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Spot blotch tolerance in barley is associated with early accumulation of hydrogen peroxide and jasmonic acid in leaves

A. Aldaoude*, M. Jawhar, Al-Shehadah, A. Shoaib

Department of Molecular Biology and Biotechnology, AECS, P.O.Box 6091 Damascus, Syria

Abstract

Spot blotch (SB), caused by the fungus *Cochliobolus sativus*, is an economically important disease of barley worldwide. Barley plants combat SB infection by eliciting a wide array of signaling pathways. To *understand barley defense mechanisms* towards this disease, the involvement of hydrogen peroxide (H_2O_2) and Jasmonic acid (JA) in tolerant 'Banteng' and susceptible 'WI2291' cultivars at early points of infection with the virulent *C. sativus* pathotype Pt4 was investigated. Data showed increasing accumulation of H_2O_2 and JA 24 hours post inoculation (hpi) as compared with the non-inoculated controls. It is noteworthy that JA signaling was activated in parallel with H_2O_2 signaling up to 48h and then decreased at 72h in both cultivars. However, data revealed higher levels of H_2O_2 (0.92 µmol/g FW) in the tolerant cv. 'Banteng' as compared with the susceptible one 'WI2291' (0.33 µmol/g FW) 72hpi. The obtained results suggest that H_2O_2 and JA accumulation might have important role during barley - *C. sativus* interaction.

Keywords: Barley, *Cochliobolus sativus*, hydrogen peroxide, Jasmonic acid, interaction

1.Introduction

Cochliobolus sativus (Ito & Kurib.) Drechsl. ex Dast. [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], the cause of SB, is an important pathogen that causes significant yield losses of barley (*Hordeum vulgare*) worldwide [2]. It is a hemibiotrophic fungus as posses both biotrophic and necrotrophic phases. Biotrophic growth phase is primarily confined to a single epidermal cell invaded by infection hyphae, whereas the necrotrophic growth phase starts upon attack of the mesophyll tissue followed by host cell death, which appears to be a result of toxin secretion [12].

The two *C. sativus* phases are not only different in their nutrient uptake strategies, but also in their virulence strategies and the disease symptoms they cause. Hence, barley plants have to orchestrate various defense mechanisms against pathogens with contrasting lifestyles. For this, in depth knowledge on the interaction between barley and *C. sativus* interaction at the genetic level is required.

The interaction between barley plants and *C. staivus* is a complex process since different defense pathways have been implicated in this process [12], which has been found to be regulated by a concerted expression of various plant signaling pathways including phytohormones and reactive oxygen species (ROS) molecules [1,9]. However, many of their specific functions are still unknown.

Different works demonstrated that JA mediated signaling pathways are mainly related to plant resistance, prompting plant defenses to external damage and fungal pathogen infections (reviewed in Thaler *et al.*, 2012) [17]. Biosynthesis of JA has been investigated in different monocotyledonous plants, and to a somewhat limited extent in monocot ones. So far, different relationships between the JA synthesis and other metabolic pathways have been reported (reviewed in Li *et al.*, 2021) [14]. Moreover, JA had the ability to induce H_2O_2 accumulation and activation of the defense system in plants [20].

Corresponding authors: ascientific@aec.org.sy

Currently, the understanding of complex regulatory networks and metabolic processes after barley infection with *C. sativus* are still very limited. To complete the picture of barley biochemical responses drawn [1], the current study investigated the possible changes of endogenous H_2O_2 and JA during barley interaction with *C. sativus* at different time points.

2. Materials and methods

2.1. Plant growth

Barley seeds of SB-tolerant 'Banteng' and susceptible 'WI 2291' cultivars [5,6] were grown in plastic pots (20-cm in diameter) filled with sterilized peat moss in three replicates, and each replicate consisted of 10 plants. The pots were placed in a greenhouse at 20 ± 2°C with a relative humidity 60 ± 5%.

2.2. SB inoculation

A single spore virulent isolate (Pt4) of *C. sativus* described by Arabi and Jawhar (2003) was used in the study. The fungus was grown in Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA). Conidia were collected by rinsing dishes with sterile water and the suspension was adjusted to 2×10^4 conidia/mL using hemacytometer. *Seedlings* were inoculated at the two- to three-leaf stage with the second leaf fully expanded using *a hand-held spray bottle* [25]. Inoculated barley plants were kept under greenhouse conditions at 20 ± 2°C °C with a 16 h photoperiod and relative humidity maintained at 90 ±5% . Control plants were sprayed with distilled water.

