscopic symptoms on primary leaves of 19-day-old resistant (MvHV07-17) and susceptible (MvHV118-17) barley lines 7 days after heat treatment. A part of plants remained uninoculated, and a second part was inoculated with barley powdery mildew (*Blumeria hordei*; Bh). Inoculation was performed on 12-day-old seedlings 2 h after heat treatments (35/25°C day/night for 120 h or 49°C water for 30 s). Heat-untreated plants were maintained at 20°C throughout the experiment. Photographs are from one representative experiment; three inoculation experiments were carried out with similar results. Barley line MvHV07-17 pretreated with short-term heat shock (49°C, 30 s) shows tiny white colonies and small necrotic spots. The susceptible line MvHV118-17 allows for higher infection rates after heat treatments.

symptoms in the two barley lines (MvHV07-17 and MvHV118-17) (Fig. 1).

We also estimated the levels of relative Bh biomass 2 and 7 DAI in resistant and susceptible barley in response to short- and long-term heat stress. Our results showed that HS brought about a marked increase of powdery mildew biomass in the Bh-resistant line (MvHV07-17) 2 and 7 DAI compared with the control (Fig. 2). Furthermore, long-term heat stress had no significant impact on Bh biomass in MvHV07-17, only a slight but not significant increase was detectable (Fig. 2). In contrast, both short and long-term heat stress further increased Bh biomass in the susceptible MvHV118-17 line (Fig. 2). Taken together, long-term heat stress has no significant effect on powdery mildew symptoms or biomass in the resistant line MvHV07-17; however, HS suppressed powdery mildew resistance, leading to the appearance of visible HR lesions and the emergence of powdery mildew, as well as elevated levels of Bh biomass. It should be emphasized that both HS and long-term heat stress enhanced the powdery mildew coverage and biomass in the susceptible MvHV118-17 line.

To gain deeper insight into how HS and long-term heat stress affects plant defense responses in resistant and susceptible barley, we performed microscopic examinations of infection processes in attacked epidermal cells 2 and 7 DAI. The analysis of samples collected 2 DAI from plants grown at 20°C revealed that only 1 to 2% of all conidia produced elongated secondary hyphae (ESH) (associated with successful fungal penetration and development of mature haustoria) in the resistant line MvHV07-17. However, 20% of all conidia formed ESH in the susceptible line (Fig. 3). Interestingly, the frequency of interaction sites where fungal penetration was prevented by papillae was around 70% of all attacked cells in both lines, indicating a significant level of basal resistance in the susceptible line as well (Fig. 3). A substantial difference was observed in the frequency of whole-cell DAB staining (indicating ongoing HR) in the epidermal cells between the resistant and susceptible lines. A larger proportion of attacked cells showed whole-cell DAB staining in the resistant plants (about 20%) than in the susceptible plants (about 10%) (Fig. 3). Both HS and long-term heat pretreatments led to a two- to threefold increase in the rate of successful powdery mildew penetration attempts in the susceptible plants (Fig. 3). Furthermore, the proportion of effective papilla responses fell by around a third in the susceptible plants after either HS or long-term heat treatments, and at the same time, the number of DAB-stained cells decreased slightly but not significantly in susceptible plants (Fig. 3). The ESH production rate of conidia increased strongly in resistant barley line MvHV07-17 from 1% to 15% and 40% in response to long term heat treatment and HS, respectively (Fig. 3). The rate of papilla-based penetration resistance decreased significantly in the resistant plant (MvHV07-17) in response to both HS and long-term heat stress; however, the effect of HS was much stronger. Interestingly, neither HS nor long-term heat stress affected the frequency of whole-cell H_2O_2 accumulation, which indicates that heat pretreatments did not make the attacked epidermal cells more prone to undergo cell death (Fig. 3).

Seven days after the inoculation, the barley–Bh interaction was assessed microscopically only in the resistant barley line (MvHV07-17) because at 7 DAI, the primary leaves of susceptible plants were almost completely covered with powdery mildew, especially in heat-treated leaves, which made microscopic observations difficult. The results of heat untreated resistant MvHV07-17 plants indicated that very low numbers of conidia developed ESH (Fig. 4A and B), which is similar to the results obtained at 2 DAI. Most of these cases were accompanied by the induction of multi-cell death as indicated by an accumulation of autofluorescent material in adjacent mesophyll cells (Fig. 4C and D). Both HS and long-term heat pretreatments increased the production of ESH; however, the increase was more pronounced and statistically significant only as a result of HS in the resistant barley (Fig. 4A). Strikingly, the average lesion diameter was significantly larger in MvHV07-17 plants exposed to HS compared with plants exposed to long-term heat stress and in the control plants kept at 20°C (Table 1). Importantly, the sporulation of powdery mildew with (Fig. 4E and F) or without (Fig. 4G and H) cell death was observed only on those MvHV07-17 plants that were subjected to HS (49°C for 30 s). Therefore, it seems that HS allows the fungus to complete the entire asexual infection cycle even in the Bh-resistant barley line MvHV07-17.

Both heat stress and Bh inoculation have a significant impact on plant ROS production. Therefore, we assayed H_2O_2 accumulation with DAB staining in both resistant and susceptible barley leaves. The accumulation of H_2O_2 was detected 0, 1, 2, 3, and 7 days after heat stress and/or powdery mildew inoculation. Our results showed that the level of H_2O_2 was significantly higher in response to HS (49°C 30 s) at 0 DAI compared with the control plants held at 20°C and the plants exposed to long-term heat treat-

