Model Wheat Genotypes as Tools to Uncover Effective Defense Mechanisms Against the Hemibiotrophic Fungus *Bipolaris sorokiniana*

Aloysius Ebelechukwu Ibeagha, Ralph Hückelhoven, Patrick Schäfer, Devendra Pal Singh, and Karl-Heinz Kogel

First, second, third, and fifth authors: Institute of Phytopathology and Applied Zoology, Interdisciplinary Research Centre for Environmental Sciences, Justus-Liebig-University, Heinrich-Buff-Ring 26-32, D-35392, Giessen, Germany; and fourth author: Directorate of Wheat Research (DWR), Kunjpura Road, Karnal, Haryana, 132001, India.

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ABSTRACT

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We investigated the interaction of several differentially resistant wheat genotypes with the hemibiotrophic phytopathogenic fungus *Bipolaris sorokiniana* (teleomorph *Cochliobolus sativus*). Wheat genotypes Yangmai 6, M 3 (W7976), Shanghai 4, and Chirya 7 showed higher levels of resistance compared with cv. Sonalika, used as a susceptible control. In a quantitative microscopic inspection, we found that fungal penetration into

the epidermal layer failed mostly through a cell wall-associated defense mechanism. In cases where the fungus successfully overcame epidermal defense, its spread within the mesophyll tissue (necrotrophic phase) was restricted in the more resistant genotypes. Epidermal cell wall-associated defense, spreading as well as the extent of electrolyte leakage of infected tissue, correlated well with field resistance. We propose that cellular host responses such as formation of cell wall appositions as well as the degree of early mesophyll spreading of fungal hyphae are indicative of the defense potential of the respective host genotype and, therefore, could be used for the characterization of new spot blotch resistance traits in cereals.

Cochliobolus sativus [(Ito & Kuribayashi) Drechs. ex Dastur; anamorph *Bipolaris sorokiniana* (Sacc.) Shoemaker] is a serious pathogen of wheat and barley in North and South America and several countries of Asia (7,12,19,22,29,32, for review see 21). Among the variety of symptoms produced by this pathogen on all plant parts, the foliar spot blotch phase has emerged as a major biotic stress limiting commercial production of wheat (8,10) and barley (1). In fact, spot blotch has become a major production constraint in South Asia's intensive cropping systems where nearly 12 million hectares of land under cultivation are affected (25,30). Effective control of spot blotch can be achieved by the introduction of resistant cultivars as an important component of integrated disease management (24). Hence, the search for parental stocks possessing an adequate level of resistance to *B. sorokiniana* is essential.

The objective of this study was to gather information on the mechanism(s) of resistance of elite wheat against *B. sorokiniana*. The fungus can grow on the plant without producing symptoms, but mainly causes necrotic lesions on the leaf (6). Pathogenesis includes germination of conidia on the leaf surface and formation of an appressorium at the tip of the germ tube that supports direct penetration through the host cuticle (3,20,21). The apparently biotrophic growth phase is primarily confined to a single epidermal cell invaded by infection hyphae, whereas the necrotrophic growth phase starts upon invasion of the mesophyll tissue fol-

lowed by host cell death, which appears to be a consequence of toxin secretion (2,21,26).

Few studies have been published on the cellular and molecular factors contributing to resistance to *B. sorokiniana* (6,21,28). Recently, it was demonstrated that the barley *Mlo* gene, which is a susceptibility factor supporting growth of the biotrophic fungus *Blumeria graminis* f. sp. *hordei* and is implicated in cell survival (5,11,16), contributes to barley background resistance to *B. sorokiniana* and other hemibiotrophic pathogens (18,20). The impact of host-generated hydrogen peroxide (H_2O_2) on early infection stages of *B. sorokiniana* has been analyzed by subcellular H_2O_2 detection in tissues infiltrated with diaminobenzidine (DAB). Intriguingly, H_2O_2 accumulates strongly in association with an unsuccessful fungal attack in the epidermis as well as in association with successful fungal growth in the barley mesophyll. However, the definitive role of H_2O_2 in defense to *B. sorokiniana* is still unclear (16,28). In the present study, we undertook an investigation of the defense responses of wheat genotypes Yangmai 6, M 3, Shanghai 4, and Chirya 7, all of which are being integrated in international breeding programs aimed at developing spot blotch resistant wheat cultivars. We propose that cellular host responses such as formation of cell wall appositions as well as the degree of early mesophyll spreading of fungal hyphae are indicative of the defense potential of the respective host genotype and, therefore, could be used for the characterization of new spot blotch resistance traits in cereals.

