An Improved Technique for Determining Net Blotch Resistance in Barley

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ABSTRACT

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An agar leaf disk technique is described for determining net blotch resistance in barley. Leaves of barley were collected and disinfested with different methods using alcohol and sodium hypochlorite. Twelve disinfested leaf disks were placed on 1.5% water agar in petri dishes with 0, 80, or 120 μg/ml of benzimidazole. Disinfestation of leaf disks with 5% sodium hypochlorite for 1 min, followed by three rinses (5 min) in sterile distilled water and placement on 1.5% water agar containing 80 μg/ml of benzimidazole, was considered the best treatment. This method was used to determine the genetic variability for net blotch resistance in five barley cultivars and 20 barley doubled haploids, including their two parents. Results were correlated (0.90–0.96, $P \leq 0.05$) with those from experiments with seedlings or adult plants.

Additional keywords: *Hordeum vulgare*

Net blotch is a common disease of barley (*Hordeum vulgare* L.), caused by *Drechslera teres* (Sacc.) Shoemaker (teleomorph = *Pyrenophora teres* Drechs.). The pathogen has a worldwide distribution and occurs wherever the crop is grown in temperate humid regions (12). In France, two forms of *D. teres* have been reported—*D. t.* f. sp. *teres* Snedegaard-Petersen and *D. t.* f. sp. *maculata* Snedegaard-Petersen, which differ in the symptoms incited on barley (3). The latter form is responsible for polymorphic symptoms that can be confused with other pathogens (5). Most of the barley cultivars are susceptible or very susceptible to this pathogen (1). The importance of straw in the survival and dissemination of the pathogen and its role in the development of the disease is also reported (4,9,13).

Fungicides can considerably reduce the incidence of net blotch, but the environmental and ecological problems caused by the use of fungicides have increased the interest in resistant cultivars. Reliable laboratory screening techniques can increase the effectiveness in breeding programs by reducing the expense of field testing. A technique for determining the resistance of poplar clones to *Marssonina* sp. using leaf disks inserted into plates of 2% water agar has been described (15). Similarly, a significant relationship between detached-leaf and whole-plant reaction to *D. teres* has been reported (11). The present study was initiated in an attempt to develop a technique to rapidly screen barley genotypes for resistance to *D. teres* with the leaf disk technique and to define the relationship between the results from agar leaf disks to those obtained in growth chamber and field experiments.

MATERIALS AND METHODS

Agar leaf disk test. In order to determine the best conditions for an agar leaf disk technique, leaves of the barley pure line 74F6 were collected from the field and disinfested with different treatments

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Table 1. The effect of leaf disinfestation method, concentration of benzimidazole, and days of incubation (5 or 8) in two experiments (I and II) on percentage of green area of barley leaf disks

Leaf disinfectant	Time	I						II						Presence of saprophytes
		5 days			8 days			5 days			8 days			
		0[x]	80	120	0	80	120	0	80	120	0	80	120	
Water	5 min	7.9 i[y]	14.2 i	32.1 fgh	7.6 f	13.0 f	24.2 e	8.0 e	14.7 e	26.4 d	7.4 e	13.4 e	23.9 d	***[z]
Alcohol 75 C	10 sec	34.8 e–h	47.8 cde	47.4 cde	31.5 de	49.8 bc	48.3 bc	41.1 cd	57.1 b	56.2 b	31.9 cd	49.9 ab	49.2 ab	**
Alcohol 95 C	10 sec	27.3 h	56.7 bcd	54.0 bcd	28.5 de	55.0 abc	48.1 bc	31.2 d	66.5 ab	55.8 b	28.3 cd	55.5 ab	48.4 b	0
NaOCl 1%	1 min	34.9 e–h	59.3 bc	54.0 bcd	32.9 de	55.9 ab	51.1 abc	37.1 c	65.7 ab	59.9 b	33.4 cd	56.2 ab	51.6 ab	*
NaOCl 1%	5 min	31.2 gh	52.9 bcd	58.9 bc	32.4 de	52.2 abc	53.6 abc	34.9 d	60.5 b	67.7 ab	31.4 cd	52.2 ab	57.2 ab	*
NaOCl 5%	1 min	41.7 d–g	71.8 a	64.2 ab	36.5 d	61.5 a	58.5 ab	39.9 cd	77.5 a	65.6 ab	35.8 c	61.6 a	56.0 ab	0
NaOCl 5%	5 min	26.5 h	49.8 bcd	45.4 c–f	28.9 de	51.5 abc	44.6 c	30.6 d	56.4 b	51.4 bc	27.8 cd	49.4 ab	45.3 b	0

[x] 0, 80, 120 = benzimidazole concentration (μg/ml).
[y] Values within an experiment and day followed by different letters differ at 5% level of significance (Newman-Keuls test). Percentage transformed using angular arcsine.
[z] 0, *, **, *** = None, few, moderate, and many saprophytes, respectively.

