A Rapid Detection Procedure for the *HtN* Gene Under Controlled Inoculation of Maize with *Exserohilum turcicum*

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ABSTRACT

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A technique for rapid and early detection of the *HtN* gene for resistance against *Exserohilum turcicum*, the cause of northern leaf blight, in maize (*Zea mays*) is based on quantitative inoculation (by air) with high inoculum pressure (100,000 and 200,000 conidia per square centimeter) on a leaf target, immediately followed by a long wetness period (48 hr). Under such optimal conditions for disease development, plants could be termed resistant or susceptible to *E. turcicum* as early as the five-leaf stage and 4-10 days after inoculation. In contrast to the commonly used drop inoculation, the technique suggested here does not allow the "escape" of susceptible plants as resistant. Currently, this technique is being used in our breeding program. It enables us to make practical use of the important *HtN* gene as a source for resistance to *E. turcicum*.

Northern leaf blight (NLB), caused by the fungus *Exserohilum turcicum* (Pass.) K.J. Leonard & E.G. Suggs (*Helminthosporium turcicum* Pass.), is one of the most widespread and destructive pathogens of corn (*Zea mays* L.) (6). Monogenic resistance has been identified in maize against four known *E. turcicum* biotypes (4,5,9,11,18,19). Additional sources of partial resistance have recently been identified in sweet corn germ plasm (15). A single gene for resistance, *HtN*, which has been identified in the Mexican variety Pepitilla, was shown to be an effective source of resistance to *E. turcicum* (2). The *HtN* gene confers resistance to three biotypes of *E. turcicum* and is expressed as the absence of lesions (4) or delayed lesion development (6,16). This gene, however, has been impractical in a backcross breeding program, because the disease's prolonged incubation period of up to 60 days (16) required full-season field tests for the detection of resistance. A partial solution to this problem was reported in 1983 by Leath and Pedersen (11), who suggested an inoculation technique for the rapid detection of the *HtN* gene in inbred lines of maize under greenhouse conditions (10). This method, however, allowed the escape of a few plants that remained lesion free although they did not carry the *HtN* gene. In addition, recent reports suggested that environmental conditions influence the expression of resistance to NLB, primarily resistance conferred by

HtN (12,19,20). In practical terms this means that reliable screening for resistance requires a method of inoculation under which the virulence of *E. turcicum* can be consistently assessed.

This report presents a technique for the rapid and early detection of the *HtN* gene under quantitative inoculation. The method is based on 1) a high inoculum pressure, provided by air pressure, of conidia on a leaf target area and 2) a long wetness period to provide favorable conditions for symptom development. We have used this method in our station since 1986 to select for *HtN* in thousands of genotypes derived from crosses and backcrosses (F_1–F_6) both homozygous and heterozygous for it. To introduce the method, this paper describes the resistance or susceptibility reactions of three populations representing resistant and susceptible parents and their F_1 generation.

MATERIALS AND METHODS

Plants. The resistant inbred line B73*HtN*, the susceptible inbred line IL772a, and their F_1 generation were grown in the greenhouse (15–28 C) in plastic pots containing 500 g of a mixture of peat and tuff (volcanic ash, Scoria)(1:1 v/v). Once a week plants were watered to saturation with a 0.1% 20-20-20 (N-P-K) fertilizer solution.

Pathogen and inoculation. Because only one local isolate of *E. turcicum* was previously identified in Israel as race 0 (1), this isolate was maintained continuously on maize plants (cultivar Jubilee) in a greenhouse. Isolates were collected at random from various locations to represent possible ecological races of *E. turcicum* (13). Inoculum was obtained from sporulating infected leaves on 5–8-wk-old plants, 8–10 days after inocu-

lation. Conidia were gently brushed into a small quantity of water containing two drops of Tween 20 and adjusted to a known concentration of conidia per milliliter of H_2O with the aid of a hemocytometer. Plants were inoculated at the five-leaf stage with a suspension containing 1×10^5 or 2×10^5 conidia per milliliter.