MATERIALS AND METHODS

Host and pathogen. Wheat genotypes obtained from the International Maize and Wheat Improvement Center (CIMMYT), Mexico, and the Directorate of Wheat Research (DWR), India, viz. Yangmai 6, M 3 (W7976), Shanghai 4, Chirya 7, Longmai 10, Ning 8201, Suzhoe 8, Chirya 1, Ciano T-79, and Sonalika (9)

Corresponding author: K.-H. Kogel

E-mail address: Karl-Heinz.Kogel@agr.uni-giessen.de

*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains supplemental material not included in the print edition. The online version contains a figure showing the interaction phenotypes of different wheat genotypes with *Bipolaris sorokiniana*.

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were used. The sources and pedigrees of the germ plasm are provided in Table 1.

Plants were grown in growth chambers at 18°C, 60% relative humidity, and a 16-h photoperiod (100 µE/m² s⁻¹). *B. sorokiniana* (*Bs-kn2* [20]) was originally isolated from the flag leaf of wheat Sonalika growing under natural conditions at Banaras, India, during April 1999, and is available from the culture collection of the Institute of Phytopathology and Applied Zoology, Justus-Liebig-University, Giessen, Germany. The single conidial isolate was produced following the method of Hetzler (13). Conidia from monoconidial cultures were transferred to a 30% V8 agar and incubated at room temperature under a 12-h photoperiod (100 µE/m² s⁻¹). After 7 days, petri dishes were flooded with distilled water containing 0.02% Tween 20. The conidia were scraped from the surface with a spatula and then filtered through two layers of muslin cloth to obtain a conidial suspension without mycelium.

Inoculation. Leaf segments were harvested and laid flat by fixing their ends on the surface of a steel sheet. A suspension containing approximately 20,000 conidia per ml of water supplemented with 0.02% Tween 20 was sprayed onto the segments. Inoculated segments were placed onto a 2-mm-thick layer of 0.5% water agar (adding 0.6 parts per million of benzimidazole) in a plastic dish closed tightly with a lid. This low concentration of benzimidazole delayed leaf senescence but did not affect the fungus. The dishes were placed in a growth chamber at 25°C and a 16-h photoperiod (100 µE/m² s⁻¹). The lower 4-cm portion of first leaf segments and/or the middle 6-cm portion of third leaf segments were evaluated 48 h after inoculation (hai) by a binocular microscope for the number of conidia and lesions.

Microscopy and histochemical assay for H₂O₂ detection. Epifluorescence microscopy (Axioplan Microscope, Zeiss, Jena, Germany) in combination with the fluorescent brightener Calcofluor (Sigma-Aldrich, Munich, Germany) was used to reveal the interaction phenotypes. Calcofluor has been used for cell and hyphal wall detection (27,28) and facilitates the location of fungal penetration sites in leaf tissue. Leaf fixation and microscopy were conducted as described by Hückelhoven and Kogel (15). Inoculated segments taken at 20, 40, and 60 hai were fixed in 0.15% trichloroacetic acid (wt/vol) in ethanol/chloroform (4:1, vol/vol). Fixed segments were later washed two times for 15 min with 50% ethanol, two times for 15 min with 50 mM NaOH, and three times for 10 min with distilled water followed by 30-min incubation in 0.1 M Tris-HCl (pH 8.5). After this step, segments were stained for 5 min with 0.3% (wt/vol) Calcofluor and finally washed four times for 10 min with distilled water. Leaf segments were stored in 50% glycerol. Papilla-like structures with a halo appear light blue under UV excitation (385 nm, emission 420 to 460 nm, data not shown), probably indicating cell wall alterations by the activity of fungal hydrolases. Detection of H₂O₂ was carried out by an endogenous peroxidase-dependent in situ staining procedure with

3,3-DAB (Sigma-Aldrich) (31). Leaves were infiltrated with DAB (1 mg/ml), pH 3.8 (adjusted with HCl).

Quantification of fungal development and defense responses. The number of brown necrotic lesions observed divided by the number of conidia observed under the binocular microscope was calculated and expressed as lesions per conidia (in percent). Detection of papilla-like structures and the hypersensitive reaction (HR) has been described (21,28). Whole-cell DAB staining or yellow-green whole-cell autofluorescence, respectively, under blue light excitation (excitation: 460 to 480 nm, emission: long-pass 510 nm) was used to detect the HR. To detect quantitative differences in fungal ramification within the mesophyll, a spreading factor was defined by assessing the range of the most distant tips of inter- and intracellular hyphae at one infection site (28).