Table 2. Net blotch disease severity[v] of five barley genotypes using three different methods of assessment

Genotype	Source	Seedling[w]	Adult plant[x]	Leaf disk[y]
Thibaut	France	6.8 a[z]	6.1 a	6.2 a
74F6	France	6.4 a	5.1 b	4.7 b
79-SIO-16	Syria	4.6 b	4.5 c	5.0 b
Arrivate	Iraq	2.3 c	2.0 d	3.5 c
79-SIO-10	Syria	2.3 c	2.0 d	3.3 c

[v] Severity rated on a 1-9 scale where 1 = 0–2.5, 2 = 2.6–5, 3 = 5.1–10, 4 = 11–20, 5 = 21–30, 6 = 31–40, 7 = 41–50, 8 = 51–75, and 9 = 76–100% affected leaf area.
[w] Seedlings at two-leaf stage inoculated with mycelial fragments and scored 8 days later.
[x] Field-grown plants with inoculum coming from infested crop residue and a suspension of mycelial fragments.
[y] Disks from the penultimate leaves of field-grown plants and inoculated with mycelial disks.
[z] Values followed by different letters differ at the 5% level of significance (Newman-Keuls test). Correlation coefficient (r) between leaf disk and seedling = 0.90 ($P = 0.05$), and leaf disk and adult plant = 0.96 ($P = 0.01$).

Table 3. Net blotch reactions of two barley cultivars and their 20 doubled haploids (DH) using two methods of assessment

Genotype	Seedling[x]	Leaf disk[y]
CI XG -DH 13	7.0 a[z]	7.4 a
CI XG -DH 6	6.8 a	7.7 a
CI XG -DH 14	6.5 ab	7.4 a
CI XG -DH 10	5.3 abc	6.4 ab
Golf (G)	5.2 abc	6.6 ab
CI XG -DH 12	5.2 abc	6.4 ab
CI XG -DH 15	5.2 abc	6.0 ab
CI XG -DH 16	5.0 abc	6.3 ab
CI XG -DH 5	4.8 abc	6.0 ab
CI XG -DH 9	4.7 abc	5.9 ab
CI XG -DH 11	4.3 bcd	5.5 ab
CI XG -DH 7	3.8 cde	4.9 bc
CI-5791	2.6 def	3.0 cd
CI XG -DH 8	2.5 def	3.0 cd
CI XG -DH 3	2.3 def	3.2 cd
CI XG -DH 20	2.2 def	3.1 cd
CI XG -DH 2	2.2 def	3.2 cd
CI XG -DH 1	2.1 def	3.8 cd
CI XG -DH 4	2.0 def	3.0 cd
CI XG -DH 17	2.0 def	3.0 cd
CI XG -DH 18	1.7 ef	2.6 d
CI XG -DH 19	1.2 f	2.3 d

[x] Seedlings at two-leaf stage inoculated with mycelial fragments and scored 8 days later.
[y] Disks from the penultimate leaves of field-grown plants and inoculated with mycelial disks.
[z] Values followed by different letters differ at 5% level of significance (Newman-Keuls test). Correlation coefficient (r) between leaf disk and seedling rating is 0.94 ($P = 0.01$).

of alcohol (95% ethanol) or sodium hypochlorite (Table 1), then steeped three times (5 min) in sterile distilled water. Treatments included alcohol at 70 or 90 C for 10 sec, 1% NaOCl for 1 or 5 min, and 5% NaOCl for 1 or 5 min. Untreated leaves were used as a check.

