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ORIGINAL ARTICLE

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Hydrogen Peroxide and Phenylalanine Ammonia-lyase as Signalling Molecules in Barley Leaves Challenged with *Cochliobolus sativus*

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Hydrogen peroxide (H_2O_2) and phenylalanine ammonia-lyase (*PAL*) have been reported as important *signaling molecules* during plant resistance against many fungal pathogens. In this study, the relative contributions of H_2O_2 and *PAL* were investigated at early time periods of barley infection with *Cochliobolus sativus*, the causal agent of spot blotch disease. H_2O_2 activity was observed in leaf tissues 24 hours post inoculation (hpi) and was accompanied with an increase in *PAL* expression in resistant and susceptible genotypes. However, the resistant genotype 'Banteng' contained higher levels of H_2O_2 and *PAL*, as compared with the susceptible one 'WI 2291'. Results demonstrated that the cooperative function of H_2O_2 and *PAL* in barley responses to *C. sativus* appeared to be dependent on the plant genotype, and it is hypothesized that the peak of activity of *PAL* at 48h and 72 h, and the rapid increase in H_2O_2 24 h in resistant and susceptible genotypes are considered general defense responses.

Key words: Hordeum vulgare, Cochliobolus sativus, H2O2, PAL expression, real time PCR

Spot blotch (SB) caused by the necrotrophic [*Cochliobolus sativus* (*Cs*) Drechs. ex Dastur] is a globally distributed disease of barley (*Hordeum vulgare* L.) causing significant yield losses (Rehman *et al.,* 2020). Typically, SB is characterized by discrete necrotrophic spots that extend beyond vascular bundles (Al-Sadi, 2021). Yield loss is mainly due to reductions in kernel size and weight (Kumar *et al.,* 2002). The development of new barley resistant cultivars is the best method for the successful control of this disease (Gupta *et al.,* 2001; Leng *et al.,* 2016), however, the basis of fungal resistance strategies and the early barley responses towards SB are still not clear.

Barley plants respond to SB by activating different mechanisms that are regulated through various plant signaling pathways. Hydrogen peroxide (H_2O_2) is an important reactive oxygen species (ROS) molecule that serves as a signal of oxidative stress and activation of signaling pathways as a result of the early response of the plant towards fungal pathogens attack (Juan *et al.,* 2021). H_2O_2 is engaged in genes encoding to phenylalanine lyase (*PAL*), which are extensively used as indicators of ROS responsive and oxidative stress specific signaling (Sahebani *et al.,* 2009).

The activation of phenylpropanoids under different stress factors has lead to be used as genetic markers for the initiation of plant defense responses. *PAL* catalyzes the first step in phenylpropanoid biosynthetic pathway (Yadav et al., 2020), and is involved in the synthesis of plant secondary antimicrobial substances, that are essential for plant defense responses (Kim *et l.,* 2014) and also in the biosynthesis of salicylic acid which is an essential signal engaged in plant systemic resistance (Chaman *et al.*, 2003).

Reverse transcription-polymerase chain reaction (RT-PCR) is the most reliable technique for measuring the relative expression level of a particular transcript and determining its expression after exposure to a specific alteration, such as infection by a fungal pathogen (Derveaux *et al.,* 2010). In the current work, we studied defense responses of two barley genotypes Banteng and WI 2291, which are integrated in international breeding programs aimed at developing *C. sativus* resistant barley genotypes. Banteng was described as highly resistant to SB (Arabi and Jawhar 2003; 2004), i.e. exhibited a lower level of symptom development as compared with WI2291.

To complete the picture of barley defense responses drawn (Al-Daoude *et al.,* 2013), the present work investigates possible changes of endogenous H_2O_2 and *PAL* expression after barley infection with *C. sativus* at different time intervals.

MATERIALS AND METHODS

Plant material

The most resistant German cv. Banteng and the universal Australian susceptible control cv. WI2291 to SB (Arabi and Jawhar, 2003) were used in the experiments. Plants were grown in flats filled with sterilized peatmoss with three replicates, and 10 seedlings per experimental unit. They were placed at temperatures 22°C (day) and 18°C (night) with in a 12-h light / 12-h dark cycle and relative humidity (RH > 90%).