Electrolyte leakage of leaf segments. Detached leaf segments were inoculated with 50,000 conidia per ml and incubated at 24°C and a 16-h photoperiod (30 µE/m² s⁻¹) in transparent plastic dishes containing 0.7% water agar supplemented with 0.6 parts per million of benzimidazole and closed tightly with a lid. Each treatment comprised 10 leaf samples divided into two replicates of five leaf sections each. After incubation for 48 h, a razor blade was used to cut a 2.5 cm leaf section of each leaf. The electrolyte leakage assay was carried out as described by Kwon et al. (23).

RESULTS

Initial screening for resistance. The level of seedling and adult plant resistance to *B. sorokiniana*, as assessed by lesion density and area under the disease progress curve in the field, as well as the origin and pedigrees of germ plasm are shown in Table 1. We analyzed disease symptoms caused by isolate *Bs-kn2* after conidial spray inoculation of Longmai 10, Chirya 7, Yangmai 6, Shanghai 4, M 3, and Yangmai 6. Initially, we screened detached segments from the third leaves (21-day-old seedlings) and first leaves (10-day-old seedlings) and found no differences in the interaction phenotypes on different leaf types (data not shown). Figure 1 shows data obtained in an independent inoculation experiment with first leaf segments of 10-day-old wheat seedlings. Sonalika exhibited highest and Yangmai 6 exhibited lowest numbers of spot blotch lesions per leaf segment while Longmai 10, Chirya 7, M 3, and Shanghai 4 showed an intermediate response (Fig. 1).

Pre-infection defense responses. A microscopic survey showed that the fungus penetrates epidermal cells through the anticlinal cell walls and subsequently invades mesophyll tissue from hyphae produced inside epidermal cells. We termed fungal penetration into epidermal cells “successful penetration” and invasion of the mesophyll tissue “successful infection.”

We comparatively analyzed the epidermal penetration resistance of cvs. Sonalika, M 3, and Yangmai 6. Pre-infection defense as defined here includes nonpenetrated cell wall appositions

TABLE 1. Levels of field resistance to *Bipolaris sorokiniana*, origin, and pedigrees of wheat genotypes used in the study

Genotype	Level of field resistance ^a	Origin	Pedigree
Sonalika ^{bc}	S	India	II53.388/AN/YT54/N10B/3/LR/4/B4946.A.4.18.2.1Y/Y53/3*Y50
Chirya 1 ^d	MR	Mexico	CS/TH.CU//GLEN/3/ALD/PVN/4/NINGMAI4/OLESON//ALD/YANGMAI 4
Longmai 10 ^d	MR	China	DONGNONG 101/LONG 70.663
Chirya 7 ^d	R	Mexico	MR CS/TH.CU//GLEN/3/ALD/PVN/4/NINGMAI 4/OLESON//ALD/YANGMAI 4
Shanghai 4 ^d	MR	China	Unknown
M 3 (W7976) ^c	R	CIMMYT	Cando/R143//Mexi 's'/3/T. taushii (CI22)
Yangmai 6 ^{df}	R	China	Unknown

^a Level of field resistance according to evaluation of lesion density (lesions cm⁻²) and area under disease progress curve of seedling and adult plant resistance to spot blotch. S, susceptible; MR, moderately resistant; R, resistant.

^b Duveiller et al. (8).

^c Duveiller and García Altamirano (9).

^d E. Duveiller (personal communication).

^e D. P. Singh (unpublished data).

^f Sharma et al. (29).

(CWA) beneath sites of fungal attack and epidermal HR, both resulting in failure of fungal development and invasion of the mesophyll tissue. The highest frequency of CWA was found in Yangmai 6 at all times (20, 40, and 60 hai) (Fig. 2). The CWA frequencies increased with time suggesting that subsequent penetration attempts are less successful than early ones. The frequencies of nonpenetrated epidermal cells undergoing an HR showed no differences at 20 h among all genotypes (Fig. 3). By 40 and 60 hai, however, HR frequencies were even lower in the resistant Yangmai 6, which can be explained by the increase in CWA with newly emerging appressoria (Fig. 2). In some cases, fungal growth was arrested by a host cell death after fungal penetration (postpenetration HR [PPHR]) when infection hyphae had been formed (Fig. 4). PPHR frequencies were also lowest in Yangmai 6.

Postinfection defense responses. We determined the rates of successful fungal infection of the mesophyll layer (Fig. 5). The highest infection rate was found in Sonalika. Mesophyll tissue of M 3 was invaded at the late time point (60 hai), and Yangmai 6 was only rarely invaded by fungal hyphae. To detect quantitative

differences in fungal spreading within the mesophyll, we analyzed the spreading factor by assessing the range of the most distant tips of inter- and intracellular hyphae at each infection site. A steady increase in the spreading factor of Sonalika from 20 to 60 hai was observed and differed from that of M 3 and Yangmai 6 (Fig. 6). Yangmai 6 exerted the lowest spreading factor, clearly reflecting the spot blotch resistance under field conditions.