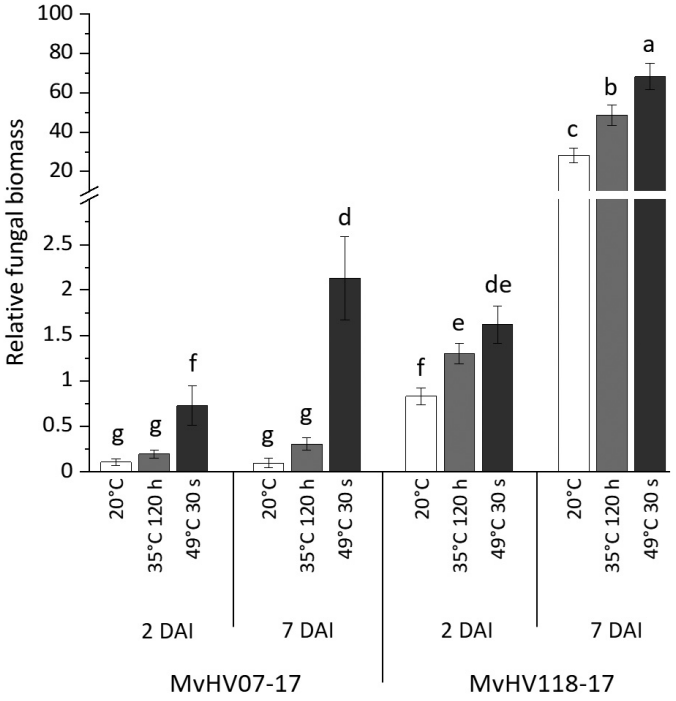

Fig. 2. Changes in barley powdery mildew (*Blumeria hordei*) biomass in response to heat stress pretreatments (35/25°C day/night for 120 h or 49°C water for 30 s) in primary leaves of resistant (MvHV07-17) and susceptible (MvHV118-17) barley lines 2 and 7 days after inoculation (DAI). Inoculation was performed on 12-day-old seedlings 2 h after heat treatments (35/25°C day/night for 120 h or 49°C water for 30 s). Plants were kept at 20°C before and after heat treatments. Control plants were maintained at 20°C. Quantification of powdery mildew DNA was carried out by real-time PCR. The abundance of *B. hordei glyceraldehyde-3-phosphate dehydrogenase* (*BhGAPDH*) was quantified and normalized using the barley reference gene *ubiquitin*. Normalized expression of *BhGAPDH* in all samples is related to that in susceptible barley (MvHV118-17) kept at 20°C at 2 DAI (arbitrary unit 1). The graph shows the average normalized relative expression of *BhGAPDH*, and error bars represent the standard deviation values of three independent experiments. Different letters indicate statistically significant differences at $P \leq 0.05$.

TABLE 1. Average lesion diameters and standard deviation (SD) values in resistant barley (MvHV07-17) leaves where mesophyll cell death was found 7 days after different heat treatments and *Blumeria hordei* inoculation[z]

Heat treatment	Average lesion diameter ± SD
20°C	103 ± 37 µm b
35/25°C day/night for 120 h	108 ± 46 µm b
49°C for 30 s	386 ± 108 µm a

[z] Different letters below average values indicate statistically significant differences at $P \leq 0.05$.

ment in both the resistant and susceptible lines (Fig. 5; Table 2). The elevation of H_2O_2 was detectable in both uninoculated and Bh-inoculated plants therefore it is probably related to HS (49°C, 30 s) pretreatment regardless of Bh inoculation. Heat pretreatments themselves did not increase the level of H_2O_2 at later time points. Bh inoculation combined with HS (49°C, 30 s) caused elevated levels of H_2O_2 1, 2, and 3 DAI in both resistant and susceptible lines; however, the level of increase was more pronounced in the resistant barley line 2 and 3 DAI (Fig. 5; Table 2). On the other hand, H_2O_2 accumulation was obvious even at 7 DAI in susceptible barley in parallel with heat-induced susceptibility to Bh (Fig. 5; Table 2). However, in case of resistant plants a significant increase of H_2O_2 accumulation was observed only on HS-pretreated leaves. Overall, HS itself increased H_2O_2 accumulation, which was further increased upon inoculation with Bh and high levels of hydrogen peroxide correlated with increased Bh biomass.

Discussion

Heat stress caused by high temperature induces an array of anatomical, physiological, and biochemical changes in plants affecting plant growth and development and modifies plant–pathogen interactions (Desaint et al. 2021; Wahid et al. 2007). Here, we found that a short-term HS (49°C, 30 s) applied shortly before powdery mildew inoculation induced susceptibility in a powdery mildew-resistant barley (MvHV07-17) line. However, according to our current results and previous work (Schwarczinger et al. 2021) long-term heat pretreatment (35°C, 120 h) has no significant impact on powdery mildew resistance in the same barley line. Our results and other studies showed that the intensity and the duration of heat exposure fundamentally influence the outcome of pathogen infections (Li et al. 2018). Resistant near-isogenic lines (NILs) of the powdery mildew susceptible barley cultivar Ingrid carry different