Infection with *C. sativus*

The highly *C. sativus* virulent pathotype Pt4 described by Arabi and Jawhar (2004) was used. The fungus was grown in Petri dishes containing potato dextrose agar (PDA, DIFCO) under 20±°C in the dark for 10 days. Seedlings were inoculated with suspension of conidia 2 x 10^4 conidia/mL using a hand-held sprayer. *C. sativus* inoculum preparation, inoculation, post-inoculation and disease records were similar to those described by Fetch and Steffenson (1999).

Detection of H_2O_2

H_2O_2 was detected in barley leaves using 3,3-diaminobenzidine (DAB) according to the protocol described by Thordal-Christenssen *et al.* (1997). The stained samples were examined using a fluorescence microscope (Olympus-ix21 station, X400, Japan). H_2O_2 was localized due to dark blue coloration in the periplasmic space of the cells. Observations were made for 25 infection sites per leaf sample collected from four to six inoculated plants. Dark-brown zones indicated the presence of H_2O_2. Hydrogen peroxide was measured using the titanium tetrachloride precipitation method as

described by Brennan and Frenkel (1977) at 0, 24, 48 and 72 hpi.

RNA isolation and *PAL* expression

Barley primary leaves were collected 0, 24, 48 and 72 hpi and homogenized with a tube pestle in liquid nitrogen. mRNA was extracted from frozen samples using Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany). The QuantiTect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis. The control samples were collected from the non- inoculated plants at the same time points. *PAL* expression was evaluated using SYBR Green Master kit (Roche, USA) in Step One system. *PAL* primers (Table 1) were designed based on NCBI (http://www.ncbi.nlm.nih.gov) database. The threshold cycle (Ct) value was determined by the real time PCR system. Seedlings inoculated with distilled water served as a control. Average Ct values were calculated from the three replications, with the ΔCT value determined by subtracting the average Ct value of genes from the Ct value of the *EF1α* gene and the equation $2^{-\Delta\Delta CT}$ was used to determine the expression levels (Livak and Schmittgen, 2001). Statistical analysis was conducted by Tukey's test at the 0.05 significance level. Values were presented as mean ± standard deviation.

RESULTS AND DISCUSSION

In this study, two barley genotypes with different reaction levels to *C. sativus* were used. As shown in Figure 1, SB severity was more in the highly susceptible genotype WI9921 compared with the resistant cv. Banteng. The results are in line with our previous observations under natural field conditions (Arabi and Jawhar, 2004).

Data showed that H_2O_2 production rate increased sharply in the resistant cv. Banteng, which got the high level of 04.5 µmol g^{-1} FW min^{-1} at 24 hpi, then reached to maximum level 09.7 µmol g^{-1} FW min^{-1} at 72 hpi. By contrast, in the susceptible cv. WI 2291 , H_2O_2 increased slowly within the first 24 h and then increased gently to the peak of 0.3.3 µmol g^{-1} FW min^{-1} at 72 hpi (Fig. 2). This might explain the high resistance level of cv. Banteng against SB disease, since it exhibited an early and quick “first oxidative burst”, likely inducing disease resistance mechanisms to set in. To further confirm this event, we examined the H_2O_2 accumulation in the two barley genotypes. Interestingly, early H_2O_2 accumulation in leaf tissues was detected in both genotypes, however, the resistant cultivar constitutively contained higher levels of H_2O_2 than the susceptible one (Fig. 2).

On the other hand, results demonstrated that *PAL* expression in the resistant cv. Banteng was higher during different time points post inoculation, and its expression increased sharply 24 hpi, by 4.2 fold increases, then decreased to basal levels 72 hpi. In the case of cv. WI 2291, there was a slower increase in *PAL* expression until 72 hpi (Fig. 3). This might be due to the formation of a rapid signaling from hydrogen peroxide for activating *PAL*, which catalyzes the first step in phenylpropanoid biosynthetic pathway against *C. sativus* infection. This step is considered to be very important as regulation point between primary and secondary metabolism (Vogt, 2010) which may be the cause of barley cell wall leakage during *C. sativus* infestation.