Twelve leaf disks (14 mm diameter) were punched from the leaves with a cork borer and placed adaxial surface up into a plastic petri dish of 1.5% water agar, containing 0, 80, or 120 μg/ml of benzimidazole. Agar in plates was 6 mm thick and poured 2 days before testing. All petri dishes were placed in a growth chamber where temperatures were 23 ± 0.5 C (day) and 18 ± 0.5 C (night) with a day length of either 16 or 12 hr. Light intensity was 90 $\mu E \cdot m^{-2} \cdot s^{-1}$. The host reaction, as percentage of green area of barley leaf disks, was noted 5 and 8 days later. A randomized block design with three replications was used. Each replication consisted of a petri dish with 12 leaf disks. The experiment was conduced twice.

Genotypes. Five barley genotypes (Table 2) and 20 doubled haploids (Table 3), including their parents, were selected and exhibited low to high degrees of resistance to net blotch in our preliminary studies. Genotypes 79-SIO-10 and 79-SIO-16 came from the International Center of Agricultural Research in Dry Areas in Syria (ICARDA). Arrivate is an Iraqi cultivar and Thibaut is a French commercial barley cultivar. Genotype 74F6 is a pure line derived from an intervarietal cross carried out in our plant breeding department. The 20 doubled haploids were obtained through a cross between the resistant line CI-5791 and Golf, a susceptible cultivar.

Seedling test. Two experiments were conducted in flats (60 × 40 × 8 cm) filled with vermiculite using a randomized complete block design with five replications. Each replication included one row of 12 seedlings per genotype. The first experiment included five genotypes, and the second one had all doubled haploids and their parents. Seedlings were irrigated with Knop's nutrient solution (1 g of $NaNO_3$, 0.25 g of KNO_3, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.25 g of KH_2PO_4, and a trace of $FeCl_3$ in 1 L of water). Temperatures were similar to the agar leaf disk tests. Light intensity was 90 $\mu E \cdot m^{-2} \cdot s^{-1}$ with a day length of 12 hr and relative humidity (RH) was 85–95%. A monoconidial isolate of *D. t. maculata* (S6-2), obtained from local cultivars, was grown for 8 days in 10% V8 agar at 23 C under continuous darkness, which promotes mycelial growth. The mycelium was ground in water with a blender for 1 min to produce 3×10^3 mycelial fragments per milliliter. Gelatin (0.25%) and the sticker Triton X-100 (one drop per 100 ml) were added.

Inoculations were performed by spraying 14-day-old seedlings at the two-leaf stage with 25 ml of the suspension of mycelial fragments. At this stage, the first leaf has stopped growing and the second extends beyond the first (14). Each flat was subsequently placed in a transparent enclosure and sprayed with distilled water to maintain saturated humidity for 48 hr. The covers were then removed. Eight days after inoculation, the approximate percentage of leaf area damaged (chlorosis and necrosis) on the first leaves of all plants was assessed.

Field experiment. Inoculated seedlings

Table 4. Sums of squares for two experiments (I and II) on the effect of leaf disinfestation, concentration of benzimidazole, day length, and days of incubation (5 or 8) on green area of barley leaf disks

Source of variation	I		II	
	5 days	8 days	5 days	8 days
Leaf disinfectant (D)	2,876.4**[z]	2,621.1**	3,821.4**	2,616.7**
Benzimidazole concentration (B)	6,433.2**	5,279.9**	8,088.6**	5,536.8**
Day length (L)	224.9 NS	50.2 NS	22.2 NS	63.1 NS
D × B	181.6**	95.8**	228.1**	101.1*
D × L	70.7 NS	54.2 NS	77.3 NS	66.4 NS
B × L	0.8 NS	30.8 NS	82.0 NS	60.2 NS
D × B × L	34.3 NS	33.6 NS	75.1 NS	39.6 NS
Error	68.5	34.2	89.0	45.74

[z] * And ** indicate significance at $P = 0.05$ and $P = 0.01$, respectively. NS = not significant.

of all five genotypes from the seedling experiment were transplanted in the field after scoring and arranged in a randomized block design with three replications. Each replication consisted of a 1.5-m row of 12 plants. The soil surrounding the seedlings was covered with naturally infested crop residues (50 g/m^2). Seedlings were then enclosed in a clear polyethylene plastic cover for 20 days to protect them and encourage infection. At growth stage 39, when the flag leaf appeared (16), the plants were sprayed with a suspension of mycelium (10 ml per plant) prepared as above. Plants were scored 14 days after inoculation on the three upper leaves.

