The use of *Cochliobolus sativus* culture filtrates to evaluate barley resistance to spot blotch

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Spot blotch (SB) caused by *Cochliobolus sativus* is a serious foliar disease that is particularly important in areas that are warm and moist during the barley growing season. In this study, the potential was assessed for using culture filtrates from potato-dextrose broth cultures of *C. sativus* in a fast, effective screening procedure for SB resistance in barley. The *C. sativus* culture filtrates were capable of eliciting SB symptoms on leaves as observed under field conditions, and no clear-cut distinction could be made between leaf symptoms produced by the culture filtrates and by the pathogen itself. Control plants treated with un-inoculated potato-dextrose broth or autoclaved water did not develop symptoms. A high correlation coefficient between the percentage of the leaf area infected (LAI) by *C. sativus* and the percentage of the necrotic leaf area (LAN) by the culture filtrate was observed ($r = 0.94$, $P < 0.001$). The established method using culture filtrates enables a fast assessment of the resistance of barley to SB and should be useful for many types of studies on this disease.

Keywords: *Bipolaris sorokiniana*, *Hordeum vulgare*, resistance test.

Cochliobolus sativus (Ito & Kurib.) Drechsler ex Dastur [anamorph: *Bipolaris sorokiniana* (Sacc.) Shoem.], the cause of spot blotch (SB), is a common foliar pathogen of barley, *Hordeum vulgare* L., a disease responsible for large economic losses in warm and moist areas in Canada and the Upper Midwest of the United States (Mathre 1990), Australia (Meldrum *et al.* 2000) and Syria (Arabi & Jawhar 2004).

The economic damage caused by SB can be avoided by using fungicides or by planting resistant genotypes, with the latter option being the more economically and environmentally appropriate solution (Gontariu & Enea 2012). Although effective, the currently used SB inoculation screening procedure (Ghazvini & Tekauz 2008) is laborious and time consuming. It permits too many escapes, is restricted to those geographic locations where the disease exists, and has slow symptoms expression. A rapid, dependable screening procedure that minimizes escapes would facilitate both commercial and research breeding programs. Additionally, under controlled conditions, large

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numbers of lines can be screened for their seedling reactions to the disease (Steffenson *et al.* 1996, Kumar *et al.* 2002).

Various fungal filtrates and/or toxins were used to screen plants for disease resistance (Fiume & Fiume 2003, Girish *et al.* 2009). *Cochliobolus sativus* can release phytotoxic compounds into the liquid medium which, upon infiltration into barley leaves, reproduces symptoms of SB disease (Gayad 1961, Olbe *et al.* 1995, Nakajima *et al.* 1998). A number of toxic metabolites have been isolated from culture filtrates of *B. sorokiniana* (Pringle 1977, Briquet *et al.* 1998). However, although these researchers demonstrated that filtrates from *C. sativus* cultures were biologically active, they did not develop systems adapted to large-scale mass-selection breeding programs. To be meaningful, the production of these toxic materials must be related to disease susceptibility of the barley and virulence of the pathogen producing them. The aim of our study was to determine if barley responses to culture filtrates of *C. sativus* could be used as a rapid, reliable assay for SB resistance.

Materials and methods

Host genotypes

The three different genotypes (WI2291, Golf and Bowman) of barley used in this study were chosen for their differential reactions to SB (Arabi & Jawhar 2003, 2007). Bowman (resistant) is an American differential genotype known to be useful for differentiating *C. sativus* virulence types (Valjavec-Gratian & Steffenson 1997). Golf (moderately resistant) is a British genotype. The universal susceptible control (cv. WI2291) from Australia was included in the experiments (Hetzler *et al.* 1990).

Inoculum preparation

After an extensive screening for over ten years in the greenhouse and in the laboratory the *C. sativus* isolate (Pt4) was proved to be the most virulent isolate to all barley genotypes available so far (Arabi & Jawhar 2003, 2007). Therefore, it was selected in this study. It was isolated from infected barley leaves showing SB symptoms and grown separately in 9-cm Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) and incubated for 10 days, at 21 ± 1 °C. Single spore cultures were established and maintained on PDA.

