A Compromised *Mlo* Pathway Affects the Response of Barley to the Necrotrophic Fungus *Bipolaris sorokiniana* (Teleomorph: *Cochliobolus sativus*) and Its Toxins

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ABSTRACT

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In search of new durable disease resistance traits in barley to control leaf spot blotch disease caused by the necrotrophic fungus *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*), we developed macroscopic and microscopic scales to judge spot blotch disease development on barley. Infection of barley was associated with cell wall penetration and accumulation of hydrogen peroxide. The latter appeared to take place in cell wall swellings under fungal penetration attempts as well as during cell death provoked by the necrotrophic pathogen. Additionally, we tested the influence of a compromised *Mlo* pathway that confers broad resistance against powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*). Powdery mildew-resistant genotypes with mutations at the *Mlo* locus

(*mlo* genotypes) showed a higher sensitivity to infiltration of toxic culture filtrate of *Bipolaris sorokiniana* as compared with wild-type barley. Mutants defective in *Ror*, a gene required for *mlo*-specified powdery mildew resistance, were also more sensitive to *Bipolaris sorokiniana* toxins than wild-type barley but showed less symptoms than *mlo5* parents. Fungal culture filtrates induced an H₂O₂ burst in all mutants, whereas wild-type (*Mlo*) barley was less sensitive. The results support the hypothesis that the barley *Mlo* gene product functions as a suppressor of cell death. Therefore, a compromised *Mlo* pathway is effective for control of biotrophic powdery mildew fungus but not for necrotrophic *Bipolaris sorokiniana*. We discuss the problem of finding resistance traits that are effective against both biotrophic and necrotrophic pathogens with emphasis on the role of the anti-oxidative system of plant cells.

Additional keywords: ascorbate, oxidative burst, wheat.

Bipolaris sorokiniana (Sacc.) Shoemaker (teleomorph: *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur) is a serious pathogen of wheat and barley in North and South America, Europe, and several countries of Asia (10,16,18,26,28). Among a variety of symptoms induced by this pathogen on all parts of the plant, the foliar spot blotch has emerged as the major biotic stress hampering commercial production of wheat (12) and barley (2,29). An effective control of spot blotch can be achieved by introduction of resistant cultivars as a major component of integrated disease management (32). Thus, identification of parental stocks possessing an adequate level of resistance to *Bipolaris sorokiniana* is urgently required.

There are only a limited number of reports describing factors of resistance to *Bipolaris sorokiniana* (16). The usual methods applied in the field and greenhouse for evaluating resistance to spot blotch are based on disease appraisal scales (1,11,40), but scores using these conventional scales are difficult to repeat because the disease establishment is influenced markedly by environmental factors (8,33). Thus, interaction phenotypes fluctuate considerably within routine greenhouse or field screening variations (34). One objective of the present study was to gather information on the defense mechanism of barley against *Bipolaris sorokiniana*. We examined microscopic subcellular changes occurring in leaf cells after inoculation with conidia and culture filtrates (CF) of *Bipo-*

laris sorokiniana under controlled conditions. These structural and chemical changes linked to interaction phenotypes may become useful and reliable components for evaluation of host resistance.

Bipolaris sorokiniana causes necrotic lesions on the leaf surface of wheat and barley (9). The mechanism of pathogenesis includes germination of conidiospores on the leaf surface and elaboration of an appressorium from the germ tube that supports direct penetration by infection hyphae through the cuticle of the host plant (4). Pathogenicity is associated with the production of toxins (31, 39). The role of toxins is confirmed by the fact that formation of necrotic lesions is triggered not only upon leaf colonization by the pathogen but also without penetration by germinating conidia (9). Further, collapse of mesophyll cells not in direct contact with developing mycelium occurs due to toxins produced by the fungus (36). CFs contain substances active in pathogenesis (21) and produce symptoms similar to those produced by the fungus itself (14,15).