Leaf disk inoculation assays. An experiment with all five genotypes and another with doubled haploids and their parents were conducted in the field by sowing seeds in a randomized block with three replications. Each replication consisted of 12 plants in a 1.5-m row. At growth stages 22, 32, and 35 (16), plants were protected from all diseases by application of a 0.1% solution of Bayfidan D (250 g/L of triadimenol + 480 g/L of anilazine).

The second fully expanded leaf from the top of the main tiller of each plant for each genotype and replication was cut and immersed in 5% sodium hypochlorite solution for 1 min followed by three rinses (5 min) in sterile distilled water. Twelve disks from separate plants for each replication were placed in sterile plastic petri dishes with 1.5% water agar and 80 μg/ml of benzimidazole. From previous results, this treatment was chosen for the agar leaf disk inoculations. A mycelial disk (2.5 mm) of the same monoconidial isolate used in the seedling test was placed on the center of each of 10 leaf disks. Drops of sterile distilled water were similarly placed on two other disks for controls. Five drops were distributed on the surface of the agar of each petri dish to help maintain the moist environment necessary for symptom development. All petri dishes were placed in a growth chamber under the same conditions used for the agar leaf disk technique described earlier with a 16-hr photoperiod. Symptom development was scored 5 days later.

Measurements and analysis. In the two agar leaf disk experiments, the percentage of leaf area that remained green for the duration of the experimental period (5 and 8 days) was transformed with the angular arcsine transformation for a normal distribution of data (6). In all five genotype resistance experiments, the percentage of leaf area exhibiting disease symptoms was visually determined and transformed to a 1–9 scale where 1 = 0–2.5, 2 = 2.6–5, 3 = 5.1–10, 4 = 11–20, 5 = 21–30, 6 = 31–40, 7 = 41–50, 8 = 51–75, and 9 = 76–100% of the leaf area affected (chlorosis + necrosis + water soaking). With this method, no further transformation was necessary (5). The statistical analysis of the two agar leaf disk tests for 5 and 8 days were done separately. For each duration, the effect of the photoperiods (12 and 16 hr), the medium, and their interactions were determined. Analyses of variance for a randomized block design were conducted for all five genotype resistance experiments.

RESULTS AND DISCUSSION

Agar leaf disk test. Statistical analysis of results of different disinfectant agents and concentrations of benzimidazole are summarized in Table 4. The effect of leaf disinfectant, benzimidazole, and their interactions were significant at both rating times. There was no difference between photoperiods (12 and 16 hr), and the other interactions were not significant. Disinfestation of leaf disks with 5% NaOCl for 5 min and alcohol at 95 C eliminated the saprophytes, but with these treatments, the levels of chlorosis were relatively severe (Table 1). Benzimidazole concentrations also showed a highly significant effect in keeping the leaf disks green during the experiment. From our results, the following treatment is recommended: leaf disk disinfection with 5% sodium hypochlorite solution for 1 min, followed by three rinses (5 min each) in sterile distilled water and placement in sterile petri dishes on 1.5% water agar containing 80 μg/ml of benzimidazole.

Resistance to net blotch. Genetic variability for net blotch resistance in five genotypes and 20 doubled haploids and their two parents are presented in Tables 2 and 3, respectively. Rankings observed in scores using leaf disks were similar to those using seedling and whole plants. In the first experiment, Arrivate and 79-SIO-10 were the most resistant genotypes in all assays, whereas Thibaut and 74F6 were the most susceptible. Some doubled haploids were as resistant as the resistant parent (CI-5791) and others were as susceptible as Golf (Table 3). The effect of postinoculation high temperature (36 C) causing resistance breakdown has been reported (10). The relatively low and rather similar temperature of the field and growth chamber may be why the results concur with our two genotype resistance experiments.

Detached leaves were successfully used to determine the reaction of barley cultivars to *D. teres* (7). Our data confirm those results with significant correlations between leaf disk and two other methods (Tables 2 and 3). We have also applied the leaf disk technique successfully in a 9 × 9 complete diallele genetic study (2). Our overall results show that the agar leaf disk method, as described, should be considered as a rapid and relatively inexpensive technique for resistance investigations. It also overcomes the disadvantages of saprophytes associated with nondisinfested leaf disks (15) and movement of host material associated with floating leaf disks (8).

In our method, leaf disks are surface-sterilized, which eliminates most of the foliar saprophytes. The constant volume of inoculum (2.5-mm mycelial disk) and the known leaf area used allow precise evaluation of variation in the degree of resistance.

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