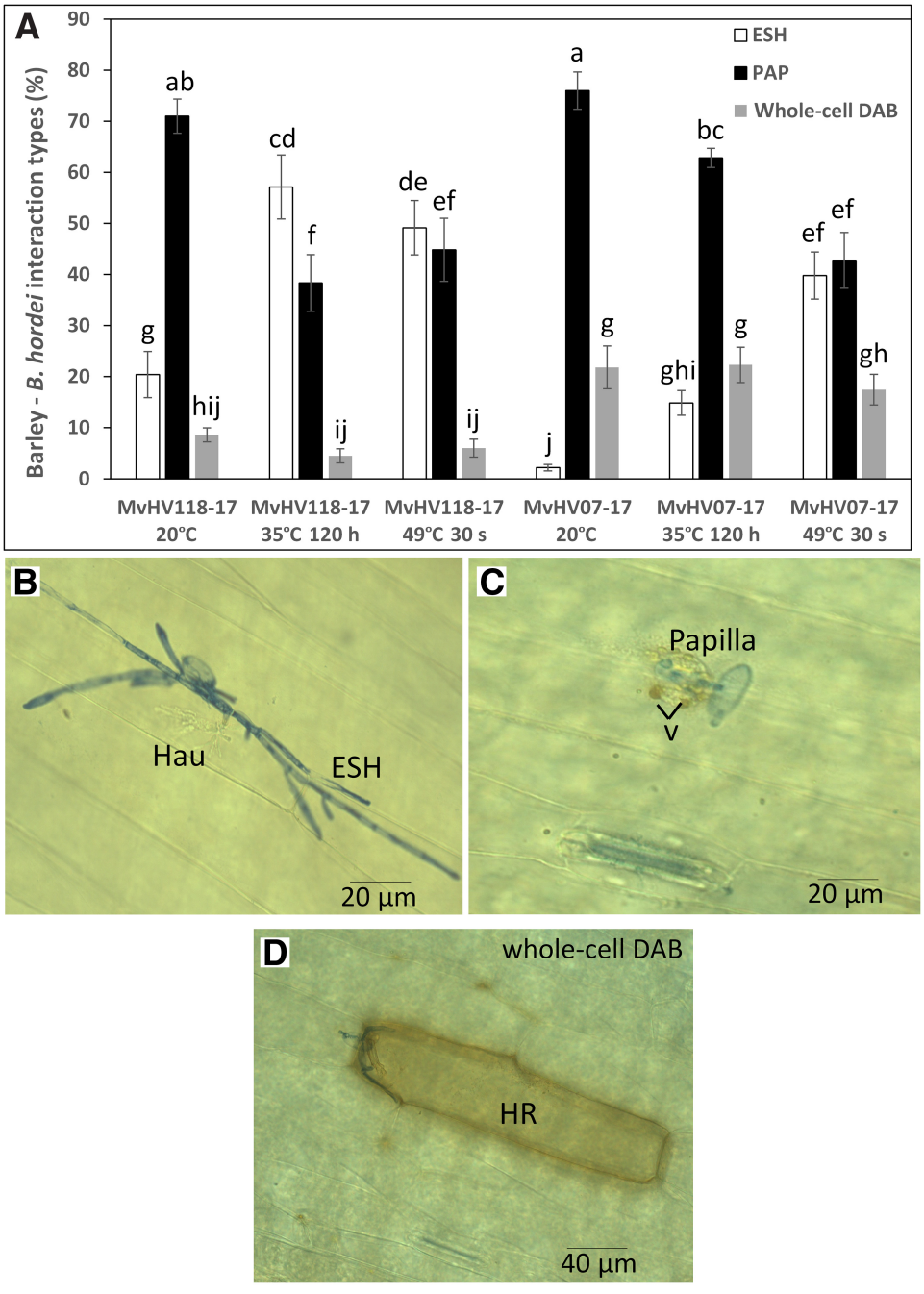

Fig. 3. A, Classes of cellular interaction phenotypes on primary leaves of 14-day-old susceptible (MvHV118-17) and resistant (MvHV07-17) barley plants 48 h after heat treatments (35/25°C day/night for 120 h or 49°C for 30 s) and inoculation with barley powdery mildew (*Blumeria hordei*; Bh). There are three categories of interactions within each heat stress pretreatment: successful Bh penetration (elongated secondary hyphae, ESH), effective papilla formation with fungal penetration failure (PAP), or whole-cell H_2O_2 accumulation as detected by 3,3′-diaminobenzidine (whole-cell DAB) in attacked barley epidermal cells. At least 100 plant/fungus interaction sites were scored in each primary leaf, and five inoculated barley leaves were examined per treatment to calculate the percentage of the respective interaction types (ESH, PAP, whole-cell DAB) within each sample. The graphs show the average of three independent experiments. Error bars represent standard deviation. Different letters above bar graphs indicate statistically significant differences at $P \leq 0.05$. **B,** Development of active haustoria (Hau) and ESH formation. **C,** Effective papilla formation in a barley epidermal cell associated with an unsuccessful penetration attempt. Papilla and vesicles (v) are stained with DAB. **D,** Whole-cell H_2O_2 accumulation detected by DAB in a barley epidermal cell attacked by Bh, indicating an ongoing hypersensitive response (HR) leading to cell death.

powdery mildew resistance genes such as *Mla12*, *Mlg*, or *mlo5*. The exposition of these NILs to HS by submerging plants in 49°C water for 20 to 50 s resulted in the appearance of powdery mildew symptoms, and the same HS further increased susceptibility in the susceptible barley cultivar Ingrid (Barna et al. 2014). Interestingly, we found that such an HS (49°C, 45 s) could partially suppress the non-host resistance of barley to wheat powdery mildew (Künstler et al. 2018). Submerging barley and wheat plants in hot water (HS, 49°C) is not a naturally occurring event. However, applying hot water treatments to inhibit plant disease resistance to different pathogens is a commonly used and well-documented method in plant biology (Aist and Israel 1977; Barna 1975; Barna et al. 2014; Hazen and Bushnell 1983; Künstler et al. 2018, 2023).

Our results showed that HS effectively reduces plant defense responses to powdery mildew infection in both resistant and susceptible barley lines, whereas the resistance-inhibiting effect of long-term heat stress was detectable only in the susceptible line. The physiological processes involved in the differential effects of heat stress can be partly explained by the results obtained in this study. Our data showed that heat stress significantly reduced papilla-mediated penetration resistance in both Bh-resistant and -susceptible interactions. However, the inhibitory effect of HS (49°C for 30 s) on penetration resistance was much more pronounced compared with the effect of long-term heat treatment at 35°C for 120 h in the resistant line MvHV07-17. The decrease in the effectiveness of penetration resistance was accompanied by a significant increase in the frequency of successful penetration events. Previous research also showed that papilla formation is strongly inhibited and Bh penetration efficiency increased in response to HS (50°C for 45 s) in partially dissected barley coleoptiles. However, previously, it has not been investigated how the inhibition of papilla formation affects the outcome of powdery mildew infection (Aist