The fourth leaf from the bottom of each plant was inoculated by either the leaf target area (LTA) method or by the common method of applying drops of a conidial suspension on the leaves. For the first method, the upper surface of the leaf was inoculated by a modified air gun quantitative inoculator (17) developed by J. Rotem (ARO, the Volcani Center, Israel, *unpublished*) for epidemiological uses. A specific number of conidia per unit of time are carried by a spray from a uniform suspension. Compressed air passes through a solenoid valve which is operated by a timer allowing air at known pressure to pass through the spraying system. The spray is directed through a funnel (30 cm in diameter) onto a specific LTA. Inoculum was applied to a target area of approximately 4.5 cm in the middle of a leaf. The air pressure and timer were adjusted to spray 0.1 ml of suspension, providing 100,000 or 200,000 conidia (when conidial concentrations were of 1×10^5 and 2×10^5 conidia per milliliter, respectively) per 1 cm^2 of leaf area. For drop inoculation, each drop of 10 μl provided 100,000 conidia when the concentration was 1×10^5 conidia per milliliter. Ten drops of inoculum were placed on the upper surface of each leaf. After inoculation, leaves were sprayed lightly with water and kept in a Percival dew chamber at 100% RH, at 20 C, for wetness periods of 12, 24, and 48 hr. Plants were then moved to a growth chamber (25 C, 100 μmol·m^{-2}·s^{-1}, 16 hr of light per day). On 5, 7, and 10 days after inoculation the size of the lesions was recorded and scaled as follows: 0 = no lesions; 1 = 0-3 mm; 2 = 4-10 mm; 3 = 10+ mm; and 4 = dead leaf. The number of lesions produced per each inoculated LTA was expressed as disease incidence and rated as follows: 0 = no symptoms; 1 = 1-3 lesions; 2 = 4-10 lesions; 3 = 10+ lesions; and 4 = dead leaf. The experiment was repeated three times and included 10 to 12 plants per treatment.

To confirm their resistance reaction under greenhouse conditions, the inocu-

lated plants were transplanted after 3 wk into 3 kg pots, and their new leaves were inoculated. The leaf whorl of each test plant was sprayed with a 5-ml conidial suspension (50,000/ml) and kept in a wet plastic bag for 24 hr. Lesion size and disease incidence were recorded as described.

RESULTS

The results of our inoculation technique indicate that the *HtN* gene can be distinguished as early as 5–10 days after inoculation (Figs. 1 and 2). A rapid response to the inoculation was detected in all three wetness periods (12, 24, and 48 hr) in both droplet (Figs. 1 and 2A, D, and G) and LTA methods (Figs. 1 and 2B, C, E, F, H, and I), regardless of the level of resistance to *E. turcicum*. However, only the susceptible inbred line IL772a, inoculated by either LTA or droplet method followed by 48 hr of wetness, showed large symptoms, high disease incidence, and eventual death of the leaves 9 days after inoculation (Figs. 1 and 2D–F). When plants were inoculated with 100,000 conidia per milliliter by droplet (100,000 conidia per drop) or

LTA (100,000/cm²), only the resistant plants showed small lesions and low disease incidence after 48 hr of wetness (Figs. 1 and 2A, B, G, and H). The higher concentration of 200,000 conidia per milliliter caused greater lesion size and disease incidence in resistant plants; yet the results were still remarkably lower than for the susceptible IL772a (Figs. 1 and 2C, F, and I). Resistance was confirmed by reinoculation of the resistant B73*HtN* and IL772a × B73*HtN* plants under greenhouse conditions (Fig. 3). Disease severity remained significantly lower on the resistant plants than on the susceptible IL772a, on which typical large symptoms developed, for up to 60 days.