However, there have been contradictory results in relation to the association between H_2O_2 and *PAL* gene induction. Delledonne *et al.* (1998) reported that H_2O_2 was not a signal for *PAL* activation in soybean cell suspensions, while in Arabidopsis and tobacco cultured cells, H_2O_2 prompted *PAL* gene expression (Desikan *et al.,* 1998). Our results might hypothesize that the peak of activity of *PAL* at 48h and 72 h, and the rapid increase in H_2O_2 at 24 h in both resistant and susceptible genotypes are general defense responses.

It is well documented that in barley leaves, *C. sativus* spores that contact the leaves surface germinate within few hours and produce appressoria from which penetration hyphae breach the leaf cuticle. We hypothesize that the *C. sativus* infection point will subsequently become the local source for ROS production that will activate the phenylpropanoid pathway, preserve or increase the levels of the antifungal compounds, which inhibit fungal development (Kumar *et al.,* 2002).

Table-1 Sequences of used primers

Gene	Gene description	Accession No.	Sequence	Amplified fragment (bp)
PAL	Phenyl alanine amino lyase	AT2G14610	CCATTGATGAAGCCAAAGCAAG ATGAGTGGGTTATCGTTGACGG	123
EF1α	Elongation foctor-1 Alapha	CV066174	GGCTGATTGTGCTGTGCTTA TGGTGGCATCCATCTTGTTA	153

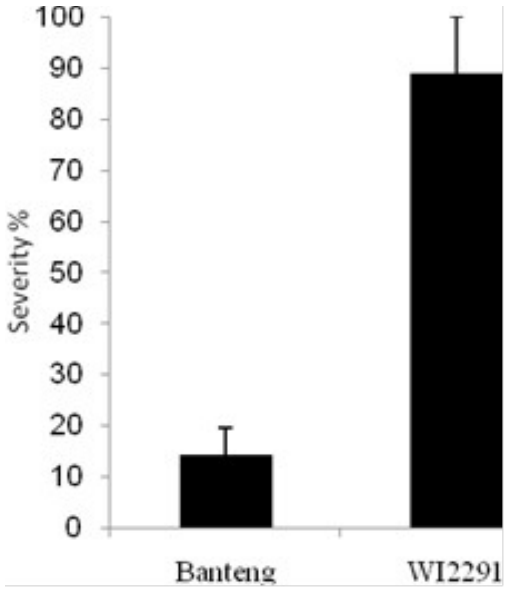

Figure 1. Disease reactions of barley (a) resistant cv. Banteng and (b) susceptible cv. WI2291 towards *C. sativus* pathogen