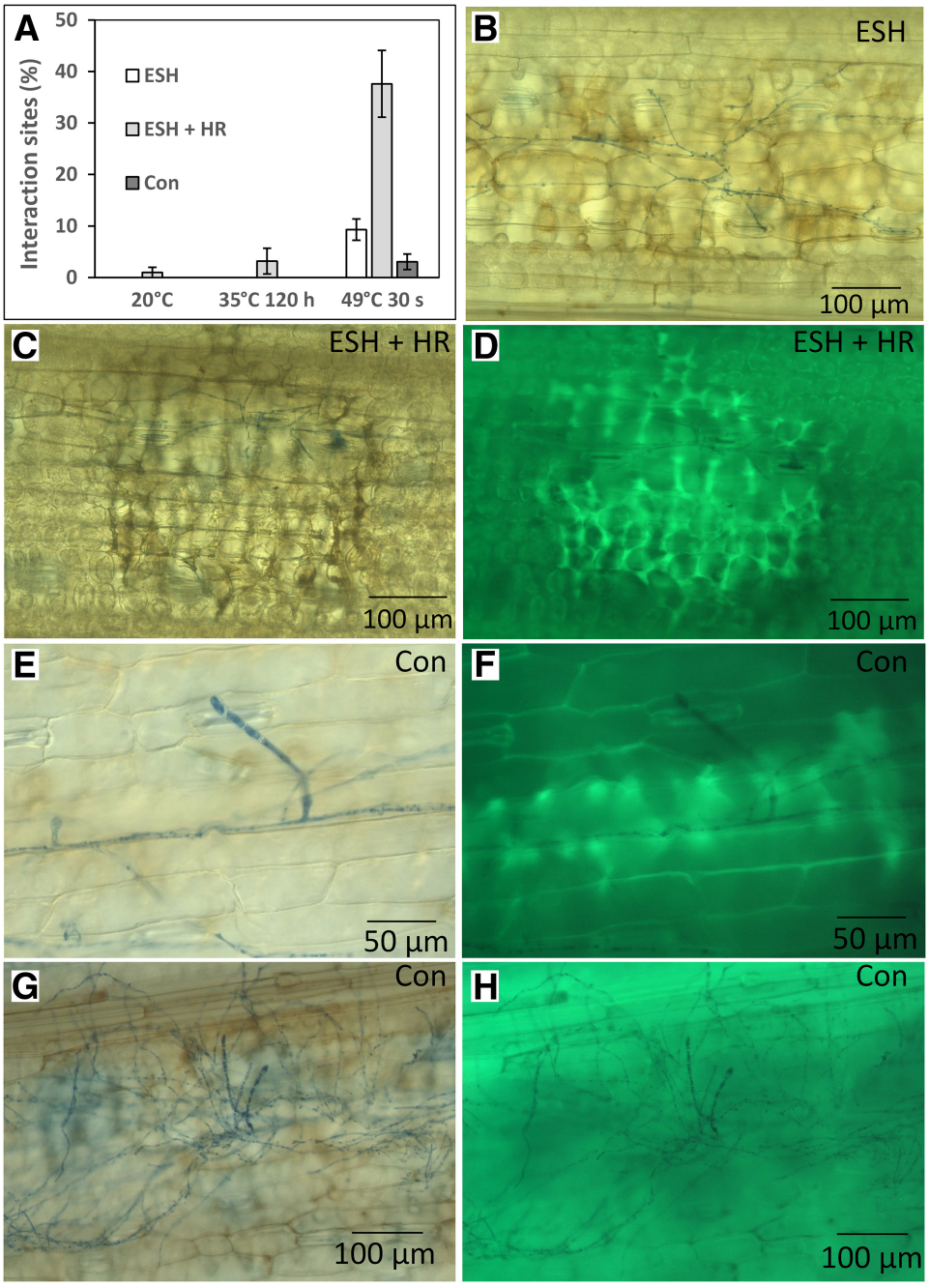

Fig. 4. A, Proportion of successful penetration sites showing elongated secondary hyphae (ESH, ESH + HR, Con) relative to the total number of interaction sites including effective papilla formation and single-cell hypersensitive response (HR). At least 100 plant/fungus interaction sites were scored in each primary leaf and five inoculated barley leaves were examined per treatment to calculate the percentage of successful penetration sites (ESH, ESH + HR, Con) within each sample. Primary leaves of 19-day-old resistant MvHV07-17 barley line were investigated 7 days after heat treatments (35/25°C day/night for 120 h or 49°C for 30 s) and inoculation with *Blumeria hordei* A6. There are three categories of interactions within each heat stress pretreatment: established colonies that produced elongated secondary hyphae and did not incite cell death (ESH), interaction sites with ESH that resulted in mesophyll cell death (ESH + HR), or ESH forming colonies with newly developed conidia (Con). **B,** A germling that did not form conidiophores (ESH). **C and D,** A micro-colony that induced multi-cell death as indicated by accumulation of H_2O_2 and autofluorescent material in underneath mesophyll cells (ESH + HR). **E and F,** Conidium formation. Cells undergoing cell death showed autofluorescence beneath the infection site (Con). **G and H,** Conidiation by an established colony. Epifluorescence microscopy did not visualize any autofluorescence of plant cells (Con). Panels C, E, and G are light micrographs and D, F, and H, respectively, are corresponding fluorescent images. H_2O_2 accumulation was detected by 3,3′-diaminobenzidine (DAB).