Inoculum disks (9-mm-diameter) were cut with a cork borer and transferred to 250-ml Erlenmeyer flasks containing 150 mL of potato dextrose broth (PDB) amended with sucrose (20 g/L) and incubated on rotary shakers at 90 rpm and 22 °C for 25 days in the dark. The inoculum disk was placed on the bent end of a glass rod to enable the flotation of the growing fungal colony. The broth cultures were then filter-sterilized by passing them, under vacuum, through filter paper (Whatman No. 541). The filtrate and PDB were used fresh or after storage at -10 °C. Control treatments were autoclaved tap water and PDB shaken and filtered in the same manner as the filtrate. In a preliminary study, five different series of filtrate dilutions were tested and

the ratio 80 % (vol/vol; filtrate/distilled water) was selected in this study on the basis of physiological criteria (virulence). Aliquots (8 ml) of the cultural filtrate were tested after 7, 15 and 25 days.

Seedling assays in growth cabinets

Barley seeds were surface-sterilized with 5 % sodium hypochlorite (NaOCL) for 5 min and then washed three times in sterile distilled water. They were planted in pots filled with sterilized peatmoss, and arranged in a randomized complete block design with five replicates. A full replicate consisted of the 5 pots of each genotype. Pots were placed in a growth chamber at temperatures 22 ± 1 °C (day) and 17 ± 1 °C (night) with a daylength of 12 h and a relative humidity of 80–90 %. Plants were inoculated at growth stage (GS) 12 (Zadoks *et al*. 1974) by uniformly spraying each plant with 0.2 mL of culture filtrate dilution with a hand-held spray bottle. Plants were then placed in the dark at 95–100 % R.H. for the first 18 h.

On the other hand, the conidial suspension was adjusted to 2×10^4 conidia/mL using hemacytometer counts of conidia to provide estimates of the inoculum concentration. A surface active agent (polyoxyethylene-20-sorbitan monolaurate) was added (100 µL/L) to the conidial suspension to facilitate dispersion of the inoculum over the leaf surfaces. A similar experimental design was performed as mentioned above. Non-inoculated control plants were infiltrated with potato dextrose broth and distilled water.

At 10 days after inoculation–infiltration, three leaves showing the highest leaf area infected (LAI) by the pathogen (as estimated by visual observation) and another three showing the highest necrotic leaf area (LAN) by the culture filtrate per plant genotype were detached from the plants, and the areas of the visible LAI/LAN were traced on thin transparent plastic sheets with black ink.

Disease assessment

The percentage of the leaf area infected (LAI) by *C. sativus* and the percentage of the necrotic leaf area (LAN) by the culture filtrate in both methods using a numerical scale of 0 to 4, where 0 (symptoms free), 1 (lesions covering 10–25 % of leaf infected area), 2 (lesions covering 25–50 %), 3 (lesions covering 50–75 %) and 4 (lesions covering 75–100 % of leaf infected area) were noted. Results were subjected to an analysis of variance (Anonymous 1996) using the super ANOVA computer package to determine whether there was a significant test × genotype interaction. The correlations coefficient between the LAI by *C. sativus* and the LAN by the culture filtrate was calculated with the SAS General Linear Models Procedure (SAS Institute, Cary, NC, USA).

Results and discussion

The first signs of uptake of toxic metabolites by barley tissues, characterized by a solid dark brown necrotic lesion were observed 48 h after spray-

ing with 80 % filtrate solutions. Mature lesions that appeared about 72 h postinoculation typically were black brown with expanding chlorosis (the "classic" spot blotch lesion). Non-inoculated culture medium had no effect on barley tissue suggesting that the symptoms on inoculated leaves were caused by fungal metabolites (data not shown). The responses of barley genotypes to *C. sativus* filtrates were similar to those observed in preliminary greenhouse and field trials (Arabi & Jawhar 2003, 2004).