In an attempt to identify new sources for durable disease resistance against *Bipolaris sorokiniana* in barley, we have analyzed the effect of mutations at the *Mlo* locus and functionally related genes on this interaction. The role of the *Mlo* locus in controlling the powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*) has been comprehensively documented (6,25,41). Recessive *mlo* alleles, of which most were generated by mutagenesis, confer resistance to *Blumeria graminis*. This *mlo* resistance is monogenic, race-nonspecific, and durable. Owing to the durability, *mlo*-resistant genotypes have become widely cultivated in many European countries, covering 700,000 ha or 30% of the spring barley area (25).

It has been argued that the *mlo*-resistance mechanism is limited to pathogens that infect living epidermal cells (biotrophic), there-

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by affecting fungal infection via rapid and excessive deposition of materials below the attacked site so intrusion is prevented. This kind of penetration resistance is associated with a highly localized accumulation of H_2O_2 in these deposits (22). H_2O_2 is involved in cross-linking reactions of phenolic compounds (45) and proteins (42) to enhance cell wall resistance against hydrolytic enzymes and mechanical pressure (39). Furthermore, it has been argued that *mlo*-resistant barley reacts to pathogens other than *Blumeria graminis* in the same way as wild-type varieties (25). However, recent investigations carried out with the necrotrophic pathogen *Magnaporthe grisea* revealed increased susceptibility of *mlo* genotypes as compared with plants carrying the dominant wild-type

gene *Mlo*, in clear contrast to their interactions with the biotrophic pathogen *Blumeria graminis* (24). In the present study, we demonstrate that powdery mildew-resistant genotypes with a compromised *Mlo* pathway exhibit enhanced sensitivity to *Bipolaris sorokiniana* toxins as compared with wild-type barley. Sensitivity was associated with a massive accumulation of H_2O_2 . We propose that these cellular host responses could be useful for identification of new resistance traits in cereals that are more effective toward *Bipolaris sorokiniana*.

MATERIALS AND METHODS

Host and pathogen. Barley genotype Ingrid (wild-type *Mlo*), an Ingrid backcross line (*I-mlo5*) bearing the recessive *mlo5* allele, and a mutant A89 bearing the recessive allele *ror1-2* in the *I-mlo5* background were used. Origin and description of these lines have been reported (13). Plants were grown in a growth chamber at 18°C, 60% relative humidity, and a 16-h photoperiod (100 μ E).

The *Bipolaris sorokiniana* culture was isolated from the boot leaf of wheat cv. Sonalika growing under natural conditions at Banaras, eastern India, during April 1999. A single conidial isolate was produced after incubation of infected leaf material bearing characteristic disease symptoms of spot blotch in a moist chamber at 20°C for 2 to 3 days and subsequently following (20). Conidia from monoconidial cultures were transferred to 30% V8 agar and incubated at room temperature under a 12-h photoperiod. After 7 days, petri dishes were flooded with sterile distilled water containing 0.02% Tween 20, and conidia were scraped from the surface with a spatula before filtration through two layers of muslin cloth to obtain an inoculum suspension without mycelium.

Inoculation. Primary leaf segments (6 cm long) harvested from 7-day-old plants were laid flat by fixing their ends on surface of a steel sheet. A suspension containing approximately 20,000 spores per ml water plus 0.02% Tween 20 was sprayed onto the segments. Inoculated segments were immediately placed onto a 2-mm thick layer of benzimidazole 0.5% water agar (0.6 parts per million of benzimidazole) in a plastic dish closed tightly with a lid.

Production of toxic CF, toxin infiltration into leaves, and scoring for toxin-induced necrosis. One-milliliter aliquots of a conidial suspension (10,000 spores per ml) were transferred to 250-ml Erlenmeyer flasks containing 50 ml of Fries medium supplemented with 0.1% yeast extract, as previously described (3). Flasks were incubated at 25°C in the dark without agitation for 3 weeks. CF-containing toxins were filtered through Whatman No. 1 filter paper, and filtrates were centrifuged at $3,500 \times g$ for 20 min. The supernatant was passed through a 0.45- μ m millipore membrane, adjusted to pH 6.5 with 1 N NaOH and stored at 4°C.