and Israel 1977; Stolzenburg et al. 1984). Others have found that HS pretreatment (50°C for 30 s) of cucumber leaves enabled the generation of penetration hyphae and plant infection by the pathogenicity-deficient *aph1* mutant of *Colletotrichum lagenarium*, which fails to develop intracellular penetration hyphae into the host epidermis at optimal temperatures (Takano et al. 2006). Moreover, an HS (55°C for 45 s applied 30 min before inoculation) completely inhibited resistance associated with the HR in a powdery mildew inoculated barley line carrying the *Mla*-resistance gene (Hazen and Bushnell 1983). It has been presented that heat stress at 33°C for 2 weeks suppressed *Cf4* and *Cf9* resistance gene-mediated HR in tomato expressing the matching avirulence proteins (AVR9) of *Cladosporium fulvum* (de Jong et al. 2002). Furthermore, prolonged high temperatures (30°C for 30 days) suppressed the transcription of *RPW8.1* and *RPW8.2* resistance genes, reduced spontaneous localized necrosis on leaves, and suppressed powdery mildew resistance of *Arabidopsis thaliana* in response to *Erysiphe cichoracearum* infection (Xiao et al. 2003). Overall, it can be concluded that the inhibition of penetration resistance is compensated by the higher rate of single-cell HR occurring in resistant barley epidermis, compared with susceptible plants, which displayed no such effect in response to the heat treatment at 2 DAI. On the other hand, at 7 DAI, single-cell epidermal HRs were reduced in resistant barley in response to HS and long-term heat stress, whereas the negative effects of HS (49°C for 30 s) was much more pronounced. This resulted in a significant increase in cell death in mesophyll cells surrounding Bh penetration sites. Interestingly, as a result of HS, the size of necrosis in barley mesophyll cells increased significantly, and visible necrotic symptoms appeared along with Bh sporulation in resistant barley. Others have observed that HS increased the size of necrotic lesions

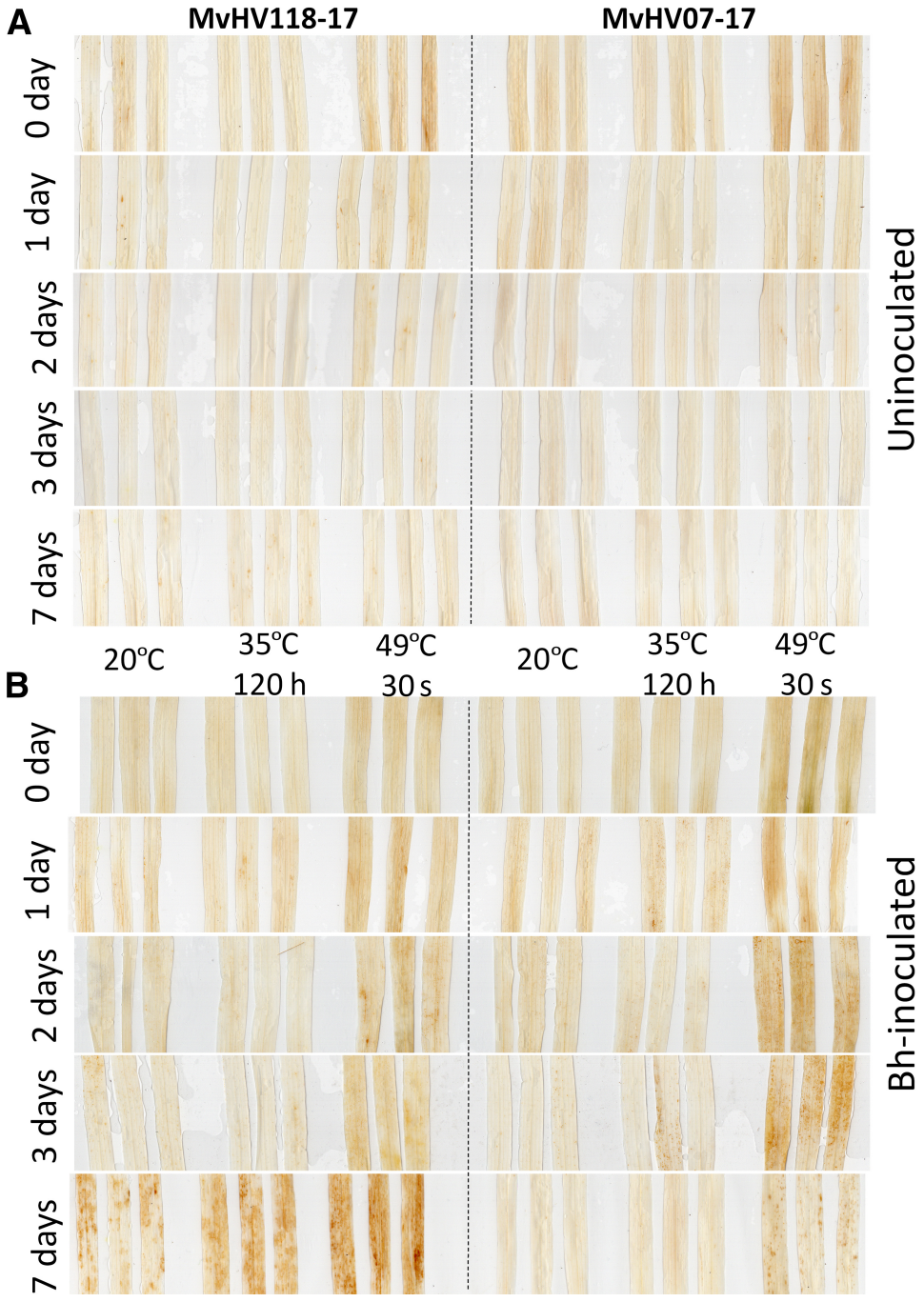

Fig. 5. Hydrogen peroxide accumulation detected by 3,3′-diaminobenzidine (DAB) following heat stress pretreatments (35/25°C day/night for 120 h or 49°C for 30 s) in *Blumeria hordei* (Bh)-resistant MvHV07-17 and susceptible MvHV118-17 barley lines 0, 1, 2, 3, and 7 days after heat stress. **A and B,** Uninoculated control and Bh-inoculated leaves, respectively. Control plants were kept at 20°C. Photographs are from one representative experiment; the experiment was repeated three times with similar results.

in resistant NILs of barley cultivar Ingrid and stimulated fungal sporulation (Barna et al. 2014). Moreover, as a result of HS, visible necrotic symptoms appeared during the non-host interaction of barley with wheat powdery mildew (Künstler et al. 2018).

