

## SHORT COMMUNICATION

HYDROGEN PEROXIDE INDUCTION IN BARLEY- *COCHLIOBOLUS SATIVUS* INTERACTION

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## SUMMARY

The fungus *Cochliobolus sativus* is the causal agent of Barley spot blotch (SB) disease. In order to better understand barley physiological reaction towards this disease, hydrogen peroxide ( $H_2O_2$ ) and ion fluxes in two different genotypes inoculated with a virulent isolate of *C. sativus* were investigated. Results showed that SB development was directly dependent on  $H_2O_2$  generation and accumulation during infection. Significant differences ( $P < 0.001$ ) in  $H_2O_2$  values were found among different time points, with values being consistently higher in the resistant genotype. The interaction between host leaves and pathogen produced a marked increase in electrolyte leakage from the susceptible genotype compared with the resistant one. The information obtained from this study highlights crucial points that can guide hypothesis-driven research to elucidate the molecular mechanisms involved in *C. sativus*-barley interaction

*Key words:* *Cochliobolus sativus*, barley, hydrogen peroxide, electrolyte leakage.

Spot blotch (SB) caused by the fungus *Cochliobolus sativus* (Ito et Kurib.) Drechs. ex Dast. [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], is a serious foliar disease of barley, particularly important in warm and moist areas during the growing season (Mathre, 1990). SB is characterized by distinct necrotrophic spots that extend beyond the vascular bundles. Yield loss results from reduction in kernel size and weight (Kumar *et al.*, 2002). Effective control of SB can be achieved by the introduction of resistant cultivars as a key component of integrated disease management (Ghazvini and Tekauz, 2008). Hence, the search for parental stocks possessing an adequate level of resistance to *C. sativus* is essential.

Reactive oxygen species (ROS), especially hydrogen peroxide ( $H_2O_2$ ) have been suggested as antimicrobial agents during plant defense response (Forman *et al.*, 2003). Moreover, the superoxide anions ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ), have been the primary ROS detected during the hypersensitive response (HR) to fungi and other pathogens (Ying *et al.*, 2010). Additionally, changes in ion fluxes are among the first measurable events during the initial establishment of HR and provide a robust method for tracking and quantifying cell collapse (Wisniewska *et al.*, 1998).

The primary objective of this research was to better understand the interaction between the fungal pathogen *C. sativus* and barley via the electrolyte leakage and accumulation of  $H_2O_2$ . Defense responses were investigated of two barley genotypes (Banteng and WI 2291) which are being integrated in international breeding programs aimed at developing SB-resistant barley cultivars.

After an extensive greenhouse and laboratory screening lasted over 10 years, the cv. Banteng proved to be the most resistant genotype to all SB isolates available so far; therefore, it was selected for this study together with the universal susceptible control cv. WI2291. Plants were grown in plastic flats (60×40×8 cm) filled with sterilized peatmoss and arranged in a randomized complete block design with three replicates. Each experimental unit consisted of 10 seedlings per genotype. A full replicate consisted of 10 pots inoculated with the major Syrian *C. sativus* pathotype (Pt4). Pots were placed in a growth chamber at temperatures of 22±1°C (day) and 17±1°C (night) with 12 h photoperiod and 80-90% relative humidity (RH).

Pt4 is the most virulent of 117 isolates collected between 1998 and 2004 from naturally infected barley in different regions of Syria (Arabi and Jawhar, 2003). Fungal mycelia were transferred from a stock culture into Petri dishes containing potato dextrose agar (Difco, USA) and incubated for 10 days at 21±1°C in the dark. Conidia were collected with 10 ml of sterile distilled water and the suspension was adjusted to 2×10<sup>4</sup> conidia/ml using an haemocytometer. A surfactant (polyoxyethylene-20-sorbitan monolaurate) was added (100 µl/l) to the conidial suspension to facilitate dispersion of the inoculum over the leaf surface.

**Table 1.** Electrolytic leakage of two barley genotypes after different time points of inoculation with *Cochliobolus sativus*, isolate Pt4.

Genotype	Origin	Disease severity	Time after inoculation				
			0 h	8 h	24 h	48 h	72 h
Banteng	Germany	13.6b	4.6b	6.3b	15.9b	24.6b	60.3b
WI2291	Australia	93.1a	6.2a	25.8a	48.3a	91.7a	189.1a

Disease severity was assessed as a percentage of leaf area exhibiting disease symptoms (combination of necrosis and chlorosis) according to Steffenson and Fetch (1996). The electrolyte leakage assay was carried out as described by Kwon *et al.* (1996). Values followed by different letters are significantly different at  $P < 0.001$  according to Newman-Keuls test.

Plants were inoculated at growth stage (GS) 12 (Zadoks *et al.*, 1974) by spraying each plant with 25 ml of conidial suspension with a hand-held spray bottle. Inoculated plants were kept in a mist chamber at 18 °C in the darkness to maintain RH at 100% for 48 h. After the mist period, inoculated plants were moved back to the greenhouse with alternating temperature of 21°C day and 17°C night. Plants inoculated with sterile distilled water served as control. Disease severity was assessed on the second leaves 14 days post inoculation, and expressed as the percentage of leaf area exhibiting disease symptoms (combination of necrosis and chlorosis) according to Steffenson and Fetch (1996).