DISCUSSION

The *HtN* gene might be used as an effective source of resistance to *E. turcicum*. However, the prolonged incubation period, which requires the use of full-season field tests, is a limiting factor in utilizing this gene (6,10). The effects of environmental conditions on the expression of this pathogen cause additional reliability problems in screening for re-

sistance (12,19,20). The use of the LTA method for quantitative inoculation in a controlled environment enabled us to rapidly and reliably detect the *HtN* gene in maize. Our results indicate that plants susceptible or resistant to *E. turcicum* could be distinguished based on disease rating as early as the five-leaf stage.

The general tendency of corn toward increased susceptibility to necrotrophic fungal foliar pathogens at reduced light intensity has been reported for *E. turcicum* (12); southern leaf blight, caused by *Bipolaris maydis* (Nisikado & Miyake) Shoemaker (8); and *Colletotrichum graminicola* (Ces.) G.W. Wils. (21). For *E. turcicum*, low temperatures (22/18 C day/night) during the first 48–72 hr after inoculation enable the fungus to penetrate slowly through the leaf mesophyll and parenchyma of the susceptible plants (3,7). An increase in lesion numbers of *E. turcicum* was observed with lengthening wetness periods (from 5 to 12 hr) and with increasing inoculation concentration (from 1,500 to 10,000 conidia per milliliter) sprayed on plants (14). Young plants were found to be more susceptible than older ones to infection by *E. turci-*
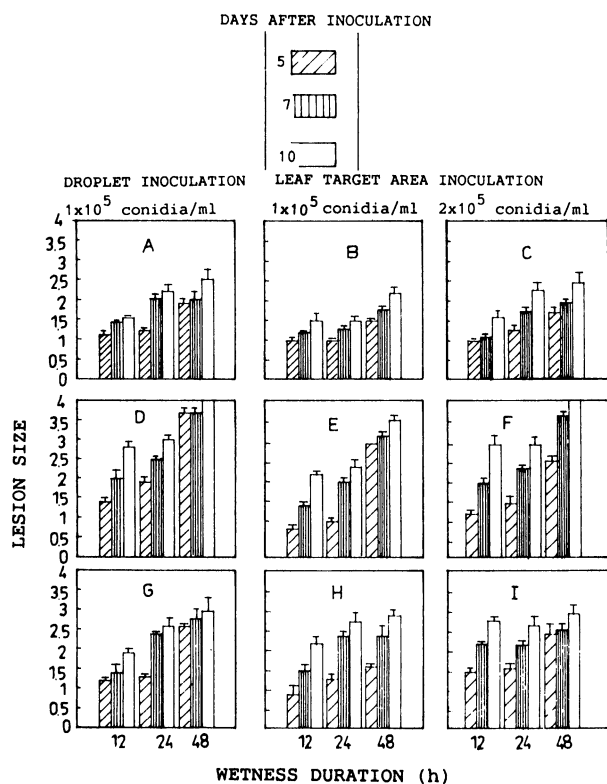