Several studies have reported that a the ROS H_2O_2 plays a diverse role in the first line of plant defense to pathogens, promoting both effective papilla formation and activation of hypersensitive cell death (Ali et al. 2018; Kuźniak and Urbanek 2000; Mansoor et al. 2022). The accumulation of H_2O_2 in papillae and in cytosolic vesicles near the papillae is necessary for the inhibition of Bh penetration (Hückelhoven et al. 1999). H_2O_2 contributes to strengthening the plant cell wall by participating in lignification and oxidative cross-linking of hydroxyproline-rich proteins (Babosha et al. 2020). Moreover, enzymatic removal of H_2O_2 with catalase significantly increased fungal penetration efficiency (Mellersh et al. 2002). Vesicle-like structures containing H_2O_2, peroxidase and phenolic material for cell wall toughening are often present near effective papillae in resistant barley genotypes (Hückelhoven et al. 1999). Actin filaments are important routes for intracellular vesicle transport, and it is known that the actin cytoskeleton is differentially reorganized in susceptible and resistant barley. Actin filaments focusing toward attempted penetration sites were closely associated with inhibition of Bh penetration (Opalski et al. 2005). However, heat stress affects the cytoskeleton, causing thereby alterations in the process of vesicular transport and cell wall deposition (Müller et al. 2007; Parrotta et al. 2016; Stolzenburg et al. 1984). Our results showed that HS itself causes H_2O_2 accumulation, which is further increased upon inoculation with Bh. In addition, elevated levels of H_2O_2 in both resistant and susceptible barley are correlated with HS-induced enhanced susceptibility reflected by increased Bh biomass. Several reports have demonstrated a significant increase in DAB staining at late stages of powdery mildew infection in susceptible plants that may be considered an important marker for tissue damage caused by pathogen attack (Bhosle et al. 2019; Tek et al. 2022). It can be assumed that the vesicular transport that occurs as a result of heat stress is damaged in these plants and hydrogen peroxide is not transported and used during plant defense responses (papilla formation and HR). Therefore, the plant becomes more susceptible even at high in planta levels of hydrogen peroxide. According to previous studies it can be anticipated that catalases are the primary enzymatic detoxifiers of hydrogen peroxide and catalase activity decreases during 47 to 50°C heat stress (Anderson 2002), which may also explain the elevated levels of hydrogen peroxide in heat-pretreated and inoculated plants.

Overall, the present study shows that although long-term heat stress (35°C for 120 h) enhanced penetration efficiency in both Bh-resistant (MvHV07-17) and -susceptible (MvHV118-17) barley lines, a significant increase in Bh biomass was experienced only in MvHV118-17, in parallel with more pronounced powdery mildew symptoms as previously described (Schwarczinger et al. 2021). We could confirm that the effect of long-term heat stress was limited in the resistant MvHV07-17 barley because Bh biomass did not change significantly and no visible powdery mildew growth was detectable even at 7 DAI (Schwarczinger et al. 2021). On the other hand, here, we demonstrate that HS compromised defense responses against the barley powdery mildew pathogen (Bh) in both the resistant and susceptible barley lines MvHV07-17 and MvHV118-17, respectively. HS-induced enhanced susceptibility was manifested by an exacerbation of disease symptoms and elevated levels of Bh biomass. Moreover, HS allowed the fungus to complete the entire asexual infection cycle, even in the Bh-resistant barley line MvHV07-17. The present study emphasizes the possible significance of a HS in compromising crop disease resistance and may contribute to a better understanding of plant defense responses to Bh in barley exposed to HS versus long-term heat stress.

Literature Cited

Aist, J. R., and Israel, H. W. 1977. Effects of heat-shock inhibition of papilla formation on compatible host penetration by two obligate parasites. Physiol. Plant Pathol. 10:13-20.

Alessandri, A., De Felice, M., Zeng, N., Mariotti, A., Pan, Y., Cherchi, A., Lee, J.-Y., Wang, B., Ha, K.-J., Ruti, P., and Artale, V. 2014. Robust assessment of the expansion and retreat of Mediterranean climate in the 21st century. Sci. Rep. 4:7211.

Ali, M., Cheng, Z., Ahmad, H., and Hayat, S. 2018. Reactive oxygen species (ROS) as defenses against a broad range of plant fungal infections and case study on ROS employed by crops against *Verticillium dahliae* wilts. J. Plant Interact. 13:353-363.

Anderson, J. A. 2002. Catalase activity, hydrogen peroxide content and thermotolerance of pepper leaves. Sci. Hortic. 95:277-284.

Babosha, A. V., Ryabchenko, A. S., Avetisyan, G. A., and Avetisyan, T. V. 2020. Visualization of the halo region in plant–powdery mildew interactions by cryoscanning electron microscopy. J. Plant Pathol. 102:103-111.

Bai, S., Liu, J., Chang, C., Zhang, L., Maekawa, T., Wang, Q., Xiao, W., Liu, Y., Chai, J., Takken, F. L. W., Schulze-Lefert, P., and Shen, Q.-H. 2012. Structure-function analysis of barley NLR immune receptor MLA10 reveals its cell compartment specific activity in cell death and disease resistance. PLoS Pathog. 8:e1002752.

Barna, B. 1975. Effect of heat predisposition and benzimidazole treatment on wheat stem rust resistance. Acta Phytopathol. Acad. Sci. Hung. 10:1-6.

Barna, B., Harrach, B., Viczián, O., and Fodor, J. 2014. Heat induced susceptibility of barley lines with various types of resistance genes to powdery mildew. Acta Phytopathol. Entomol. Hung. 49:177-188.

Bento, V. A., Ribeiro, A. F. S., Russo, A., Gouveia, C. M., Cardoso, R. M., and Soares, P. M. M. 2021. The impact of climate change in wheat and barley yields in the Iberian Peninsula. Sci. Rep. 11:15484.