2.3. Detection of H_2O_2

H_2O_2 in barley leaf tissues was detected using the method described by Thordal-Christensen *et al.* (1997) [18]. Leaf samples were stained with DAB (3, 30-diaminobenzidinetetrahydrochloride) and with Calcofluor White. The stained samples were examined under a fluorescence microscope (Olympus-ix21 station, X400, Japan). Observations were made at 0, 24, 48 and 72 hpi for at least 10 infection sites per leaf sample collected from two to four infected plants. H_2O_2 concentration was measured as µmol/g fresh weight (FW).

2.4. Quantification of JA in plant samples

JA was analyzed at different time points 0, 24, 48 and 72 hpi following the method of Trapp *et al.* (2014) [19]. Briefly, 100 mg of barley seedlings was ground in liquid nitrogen and the extraction was performed using 1.0 mL of ethyl acetate, dichloromethane, isopropanol, MeOH:H_2O. Samples were centrifuged at 16,000 g for 5 min. The supernatant phase was transferred into a new tube and was passed through carbon-packed solid phase extraction tubes, dried in speed vac. After drying, 1000µL of MeOH were added to each sample, homogenized under vortex and centrifuged for 10 min at 16,000 g. The supernatant was applied on a thin layer chromatography plate (Silica gel on TLC Al foil with fluorescence indicator (254 nm) using automatic TLC sampler 4 (Camag, Switezerland). TLC plate was developed in a glass chamber using isopropanol:ammonia: water (9:1:1, v/v) as development solvent. The plate was dried and read using TLC Scanner 3 (Camag, Switezerland). Changes in JA were compared to the control for the same day. Three replications were performed for each time point.

2.5. Data analysis

The data obtained from three replicates of H_2O_2 and JA was used for analysis. Comparison of means between cultivars was performed using Statview program [3].

3. Results and Discussion

In this study, two barley cultivars Banteng and WI2291 with different resistance levels to *C. sativus* infection were used. SB severity was always higher in the susceptible cultivar as compared with the tolerant one (Table 1). These observations are in agreement with those recorded under natural field infections [6].

Table 1. SB symptoms on two barley cultivars used in this work

Cultivar	Origin	Disease* Reaction	SB symptoms
Banteng	Germany	T	Small round to oblong dark brown necrotic lesions
WI2291	Australia	S	Solid dark brown necrotic lesions with expanding chlorosis

*Disease reaction and symptoms as described by Arabi and Jawhar (2003; 2004) [5,3]. T: Tolerant and S: susceptible.

In order to get insight into the early responses of H_2O_2 and JA in barley inoculated with *C. sativus* Pt4, four different time periods were investigated. Seedlings sprayed with distilled water were also analyzed at 0h. Data showed that penetration resistance at the early stage (24 h) is closely associated with local generation of H_2O_2 in cell wall appositions visualized by DAB staining. H_2O_2 was localized due to a dark blue coloration in the periplasmic space of the plant cells (Fig. 1).

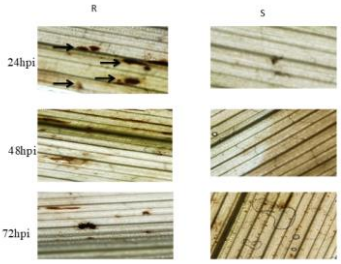

Figure 1. Microscopic images of H_2O_2 accumulation in tissues of tolerant (T) and susceptible (S) barley leaves infected with *C. sativus* at different time points of inoculation.

This earliest detection of H_2O_2 might be in relation to the first signs of hypersensitive response (HR), known to be observed after inoculation [23].

Significant differences ($P<0.001$) in H_2O_2 values were found among time periods, with values being consistently higher in the tolerant cultivar (Fig. 2). The high accumulation of H_2O_2 recorded in the tolerant cv. 'Banteng' (0.92 µmol/g FW) and its reduced expression in the susceptible ones 'WI2291' (0.33 µmol/g FW) might suggest that H_2O_2 can play an important role in barley resistance towards *C. sativus* pathogen.

Our results are in agreement with previous works which reported that higher level of H_2O_2 was observed in a wheat tolerant cultivar than that of the susceptible ones after infection with *Septoria tritici* [21], and in banana after infection with *Fusarium oxysporum* [13]. Lin *et al.* (2009) [15] reported that although H_2O_2 is essential for signaling and plant defense against pathogen infection, it can react with metal ions to form a hydroxyl radical (OH•) or with the superoxide (O2) radical, which is very toxic to pathogens in addition to enhancing the cytotoxicity of H_2O_2. Oxidative stress can cause damage to lipids, pigments, cellular proteins and nucleic acids [4].