Tab. 1. Symptoms severity scores and percentage of plants rated with culture-filtrate and the percentage of plants rated by inoculation with *Cochliobolus sativus* on barley

Genotype	Source	Symptoms severity score*	% SB severity	
			Culture -filtrate assay**	Conidial suspension
Golf	England	2.0	38.4	29.1
WI 2291	Australia	4.0	89.9	93.5
Bowman	USA	1.0	16.1	17.3
LSD		0.41	0.5	0.37

* Based on four-point severity scale where 1 = lowest and 4 = highest.
** Mean of SB severity of three barley genotypes challenged with a 80 % dilution of filtrate

Tab. 2. Reaction of barley seedlings to filtrates from *Cochliobolus sativus* of different ages

Treatment	Barley genotypes		
	Golf	WI2291	Bowman
Water	0.0	0.0	0.0
PDB	0.0	0.0	0.0
Culture filtrate (7 days)	15.2	24.6	1.2
Culture filtrate (15 days)	33.5	66.8	8.6
Culture filtrate (25 days)	57.3	94.3	19.6
LSD (5 %)			

The reactions of the three barley genotypes tested for SB resistance for both culture filtrate and *C. sativus* are reported in Tab. 1. However, differences in severity levels were detected among the different genotypes, with the severity values being consistently higher in the susceptible genotypes in both experiments (Tab. 1). Inoculation with the *C. sativus* virulent pathotype Pt4 increased the SB severity on the susceptible genotypes in culture filtrate and conidial suspension experiments. Both methods showed that WI 2291 was susceptible; Golf was moderately susceptible, whereas, Bowman was resistant (Tab. 1). The ratings of genotypes shown to be either resistant or susceptible to SB were quite consistent among different tests. The experiments were repeated five times and showed similar outcomes. Therefore, the results of one experiment are presented here (Tab. 1).

On the other hand, the age of *C. sativus* cultures was important (Tab. 2). Filtrates obtained after 15 and 25 days of fungal growth caused very severe

symptoms on seedling leaves of the susceptible cv. WI2291. Control plants treated with un-inoculated potato-dextrose broth or autoclaved water did not develop symptoms (Tab. 2). A high correlation coefficient between the LAI and the LAN was observed (r = 0.94, P < 0.001) (Fig. 1). Similar results have been found on cotton (Mehta & Brogin 2000) and tomato (Fiume & Fiume 2003).

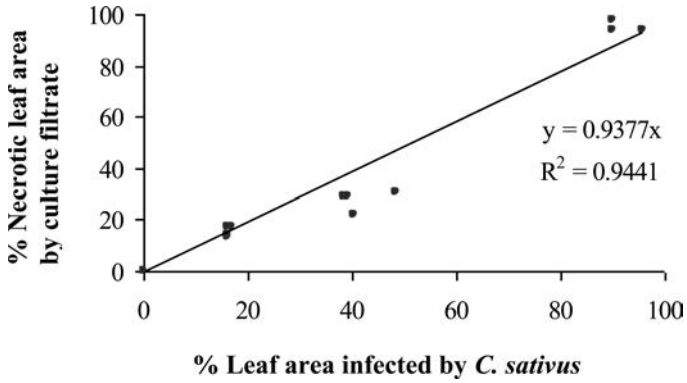

Fig. 1. Relationship between percentage of leaf area infected by *Cochliobolus sativus* and percentage of necrotic leaf area by its culture filtrate of seedling plants of three barley genotypes.

The results show that culture filtrates have potential for use in screening barley for resistance to SB. Susceptible barley genotypes developed symptoms typical for SB infected barley in the field (Arabi & Jawhar 2004). This supports the assumption that the culture filtrate is challenging the same host systems as those challenged by fungal inoculation. The method yielded promising results for screening resistant plants to SB by exposing barley seedlings to 80 % culture filtrates from 25-day-old *C. sativus* cultures. Hence, it seems to be a valid and rapid laboratory test for evaluating the response of barley plants to SB. Further research is needed to isolate and identify structurally the toxic metabolites produced by *C. sativus in vitro*, since the chemical characterization can contribute to an understanding of the physiological role of a toxin (Kumar *et al*. 2002).

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