TABLE 2. Intensity of 3,3′-diaminobenzidine staining for H_2O_2 accumulation in uninoculated and *Blumeria hordei* (Bh)-inoculated leaves of susceptible MvHV118-17 and resistant MvHV07-17 barley lines 0, 1, 2, 3, and 7 days following heat stress pretreatments (35/25°C day/night for 120 h or 49°C for 30 s)[z]

	MvHV118-17			MvHV07-17		
Days	20°C	35°C for 120 h	49°C for 30 s	20°C	35°C for 120 h	49°C for 30 s
Uninoculated						
0 day	6.5 ± 1.4 i–l	4.8 ± 1.1 k–l	11.5 ± 1.6 c–g	7.1 ± 1.9 h–l	6.5 ± 1.2 i–l	14.3 ± 1.3 c–g
1 day	6.3 ± 1.6 i–l	5.5 ± 1.4 j–l	9.3 ± 1.4 e–j	7.0 ± 2.3 h–l	5.6 ± 1.7 i–l	8.9 ± 1.2 f–k
2 days	4.8 ± 1.9 k–l	5.6 ± 1.3 i–l	5.4 ± 1.2 j–l	6.8 ± 1.0 h–l	5.2 ± 1.4 j–l	6.2 ± 1.3 i–l
3 days	5.9 ± 1.6 i–l	6.1 ± 2.2 i–l	5.8 ± 2.0 i–l	6.7 ± 1.7 h–l	5.8 ± 1.5 i–l	5.5 ± 1.0 j–l
7 days	5.8 ± 2.0 i–l	5.0 ± 1.2 j–l	6.3 ± 1.2 i–l	5.6 ± 1.4 j–l	5.8 ± 1.5 i–l	5.4 ± 1.6 j–l
Bh-inoculated						
0 day	6.2 ± 1.6 i–l	5.2 ± 1.3 j–l	10.5 ± 1.5 c–i	6.1 ± 1.5 i–l	6.5 ± 1.4 i–l	14.0 ± 1.5 b–d
1 day	4.9 ± 1.9 k–l	5.7 ± 1.7 i–l	14.0 ± 2.3 b–d	6.9 ± 1.8 h–l	7.0 ± 1.0 h–l	11.0 ± 2.2 c–h
2 days	5.4 ± 1.2 j–l	4.2 ± 1.5 l	11.5 ± 2.0 c–g	7.3 ± 1.4 g–l	4.9 ± 1.8 k–l	17.5 ± 2.1 b
3 days	7.9 ± 1.7 g–l	8.6 ± 1.5 g–k	13.3 ± 1.4 b–e	6.7 ± 0.7 h–l	9.3 ± 1.6 e–j	18.8 ± 2.5 b
7 days	12.9 ± 1.5 c–f	18.7 ± 2.8 b	28.3 ± 3.9 a	5.6 ± 2.2 i–l	4.9 ± 1.9 k–l	9.9 ± 1.5 d–i

[z] Control plants were kept at 20°C. The average staining intensity of leaves was expressed as a percentage of maximum pixel intensity found in the image. Error values represent standard deviation. Different letters below average values indicate statistically significant differences at $P \leq 0.05$.

Bhosle, S. M., Marathe, N., and Makandar, R. 2019. The *er2* gene resistance against powdery mildew infection is associated with enhanced antioxidative protection and defense gene expression. Physiol. Mol. Plant Pathol. 106: 253-262.

de Jong, C. F., Takken, F. L. W., Cai, X., de Wit, P. J. G. M., and Joosten, M. H. A. J. 2002. Attenuation of Cf-mediated defense responses at elevated temperatures correlates with a decrease in elicitor-binding sites. Mol. Plant-Microbe Interact. 15:1040-1049.

Desaint, H., Aoun, N., Deslandes, L., Vailleau, F., Roux, F., and Berthomé, R. 2021. Fight hard or die trying: When plants face pathogens under heat stress. New Phytol. 229:712-734.

Han, S.-H., Kim, J. Y., Lee, J.-H., and Park, C.-M. 2021. Safeguarding genome integrity under heat stress in plants. J. Exp. Bot. 72:7421-7435.

Hazen, B. E., and Bushnell, W. R. 1983. Inhibition of the hypersensitive reaction in barley to powdery mildew by heat shock and cytochalasin B. Physiol. Plant Pathol. 23:421-438.

Hückelhoven, R., Fodor, J., Preis, C., and Kogel, K.-H. 1999. Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. Plant Physiol. 119:1251-1260.

Hückelhoven, R., and Kogel, K.-H. 1998. Tissue-specific superoxide generation at interaction sites in resistant and susceptible near-isogenic barley lines attacked by the powdery mildew fungus (*Erysiphe graminis* f. sp. *hordei*). Mol. Plant-Microbe Interact. 11:292-300.

Hückelhoven, R., and Kogel, K.-H. 2003. Reactive oxygen intermediates in plant-microbe interactions: Who is who in powdery mildew resistance? Planta 216:891-902.

Jagadish, S. V. K., Way, D. A., and Sharkey, T. D. 2021. Plant heat stress: Concepts directing future research. Plant. Cell Environ. 44:1992-2005.

Kim, H., O'Connell, R., Maekawa-Yoshikawa, M., Uemura, T., Neumann, U., and Schulze-Lefert, P. 2014. The powdery mildew resistance protein RPW8.2 is carried on VAMP721/722 vesicles to the extrahaustorial membrane of haustorial complexes. Plant J. 79:835-847.

Koga, H., Bushnell, W. R., and Zeyen, R. J. 1990. Specificity of cell type and timing of events associated with papilla formation and the hypersensitive reaction in leaves of *Hordeum vulgare* attacked by *Erysiphe graminis* f. sp. *hordei*. Can. J. Bot. 68:2344-2352.

Koga, H., Zeyen, R. J., Bushnell, W. R., and Ahlstrand, G. G. 1988. Hypersensitive cell death, autofluorescence, and insoluble silicon accumulation in barley leaf epidermal cells under attack by *Erysiphe graminis* f. sp. *hordei*. Physiol. Mol. Plant Pathol. 32:395-409.