Electrolyte leakage. We evaluated the extent of host cell collapse by measuring electrolyte leakage in leaf segments (Table 2). We found a close association between the extent of electrolyte leakage and field susceptibility. Wheat genotypes developing severe necrosis in response to fungal attack exhibited enhanced ion leakage into the apoplastic fluid. Leakage was extremely high in the susceptible genotype Sonalika.

DISCUSSION

The objective of this study was to elucidate cellular defense responses to *B. sorokiniana* in wheat genotypes currently used

Fig. 1. Relative inoculum efficiency on different wheat genotypes. Binocular microscopy evaluation of spot blotch formation of *Bipolaris sorokiniana* isolate *Bs-kn2* on 10-day-old first leaves from various wheat genotypes at 48 h after inoculation. Displayed is the percentage of conidia causing a visible necrotic lesion on a 4-cm-long leaf segment after spray inoculation with a solution of 20,000 conidia per ml. Ten leaf segments from each genotype were used with the mean from three independent replications reported. The experiment was repeated two times with similar results. Vertical bars represent standard deviation of the means.

Fig. 2. Relative cell wall apposition formation in spot blotch-inoculated select wheat genotypes. Frequencies of cell wall appositions (CWAs) formed in response to *Bipolaris sorokiniana*, isolate *Bs-kn2*, in wheat genotypes Sonalika, M 3, and Yangmai 6 at 20, 40, and 60 h after inoculation (hai). Leaf segments of 10-day-old first leaves were inoculated with 50,000 *B. sorokiniana* conidia per ml. Thirty interaction sites were evaluated per leaf. The mean of three leaves for each genotype and inspection time point is displayed. The experiment was repeated two times with similar results. Vertical bars represent standard deviation of the mean.

Fig. 3. Relative prepenetration hypersensitive reactions (HR) in spot blotch-inoculated select wheat genotypes. Frequencies of HR in nonpenetrated epidermal cells of wheat genotypes Sonalika, M 3, and Yangmai 6 in response to *Bipolaris sorokiniana*, isolate *Bs-kn2*, at 20, 40, and 60 h after inoculation (hai) as measured by epifluorescence microscopy. Thirty interaction sites were evaluated per leaf. The mean of three leaves for each genotype and inspection time point is displayed. The experiment was repeated two times with similar results. Vertical bars represent standard deviation of the mean.

Fig. 4. Relative postpenetration hypersensitive reactions (PPHR) in spot blotch-inoculated select wheat genotypes. Frequencies of PPHR in attacked epidermal cells of wheat genotypes Sonalika, M 3, and Yangmai 6 in response to *Bipolaris sorokiniana*, isolate *Bs-kn2*, at 20, 40, and 60 h after inoculation (hai). PPHR was observed as whole-cell autofluorescence of penetrated cells leading to encapsulation of fungal infection hyphae and a subsequent arrest of fungal development. Thirty interaction sites were evaluated per leaf. The mean of three leaves for each genotype and inspection time point is displayed. The experiment was repeated two times with similar results. Vertical bars represent standard deviation of the mean.

in international breeding programs. Our data indicate close associations between adult plant resistance in the field and reduced fungal penetration into epidermal cells, reduced infection of and spreading within mesophyll tissue as well as low ion leakage.

B. sorokiniana exerts a hemibiotrophic life style in its interaction with barley; that is, it shows distinct biotrophic and subsequent necrotrophic growth phases (28). The same applies for its interaction with wheat as demonstrated here. We show that resistance of wheat to *B. sorokiniana* is primarily attributed to penetration resistance due to a CWA-associated defense mechanism. Moreover, we detected two types of cell death: a frequent HR of attacked but nonpenetrated cells and, more rarely, a PPHR leading to an encapsulation of infection hyphae. HR frequencies, however, do not explain differences in resistance to *B. sorokiniana* among the genotypes. Rather consistently, HR occurs in all genotypes at a similar extent. Therefore, one can speculate that HR is a component of basal resistance to *B. sorokiniana* in wheat.

Penetration resistance at the epidermal stage is closely associated with local generation of H_2O_2 in CWA and/or in whole cells, visualized by DAB staining. This finding is reminiscent of defense responses of cereals toward the biotrophic powdery mildew fungus, *Blumeria graminis* (14,17), thus supporting the view that *B. sorokiniana*, at very early infection stages, exerts a biotrophic life style (6,28).