Pure and water-diluted CF (1:10, 1:100, and 1:500, vol/vol) was injected into primary leaves of plants (19). Leaves injected with

Fig. 1. Spot blotch symptoms on barley leaves; representative spot blotch lesions on primary leaves of barley. **A**, Reaction types (RT) on primary leaves 72 h after inoculation 1, 2, 3, and 4, whereby RT1 = minute necrotic dots without chlorosis; RT2 = round or irregular necrotic spots without chlorosis; RT3 = round or irregular necrotic spots with chlorosis; and RT4 = larger round or irregular necrotic spots with pronounced chlorosis. **B**, Scale for scoring intensity of spot blotch on tertiary leaves 96 h after inoculation: 0 = free of disease spots (highly resistant); 1 = necrotic spots with dominance of RT1, up to 10% leaf area involved (resistant); 2 = necrotic spots with dominance of RT1 and RT2, chlorosis absent, up to 50% leaf area involved (moderately resistant); 3 = necrotic spots with dominance of RT3, moderate chlorosis, up to 50% leaf area involved (susceptible); and 4 = necrotic spots with dominance of RT4, severe chlorosis, >50% leaf area involved (highly susceptible). **C**, Scale for scoring intensity of necrosis on primary leaves 96 h after injecting culture filtrate of *Bipolaris sorokiniana*: 0 = no symptoms; 1 = faint necrosis in the infiltrated area; 2 = scattered, small necrotic spots in the infiltrated area; 3 = both necrosis and chlorosis in the infiltrated area; and 4 = continuous and severe necrosis in the infiltrated area.

TABLE 1. Influence of a compromised *Mlo* pathway on host reaction to culture filtrate of *Bipolaris sorokiniana* injected into 7-day-old primary leaves of differentially reacting barley lines^a

Genotype	Culture filtrate (water dilution)				Fries medium
	1:1	1:10	1:100	1:500	1:1
Ingrid (<i>Mlo Ror1</i>)	1.8	1.4	0.4	0.0	0.4
<i>I-mlo5</i> (<i>mlo5 Ror1</i>)	4.0	3.8	1.4	0.4	0.4
A89 (<i>mlo5 ror1-2</i>)	2.8	2.6	1.0	0.0	0.4
LSD	1.2	0.9	0.6	0.02	

^a Estimated following Tomas and Bockus (42) with modifications (Fig. 1C). Injection with 1:10, 1:100, and 1:500 dilutions of Fries medium as well as water alone produced no visible symptoms. Difference among genotypes at $P = 0.05$ according to Fisher's least significant difference (LSD) test, analysis of variance based on mean values of three replications. Each replication represented a set of five leaves with replication reading being mean reaction score (0 to 4) of five leaves.

water and Fries medium served as controls. After injection, plants were placed in a growth chamber (25°C and 16-h photoperiod). Toxic symptoms developed after 4 days of infiltration were macroscopically evaluated on the basis of a rating scale proposed by Tomas and Bockus (43), with modifications illustrated in Figure 1C.

Microscopic analysis and histochemical assay for H₂O₂ detection. Histochemical detection of H₂O₂ was carried out by an endogenous peroxidase-dependent in situ histochemical staining procedure with 3,3-diaminobenzidine (DAB) (42). The specificity of staining was verified by adding ascorbate, an H₂O₂ scavenger. Leaves were infiltrated with indicated concentrations of ascorbate mixed with DAB (1 mg/ml) in the ratio of 1:1 (vol/vol). Clearance

and storage of leaf segments, staining of fungal structures, and microscopy were done following the methods (23).