Binocular microscopy evaluation of SB induction by isolate Pt4 was carried out on 10-day-old second leaves from the two barley genotypes 48 h post inoculation and expressed as the percentage of conidia causing a visible necrotic lesion on a 4-cm-long leaf segment. Ten leaf segments from each genotype were used and the mean from three independent replications reported. The experiment was repeated twice.

H<sub>2</sub>O<sub>2</sub> was detected by an endogenous peroxidase-dependent *in situ* staining procedure with 3,3-DAB (Sigma-Aldrich, USA) (Thordal-Christensen *et al.*, 1997), infiltrating leaves with DAB (1 mg/ml) pH 3.8 (adjusted with HCl) and was measured using the titanium tetrachloride precipitation method (Brennan and Frenkel, 1977) at 8, 24, 48 and 72 h after inoculation. Whole-cell DAB staining or yellow-green whole-cell auto fluorescence under blue light excitation (480 nm, emission: long pass 500 nm) were used for HR detection.

Electrolyte leakage assay for the different time points was carried out as described by Kwon *et al.* (1996). Comparison of means between genotypes was performed using the Statview program (Anonymous, 1996).

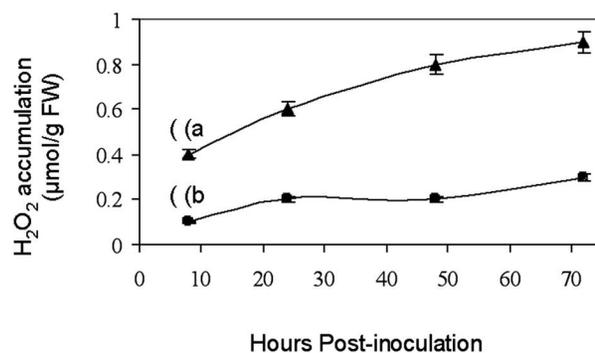
Disease severity was always more obvious in the highly susceptible cv. WI9921 compared with the resistant cv. Banteng. Infection responses of barley genotypes and their origin are shown in Table 1. Schäfer *et al.* (2004) reported that fungus development on tissue seems to be indicative of plant potential to contain pathogen spread. Hence, the spreading factor is a reliable character for the

resistance potential of a cultivar. Our experiment was repeated twice and had similar results.

Table 1 shows a marked increase in electrolyte leakage following the interaction between barley leaves and *C. sativus*. At 72 h post inoculation, this increase was much higher in the susceptible genotype (189.1) than in the resistant one (60.3).

Significant differences ( $P < 0.001$ ) in H<sub>2</sub>O<sub>2</sub> values were found among time points, with values being consistently higher in the resistant genotype Banteng (Fig. 1). Detection of H<sub>2</sub>O<sub>2</sub> might be related to the HR earliest signs after inoculation (Shinogi *et al.*, 2003). Similar results were found in rice (Xiong and Yang, 2003) and wheat (Hulbert *et al.*, 2007).

Penetration resistance at the early stage (8 h) is closely associated with local generation of H<sub>2</sub>O<sub>2</sub> in cell wall appositions visualized by DAB staining (Fig. 1). This finding is reminiscent of defense responses of cereals toward the biotrophic powdery mildew fungus *Blumeria graminis* (Hückelhoven *et al.*, 1999), and might support the potential of H<sub>2</sub>O<sub>2</sub> in relation to the earliest HR signs post inoculation. The fact that the *C. sativus* is heminecrotrophic with initial biotrophic stage during disease development reflects a pathogenic strategy to cope with plant cell death-associated defense (Schäfer *et al.*,



**Fig. 1.** H<sub>2</sub>O<sub>2</sub> accumulation in two barley genotypes (a) Banteng and (b) WI2291 inoculated with *C. sativus*. Leaf tissue from infected leaves was assayed 8-72 h, after infection. H<sub>2</sub>O<sub>2</sub> levels are shown as means per gram fresh weight (FW).

2004). However, it is unclear whether during the necrotrophic phase host-generated  $H_2O_2$  contributes to resistance or to successful pathogenesis. Increase of  $H_2O_2$  levels can affect plant defense in several ways, presumably by stimulating cross-linking of cell wall proline-rich proteins (Bradley *et al.*, 1992), and by inducing several plant genes involved in cellular protection and defense (Mehdy, 1994; Forman *et al.*, 2003).

In the present study, we have shown the impact of barley-generated  $H_2O_2$  on early infection stages of *C. sativus*. The ability of  $H_2O_2$  to induce electrolyte leakage depends on the barley resistance level to the pathogen at different time points. Rapid generation of  $H_2O_2$  during the oxidative burst after 8 h of infection suggests its major role as a central component of barley defense responses to *C. sativus* challenge.

## ACKNOWLEDGEMENTS

The authors thank the Director General of AECS and the Head of the Molecular Biology and Biotechnology Department for their continuous support throughout this work.

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