The fact that *C. sativus* is a heminecrotrophic with an initial biotrophic stage during SB development would reflect its strategy to cope with barley plant cell death-related defense [22]. It is suggested from this investigation that barley-generated H_2O_2 during the necrotrophic phase contributes to resistance, since a large number of H_2O_2 cells were localized in the tolerant cv.'Banteng' as compare with the susceptible one. However, the high accumulation of H_2O_2 can affect plant defense in several ways, most probably by stimulating cross-linking of proline-rich proteins of the cell [7], and by activating different genes involved in defense mechnanisms [9].

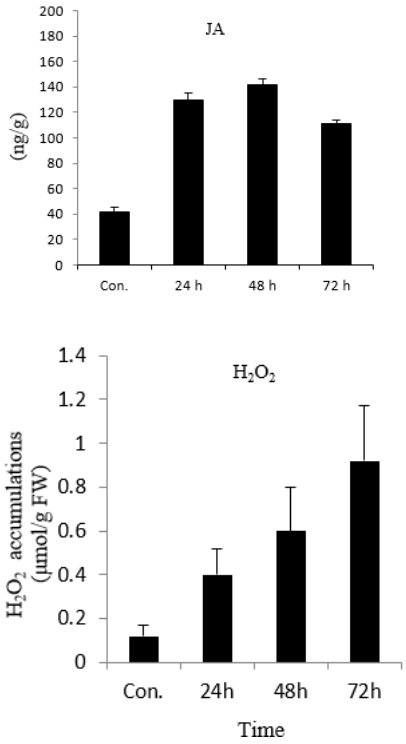

Figure 2. Changes in JA and H_2O_2 in barley tolerant 'Banteng' plants' infected with *C. sativus.* Data represents the mean of three replicates SE

On the other hand, data showed that JA contents of tolerant and susceptible barley leaves increased 24hpi in comparison with non-inoculated plants at the start of the experiment (Fig. 2). However, although JA signaling was activated in parallel with H_2O_2 signaling up to 48h, it decreased at 72h in both cultivars (Figs. 1,2 and 3).This in agreement with Ellis *et al.* (2002) [8] who reported that JA signaling pathways increased after infection of *Arabidopsis* by *Pseudomonas syringae*. Glazebrook (2005) [10] and Wasternack (2007) [24] reported that JA

signaling has a basic roles in plant resistance against necrotrophs. Furthermore, it has been found that JA accumulation may result following oxidative membrane damage, and it does occur after the initial oxidative burst [16]. Kauss *et al.* (1994) [11] documented that JA can potentiate the oxidative burst in elicitor-treated parsley suspension cells.

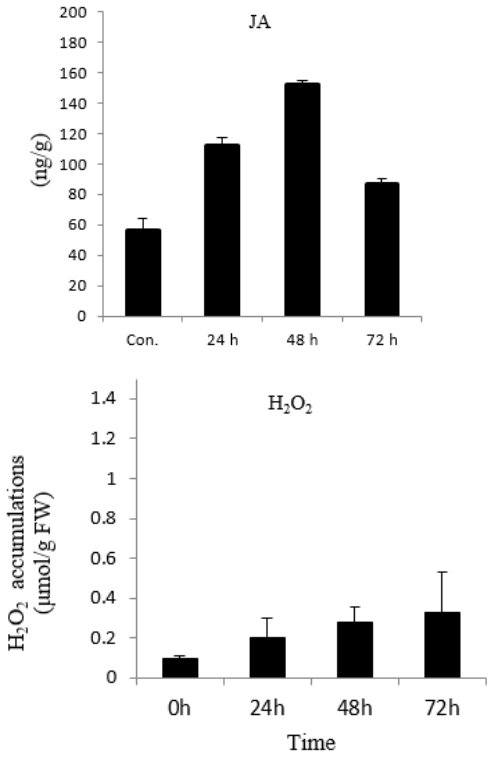

Figure 3. Changes in JA and H_2O_2 in barley susceptible 'WI 2291' plants' infected with *C. sativus.* Data represents the mean of three replicates SE.

4.Conclusion

This study illustrated that significant increases in JA and H_2O_2 were found upon barley challenged with *C. sativus*, with values being consistently higher in the tolerant cultivar. The patterns of H_2O_2accumulation clearly differentiated during the time course of inoculation and corresponded to JA in tolerant and susceptible barley, which could indicate that these changes might have roles in barley – *C. sativus* interactions. This study serves to broaden our understanding of the molecular cellular basis of barley–*C. sativus* interactions but more studies, including also more susceptible and tolerant barley cultivars, are needed to better understand the defense mechanisms.

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Compliance with Ethics Requirements: Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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