RESULTS

Predominating macroscopic interaction phenotypes. Disease symptoms caused by *Bipolaris sorokiniana* after conidial infection were analyzed on primary leaf segments and tertiary leaves at 72 and 96 h after inoculation, respectively. Lesions representing reaction types (RT) (Fig. 1A, RT1 to RT4) appeared simultaneously but in variable proportions on leaf surfaces of the different barley genotypes. Differential responses of barley were supported by the late disease score (96 h after inoculation) on

Fig. 2. Light micrographs of barley leaf cell responses to invasion by conidia (c) of *Bipolaris sorokiniana*. Leaves were injected with 3,3-diaminobenzidine (DAB) to detect H₂O₂ 2 h before fixation of leaf segments. **A**, Cell wall swelling (cws) at a site of attempted penetration of an epidermal cell by an appressorium (app) formed at the tip of germ tube (gt); inset shows closer view of a cws. **B**, Spherical brown spots (sbs) on epidermal cell wall in the vicinity of a germinated conidium (out of focus). **C**, Successful penetration evidenced by the presence of intracellular hyphae (ich) arising from the appressorium formed at the tip of germ tube. **D**, Evenly DAB-stained epidermal (ese) and mesophyll (esm) cells in the vicinity of a germinated conidium showing bipolar germ tubes typical of *Bipolaris sorokiniana*. **E**, Localized dark browning (ldb) of epidermal cell walls. **F**, Irregular brown mesophyll cells (ibm) appearing by 48 h after inoculation in the vicinity of a conidium-bearing germ tube and appressorium.

tertiary leaves, as illustrated in Figure 1B. When we tested barley cv. Ingrid *mlo*-backcross lines showing race nonspecific resistance to *Blumeria graminis* f. sp. *hordei*, we unexpectedly detected increased susceptibility to *Bipolaris sorokiniana* as compared with the wild-type barley (*Mlo* genotype, data not shown). However, during these studies infection phenotypes shown in Figure 1A and B varied from experiment to experiment although care was taken to accurately control conditions. Therefore, a more detailed analysis was conducted to find interaction characteristics that may serve as a more reliable tool to search for resistant barley cultivars.

Mutations in genes functionally related to the *Mlo* pathway affect sensitivity of barley to toxins of *Bipolaris sorokiniana*. Pure and water-diluted CF induced necrosis of various intensities, visible 96 h after infiltration into barley primary leaves (Fig. 1C). A faint chlorosis developed in leaves of all three genotypes infiltrated with Fries medium, possibly due to adverse effects of various salts and high amounts of sugar. Leaves injected with water did not show any abnormal symptoms. We treated genotypes differing at the *Mlo* locus with the toxic CF. The line *I-mlo5* was the most sensitive to CF (Table 1). Its leaves showed a significantly higher intensity of necrosis at all levels of CF dilutions. On the contrary, leaves of cv. Ingrid were the least sensitive. A89 showed intermediate sensitivity to CF. A89 bears a mutation in the gene *Ror1* that is required for *mlo*-specified resistance to the powdery mildew fungus and shows moderate susceptibility to powdery mildew (13).

Predominating microscopic interaction phenotypes. In order to study the interaction of barley with *Bipolaris sorokiniana* in more detail, we analyzed fungal development and the cellular plant reactions at early interaction stages after inoculation with a conidial suspension. Barley cv. Ingrid reacted to conidia germination and appressoria formation with the development of local cell wall swellings beneath fungal structures (Fig. 2A). Additionally, spherical brown spots were visible near fungal structures. These spots were less localized than cell wall swellings in the vicinity of germinated fungal spores. Although these structures were already visible in fixed leaves without staining, they became more easily detectable if the leaves were stained with DAB to detect endogenous H_2O_2 accumulation (Fig. 2A, inset and B). These structural plant cell wall alterations had increasing frequencies from 24 to 48 h after inoculation. Penetration, which was the prerequisite for development of intracellular hyphae arising from appressoria

(Fig. 2C), was rarely detected at 24 h after inoculation but more frequently from 36 h after inoculation onward (Fig. 3).

Localization of H_2O_2 . A DAB injection method was used to detect pathogenesis-related accumulation of H_2O_2 . Evenly brown-stained epidermal and mesophyll cells were detected at 8 h after inoculation onward, with an increasing number during the interaction (Fig. 2D). Between 24 and 48 h after inoculation, the number of brown mesophyll cells at interaction sites increased especially (Fig. 4). Epidermal cells showing irregularly intensive DAB-stained cell wall zones (Fig. 2E) were present in a similar number during early interaction stages from 24 to 48 h after inoculation (Fig. 4). Mesophyll cells with intensive DAB staining, predominantly in chloroplasts (Fig. 2F), were visible at 48 h after inoculation when cells started to collapse.