Once the fungus has successfully colonized epidermal cells, blocking of mesophyll invasion and inhibition of spreading within the mesophyll tissue represent subsequent defense mechanism(s). It appears that invasion by itself, as well as spreading after invasion, is subject to distinct defense reactions exerted by more resistant wheat genotypes (Figs. 5 and 6). Development of the fungus within the mesophyll seems to be indicative of the potential of a plant to restrict the pathogen. Hence, the spreading factor is a reliable character for the resistance potential of a cultivar. Importantly, the spreading factor associates with lesion development (Figs. 1 and 6) as well as with the number of collapsed mesophyll cells per lesion in both wheat (data not shown) and barley (28).

Once the fungus has entered the mesophyll, cell death appears to trail the expanding hyphae, which proliferate from the site of penetration to neighboring cells. In this phase, cell death seems ineffective in blocking the pathogen. It remains unclear whether mesophyll cell death is a response under host control or, alterna-

tively, toxin-provoked and out of host control (4). Interestingly, the barley albino mutant *albostrians* expresses a white leaf phenotype that is super-susceptible to *B. sorokiniana*. In this mutant, fungal spreading within the mesophyll is strongly enhanced compared to the green leaves even though mesophyll cell death in *albostrians* was not detectable (28). This result is an argument in favor of cell death to restrict fungal development in the mesophyll. Alternatively, the speed in the occurrence of mesophyll cell death is pivotal to stop fungal cell-to-cell movement. In such a situation, the necrotrophic growth phase of *B. sorokiniana* would reflect a pathogenic strategy to cope with plant cell death-associated defense (28). It remains unclear whether during the necrotrophic phase host-generated H_2O_2 contributes to resistance or successful pathogenesis. Again, data obtained in the *albostrians* mutant indicate that H_2O_2 is not required for fungal pathogenesis (28).

Our data show that resistance to Yangmai 6 is clearly associated on the cellular level with enhanced CWA formation, reduced mesophyll invasion, and reduced spreading within the mesophyll. These cellular defense responses are also found in the other wheat lines, including highly susceptible Sonalika, though to a much lower frequency. In Yangmai 6, early defense reactions conditioned the leaf for even more effective defense against secondary infection attempts, suggesting that locally induced resistance might play a role.

Fig. 5. Relative rate of mesophyll colonization in spot blotch-inoculated wheat genotypes. Successful mesophyll infection of wheat genotypes Sonalika, M 3, and Yangmai 6 by *Bipolaris sorokiniana*, isolate *Bs-kn2*, at 20, 40, and 60 h after inoculation (hai). Fungal attack was termed "successful infection" when the intracellular or intercellular hyphae were visible in the mesophyll layer. Thirty interaction sites were evaluated per leaf. The mean of three leaves for each genotype and inspection time point is displayed. The experiment was repeated two times with similar results. Vertical bars represent standard deviation of the mean.

Fig. 6. Fungal development within the mesophyll tissue (spreading) of wheat genotypes Sonalika, M 3, and Yangmai 6. The degree of spreading of *Bipolaris sorokiniana*, isolate *Bs-kn2*, is specified as a spreading factor (μm) by assessing the range of the most distant tips of hyphae at one infection site for the time points 20, 40, and 60 h after inoculation (hai). Thirty interaction sites were evaluated per leaf. The mean of three leaves for each genotype and inspection time point is displayed. The experiment was repeated two times with similar results. Vertical bars represent standard deviation of the mean.

TABLE 2. Electrolytic leakage of mesophyll tissue of wheat genotypes after inoculation with *Bipolaris sorokiniana*, isolate *Bs-kn2*^a

Genotypes	Level of field resistance	Time after inoculation			
		0 h	24 h	48 h	72 h
Yangmai 6	R	4.7	27.8	41.2	75.8
Chirya 7	R	2.9	47.2	62.6	89.5
Longmai 10	MR	7.5	32.2	51.1	100.3
Sonalika	S	6.6	43.4	90.8	161.2

^a MR, moderately resistant; R, resistant; S, susceptible. Comparison of means between genotypes using Games-Howell test: there is a significant difference between Yangmai 6 and Sonalika at $P < 0.05$, but no significant difference between Yangmai 6, Chirya 7, and Longmai 10 at 48 and 72 h after inoculation (hai). Comparison of means between time after inoculation using paired t test of repeated measures: there is a highly significant difference at all time points in Sonalika ($P < 0.01$). Yangmai 6, Chirya 7, and Longmai 10 also showed small but significant differences with time ($P < 0.05$).

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