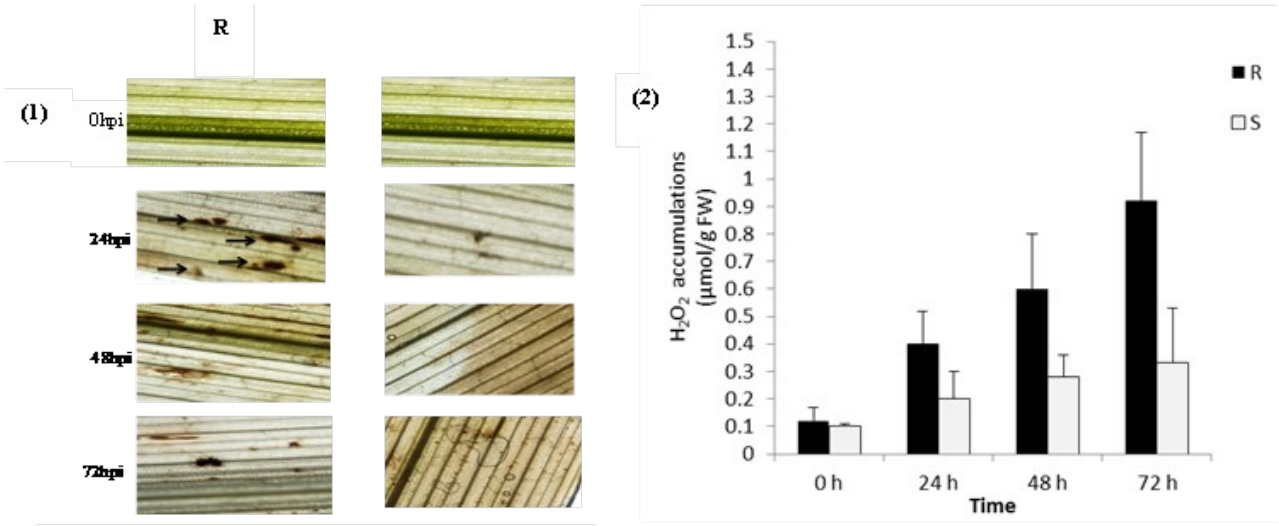

Figure 2. (1) Localization of H2O2 and (2) its changes in tissues of barley leaves; (R) resistant cv. Banteng and (S) susceptible cv. WI2291 after different time points of inoculation with *C. sativus*.

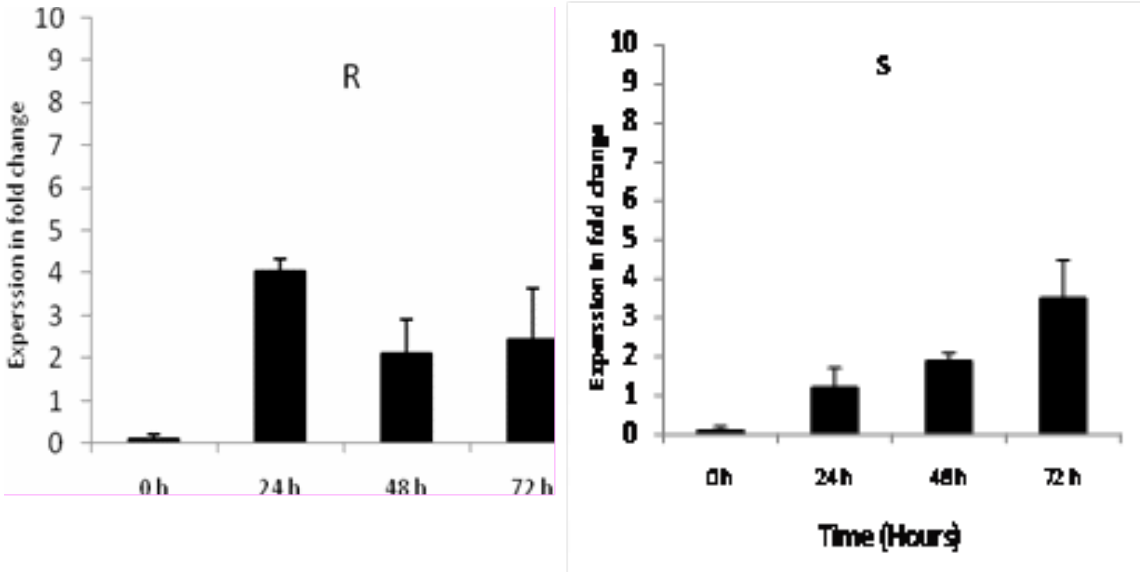

Figure 3. Relative *PAL* expression profiles in the resistant cv. Banteng (R) and in the susceptible cv. WI2291(S) during the time course following *C. sativus infection.* Error bars are representative of the standard error (Mean ± SD, *n* = 3). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 1.00).

CONCLUSIONS

This work sheds some light on the relative contributions of H_2O_2 and *PAL* during *C. sativus*– barley interactions. Results showed that their contribution to the resistance response appears to depend on the plant genotype. We can hypothesize that the peak of activity of *PAL* at 48h and 72 h, and the rapid increase in H_2O_2 at 24 h in both resistant and susceptible genotypes are general defence responses. However, since SB-infection caused spreading cellular H2O2accumulation and increased *PAL* expression, thus, H2O2 could be the common inductive factor regulating this gene through still unidentified signal transduction pathway.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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