Sensitivity to toxic CF associated with massive production of H_2O_2 . Intensity of H_2O_2 accumulation in response to injection of CF varied in the three genotypes according to toxin sensitivity (Table 1). Intense brown DAB staining of the area infiltrated with pure CF appeared most prominently in *I-mlo5* leaves, intermediate in A89, and least pronounced in cv. Ingrid (Fig. 5A).

Ascorbate inhibits H_2O_2 accumulation. DAB staining seen under the microscope (Fig. 2D) could also be detected with the naked eye as a brown lesion. A gradual decrease in the density of brown-stained lesions occurred when increasing concentration of ascorbate were included into DAB solutions (Figs. 5B and 6). Furthermore, the number of brown epidermal and mesophyll cells decreased proportionally with the amount of ascorbate infiltrated into leaves of cv. Ingrid at 36 h after inoculation (Fig. 7). Similarly, DAB staining induced by CF was completely prevented by 50 mM ascorbate when injected before 36 h after CF treatment (Fig. 5C).

DISCUSSION

The present study was undertaken to elucidate characteristics of the mechanism determining resistance and susceptibility in barley to the necrotrophic fungus *Bipolaris sorokiniana*, which constitutes an increasing threat for intensive plant growing regions worldwide. Most importantly, H_2O_2 accumulation visualized with DAB was involved in pathogenesis of spot blotch. Therefore, DAB staining may serve as a future tool to search for cereal cultivars that are resistant to *Bipolaris sorokiniana* and possibly other necrotrophic pathogens and their toxins.

Fig. 3. Cellular responses of 7-day-old primary leaves of barley line Ingrid at 24, 36, and 48 h after inoculation with conidia of *Bipolaris sorokiniana*. White columns indicate epidermal cells with swelled walls below appressoria (Fig. 2A); black columns indicate penetrated epidermal cells (Fig. 2C); striped columns indicate epidermal cells with spherical spots (Fig. 2B). A microscopic field represents a site with at least one conidium with germ tube and appressorium of *Bipolaris sorokiniana*. Data are means from three repetitions with 50 interaction sites each.

Fig. 4. Profiling of H_2O_2 accumulation in epidermal and mesophyll tissue of 7-day-old primary leaves of barley cv. Ingrid at 24, 36, and 48 h after inoculation with conidia of *Bipolaris sorokiniana*. 3,3-Diaminobenzidine (DAB) coloration profile: white columns indicate epidermal cells with localized browning (Fig. 2E); black columns indicate evenly stained epidermal cells (Fig. 2D); and striped columns indicate evenly stained mesophyll cells (Fig. 2D). Data are means from three repetitions with 50 interaction sites each.

In quest of new sources for durable disease resistance, we have analyzed the effect of mutations at the barley *Mlo* locus and functionally related genes on development of the foliar spot blotch disease. Increased susceptibility of *I-mlo5* was verified by its higher sensitivity to CF, which induced necrosis even at high dilutions (Table 1). A similar influence has been recorded for different *mlo* alleles, carried in backgrounds of the barley cvs. Ingrid and Pallas, on the necrotrophic pathogen *M. grisea* (24). We also noted an effect of the *Ror1* locus, which is required to fully express *mlo* resistance to *Blumeria graminis*. Genotype A89, bearing the mutant allele *ror1-2*, was intermediate sensitive to CF. Interestingly, the influence of the *Ror* mutation on expression of *mlo* resistance to *Blumeria graminis* is also incomplete, producing an intermediate infection phenotype. Together, our data support the view that a compromised Mlo pathway supports the development of necrotrophic pathogens in clear contrast to its effect on biotrophs.