Kolozsváriné Nagy, J., Schwarczinger, I., Király, L., Bacsó, R., Ádám, A. L., and Künstler, A. 2022. Near-isogenic barley lines show enhanced susceptibility to powdery mildew infection following high-temperature stress. Plants 11: 903.

Künstler, A., Bacsó, R., Albert, R., Barna, B., Király, Z., Hafez, Y. M., Fodor, J., Schwarczinger, I., and Király, L. 2018. Superoxide ($O_2^{\cdot -}$) accumulation contributes to symptomless (type I) nonhost resistance of plants to biotrophic pathogens. Plant Physiol. Biochem. 128:115-125.

Künstler, A., Füzék, K., Schwarczinger, I., Nagy, J. K., Bakonyi, J., Fodor, J., Hafez, Y. M., and Király, L. 2023. Heat shock-induced enhanced susceptibility of barley to *Bipolaris sorokiniana* is associated with elevated ROS production and plant defence-related gene expression. Plant Biol. 25:803-812.

Kusch, S., and Panstruga, R. 2017. *mlo*-based resistance: An apparently universal "weapon" to defeat powdery mildew disease. Mol. Plant-Microbe Interact. 30:179-189.

Kuska, M. T., Behmann, J., Großkinsky, D. K., Roitsch, T., and Mahlein, A.-K. 2018. Screening of barley resistance against powdery mildew by simultaneous high-throughput enzyme activity signature profiling and multispectral imaging. Front. Plant Sci. 9:1074.

Kuźniak, E., and Urbanek, H. 2000. The involvement of hydrogen peroxide in plant responses to stresses. Acta Physiol. Plant. 22:195-203.

Li, B., Gao, K., Ren, H., and Tang, W. 2018. Molecular mechanisms governing plant responses to high temperatures. J. Integr. Plant Biol. 60:757-779.

Mansoor, S., Ali Wani, O., Lone, J. K., Manhas, S., Kour, N., Alam, P., Ahmad, A., and Ahmad, P. 2022. Reactive oxygen species in plants: From source to sink. Antioxidants 11:225.

Mellersh, D. G., Foulds, I. V., Higgins, V. J., and Heath, M. C. 2002. H_2O_2 plays different roles in determining penetration failure in three diverse plant−fungal interactions. Plant J. 29:257-268.

Müller, J., Menzel, D., and Šamaj, J. 2007. Cell-type-specific disruption and recovery of the cytoskeleton in *Arabidopsis thaliana* epidermal root cells upon heat shock stress. Protoplasma 230:231-242.

Opalski, K. S., Schultheiss, H., Kogel, K.-H., and Hückelhoven, R. 2005. The receptor-like MLO protein and the RAC/ROP family G-protein RACB modulate actin reorganization in barley attacked by the biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. Plant J. 41: 291-303.

Parrotta, L., Faleri, C., Cresti, M., and Cai, G. 2016. Heat stress affects the cytoskeleton and the delivery of sucrose synthase in tobacco pollen tubes. Planta 243:43-63.

Pennington, H. G., Li, L., and Spanu, P. D. 2016. Identification and selection of normalization controls for quantitative transcript analysis in *Blumeria graminis*. Mol. Plant Pathol. 17:625-633.

Schmittgen, T. D., and Livak, K. J. 2008. Analyzing real-time PCR data by the comparative C_T method. Nat. Protoc. 3:1101-1108.

Schultheiss, H., Dechert, C., Király, L., Fodor, J., Michel, K., Kogel, K.-H., and Hückelhoven, R. 2003. Functional assessment of the pathogenesis-related protein PR-1b in barley. Plant Sci. 165:1275-1280.

Schwarczinger, I., Kolozsváriné Nagy, J., Király, L., Mészáros, K., Bányai, J., Kunos, V., Fodor, J., and Künstler, A. 2021. Heat stress pre-exposure may differentially modulate plant defense to powdery mildew in a resistant and susceptible barley genotype. Genes 12:776.

Stolzenburg, M. C., Aist, J. R., and Israel, H. W. 1984. The role of papillae in resistance to powdery mildew conditioned by the *ml-o* gene in barley. I correlative evidence. Physiol. Plant Pathol. 25:337-346.

Takano, Y., Takayanagi, N., Hori, H., Ikeuchi, Y., Suzuki, T., Kimura, A., and Okuno, T. 2006. A gene involved in modifying transfer RNA is required for fungal pathogenicity and stress tolerance of *Colletotrichum lagenarium*. Mol. Microbiol. 60:81-92.

Tek, M. I., Calis, O., Fidan, H., Shah, M. D., Celik, S., and Wani, S. H. 2022. CRISPR/Cas9 based *mlo*-mediated resistance against *Podosphaera xanthii* in cucumber (*Cucumis sativus* L.). Front. Plant Sci. 13:1081506.

Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D. B. 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. Plant J. 11:1187-1194.

Trujillo, M., Altschmied, L., Schweizer, P., Kogel, K.-H., and Hückelhoven, R. 2006. Respiratory Burst Oxidase Homologue A of barley contributes to penetration by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. J. Exp. Bot. 57:3781-3791.

Wahid, A., Gelani, S., Ashraf, M., and Foolad, M. R. 2007. Heat tolerance in plants: An overview. Environ. Exp. Bot. 61:199-223.

Xiao, S., Brown, S., Patrick, E., Brearley, C., and Turner, J. G. 2003. Enhanced transcription of the Arabidopsis disease resistance genes *RPW8.1* and *RPW8.2* via a salicylic acid-dependent amplification circuit is required for hypersensitive cell death. Plant Cell 15:33-45.

Zhou, R., Kong, L., Yu, X., Ottosen, C.-O., Zhao, T., Jiang, F., and Wu, Z. 2019. Oxidative damage and antioxidant mechanism in tomatoes responding to drought and heat stress. Acta Physiol. Plant. 41:20.