The most striking subcellular structural changes were swellings of epidermal cell walls below appressoria (Fig. 2A) and the formation of papilla-like structures in epidermal cell walls (Fig. 2B). Interestingly, some cell wall swellings beneath primary penetration attempts were nonpenetrated as late as 48 h after inoculation. This indicates that these plant cell wall alteration may have a function in penetration resistance to *Bipolaris sorokiniana*. Moreover, a reduced frequency of these cell wall appositions in *I-mlo5* was linked to a higher frequency of successful infection sites with fungal hyphae in epidermal cells (data not shown). Thus, formation of pathogen-induced cell wall appositions (papilla) should be an effective resistance mechanism against both *Blumeria graminis* and *Bipolaris sorokiniana*. Interestingly, helminthosporol, one of the toxins produced by *Bipolaris sorokiniana*, affects the membrane physiology and is an inhibitor of plant 1,3-beta-glucan synthase activity that is involved in cell wall fortification (5). Therefore, helminthosporol may suppress formation of plant cell wall apposition and provoke membrane dysfunction leading to necrotic cell death. Successful suppression of cell wall fortification might be involved in the penetration strategy of *Bipolaris sorokiniana*.

Molecularly, a lack of the Mlo wild-type product leads to resistance to *Blumeria graminis*, whereas its presence limits susceptibility to *Bipolaris sorokiniana*. Our data are consistent with the hypothesis that Mlo is part of a mesophyll cell survival pathway (6). Cell survival may restrict the growth of necrotrophic fungi that kill host cells before they feed from them. This hypothesis is supported by the fact that *mlo*-genotypes undergo spontaneous cell death in late developmental stages, predominantly in mesophyll cells (37). In our study, a strong accumulation of H_2O_2 , a reactive oxygen intermediate (ROI), was found, especially in mesophyll cells that appeared to collapse (Fig. 2F). In contrast, subcellular DAB staining in epidermal cell wall appositions (Fig. 2A and B) and in localized regions on epidermal cells (Fig. 2E) was linked to interaction sites with limited fungal development. Whereas the former is probably an indication for deregulated cell physiology and overtaxed anti-oxidative capacities, the latter might point to controlled changes that restrict cellular accessibility for *Bipolaris sorokiniana*.

The role of H_2O_2 in cell death induced by toxins is supported by the finding that CF provokes accumulation of H_2O_2 in barley leaves that subsequently became necrotic (Figs. 1C and 5A and C). Because *mlo* genotypes were more sensitive to CF, a role of Mlo in negative cell death control is supported. Thus, enhanced sensitivity of *mlo*-barley to *Bipolaris sorokiniana* CF seems to be a result of the character of these genotypes as accelerated cell death mutants.

ROIs are thought to play a dual role in plant resistance to pathogens. On the one hand, they are involved in induction of several defense reactions, including pathogenesis-related gene expression, phytoalexin synthesis, and the hypersensitive reaction (HR). On

Fig. 5. H_2O_2 accumulation visualized by 3,3-diaminobenzidine (DAB) in three differentially reacting barley lines in response to toxin treatment or inoculation with *Bipolaris sorokiniana*. **A**, Differential responses of 7-day-old primary leaves of barley lines 36 h after injecting pure culture filtrate of *Bipolaris sorokiniana* (toxic necrosis depicted in Figure 1C is not visible at this time). **B and C**, Deterrence effect of ascorbate on H_2O_2 accumulation on primary leaf segments of barley line *I-mlo5* seen after 2 h of infiltration with a mixture of DAB plus indicated concentrations of ascorbate at 36 h after **B**, inoculation with conidia of *Bipolaris sorokiniana* and **C**, culture filtrate infiltration.

Fig. 6. Number of disease lesions associated with brown color indicating H_2O_2 accumulation and its deterrence by ascorbate (Fig. 5B). Segments of 7-day-old primary leaves of barley genotypes cv. Ingrid were inoculated with a suspension of 20,000 conidia of *Bipolaris sorokiniana* per milliliter and injected by 36 h after inoculation with 3,3-diaminobenzidine (DAB) (1 mg/ml) or a mixture of DAB with the indicated concentrations of ascorbate. Macroscopic evaluation was performed 2 h later. Each value is mean of five leaves. Vertical bars represent standard deviation of the mean.

the other hand, their accumulation may be involved in successful pathogenesis. Although the role of H_2O_2 in cell death induction is widely accepted (7,30), its benefit for resistance strategies varies with the type of pathosystem and host tissues. It is noteworthy that ROI accumulation is closely linked with resistance of barley against the powdery mildew fungus, whereas susceptibility is associated with a nonoxidative status of cells bearing functional haustoria (22). In contrast, ROI accumulation is an indicator of successful pathogenesis in other pathosystems (17,44,46).

We found H_2O_2 in cell wall swellings beneath appressoria (Fig. 2A) and in localized cell wall zones (Fig. 2E) to be spatially linked to restricted development of *Bipolaris sorokiniana*. Because fungal penetration attempts into epidermal cells were prevented at sites of H_2O_2 accumulation, we suggest that H_2O_2 is involved in inaccessibility of epidermal barley cells. A regular brownish DAB staining of epidermal and mesophyll cells, shown in Figure 2D (less intensive than staining in collapsed mesophyll cells shown in Figure 2F), was frequently observed in all genotypes. The same cells showed autofluorescence under UV light (data not shown). In the barley–powdery mildew interaction, whole cell autofluorescence is taken as an indication of cell death (27). Epidermal cell death is an effective defense feature against powdery mildew if it occurs as a HR. In the interaction of *Bipolaris sorokiniana* and barley, HR might either occur too late to stop the fungus completely, or alternatively, the necrotrophic fungus might not be affected by cell death at all (17).

In the mesophyll tissue, a strong H_2O_2 accumulation was detected in cells that appeared to collapse (Fig. 2F). In this particular case, H_2O_2 accumulation is likely to indicate loss of cellular control over oxidative processes, and thus, a chaotic reaction rather than a programmed cell death. The loss of control is probably provoked by fungal toxins.

Victorin, the host-selective toxin produced by *C. victoriae*, the causal agent of victoria blight of oats, induces chlorosis characterized by loss of chlorophyll and the large subunit of ribulose-1,6-bisphosphatocarbonylase (38). This may further provoke photo-oxidative stress supporting cell death (35). Accordingly, high light intensities support the formation of leaf spots caused by *Bipolaris sorokiniana* on barley (8). Therefore, intensively brown-stained chloroplasts in attacked mesophyll tissue (Fig. 2F) might indicate involvement of photo-oxidative stress in necrotic cell death induced by *Bipolaris sorokiniana* toxins.

Fig. 7. Number of leaf cells associated with brown color indicating H_2O_2 accumulation and its deterrence by ascorbate. Segments of 7-day-old primary leaves of the indicated barley line were inoculated with 20,000 conidia per ml of *Bipolaris sorokiniana*. They were further injected at 36 h after inoculation with 3,3-diaminobenzidine (DAB) (1 mg/ml of water) or a mixture of DAB with the indicated amounts of ascorbate. Each value is the mean of five leaf segments with at least 30 interaction sites each. Vertical bars represent standard deviation of the mean of the total number of stained cells.

The scavenger ascorbate inhibited the color reaction significantly, demonstrating that tissue browning was due to a chemical reaction involving polymerization of DAB in the presence of H_2O_2 . A gradual decrease of brown lesions (Figs. 5B and 6) occurred on elevating the concentration of ascorbate in the DAB solution. We also tested whether ascorbate influenced interaction phenotypes. After injection of ascorbate the number of disease lesions after conidia inoculation was reduced in a dose-dependent manner. Fifty millimolars ascorbate completely inhibited lesion formation (data not shown). Because high concentrations of ascorbate also inhibited growth of *Bipolaris sorokiniana* in axenic culture on potato dextrose agar (data not shown), it is difficult to draw final conclusions about the role of H_2O_2 in pathogenesis of the fungus. However, the fact that H_2O_2 scavenging by ascorbate reduced the symptoms elicited by fungal toxins (R. Hükelhoven, L. Király, and K.-H. Kogel, *unpublished data*) suggests that this ROI may play an important role in pathogenesis.

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