Fig. 1. The effect of conidial concentration, method of inoculation, and length of wetness period on lesion development of *Exserohilum turcicum* on susceptible or resistant maize (*Zea mays*). (A), (B), and (C) = IL772a × B73*HtN*, the F₁ generation of a cross between a susceptible and a resistant parent; (D), (E), and (F) = IL772a, the susceptible line; and (G), (H), and (I) = B73*HtN*, the resistant line. Leaf target area inoculations were made with an air gun. Lesion size was scaled as follows: 1 = 0–3 mm; 2 = 3–10 mm; 3 = 10+ mm; and 4 = dead leaf. The experiment was repeated three times. Values are the mean of 10–12 plants for each treatment. Bars represent standard errors.
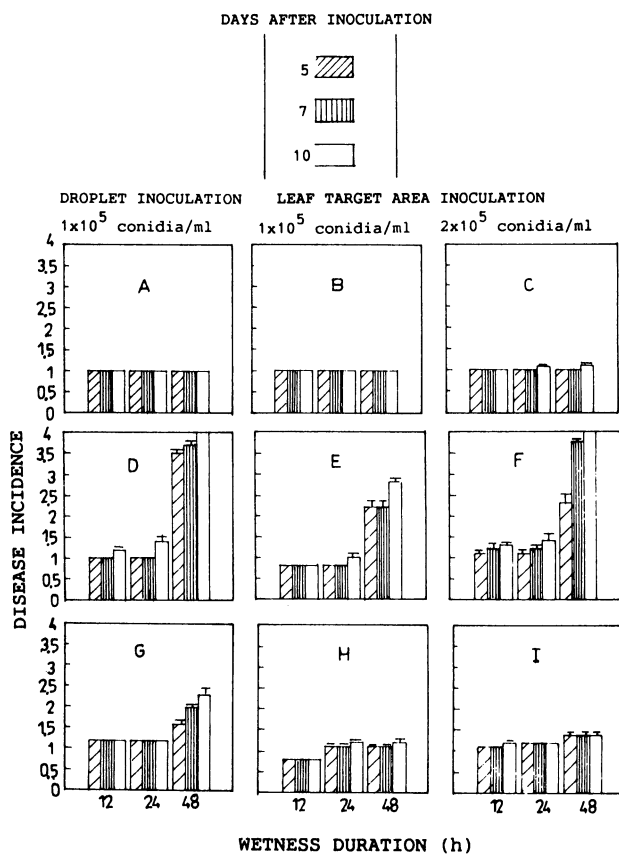

Fig. 2. The effect of conidial concentration, method of inoculation, and wetness period on disease incidence of northern leaf blight (*Exserohilum turcicum*) on susceptible or resistant maize (*Zea mays*). (A), (B), and (C) = IL772a × B73*HtN*, the F₁ generation of a cross between a susceptible and a resistant parent; (D), (E), and (F) = IL772a, the susceptible line; and (G), (H), and (I) = B73*HtN*, the resistant line. Leaf target area inoculations were made with an air gun. Disease incidence was rated as follows: 0 = no symptoms; 1 = 1–3 lesions; 2 = 4–10 lesions; 3 = 10+ lesions; and 4 = dead leaf. The experiment was repeated three times. Values are the mean of 10–12 plants for each treatment. Bars represent standard errors.
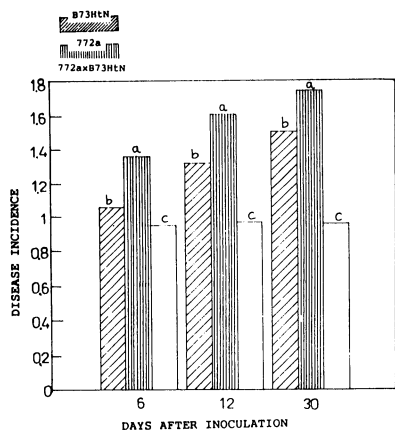
Fig. 3. Disease incidence of northern leaf blight (*Exserohilum turcicum*) on susceptible or resistant 5-wk-old maize (*Zea mays*) after artificial inoculation in the greenhouse. The leaf whorl of each of the tested plants (5 wk old) was sprayed with a 5-ml conidial suspension (50,000/ml) and kept in the greenhouse. Disease incidence, expressed as the number of lesions per plant, was rated as follows: 0 = no symptoms; 1 = few lesions; 2 = moderate number of lesions; and 3 = many lesions. Values are the mean of 40–50 plants for each genotype. Columns with the same letter are not statistically different (P = 0.05) according to Duncan's multiple range test.

cum. In this epidemiological report, a 12-hr wetness period and a suspension of 10,000 conidia per milliliter were the most extreme parameters relevant for the simulation study of disease development. However, a resistance-expression study should be conducted under extreme environmental (light, temperature, and wetness periods) and biotic (conidial concentration and plant age) conditions, all of which have a substantial influence on the host–parasite relations.

Our method is based on the combined effects of factors not previously used for resistance detection: application of a high concentration of inoculum (100,000 conidia per milliliter) followed by a long, dark wetness period (48 hr) immediately after inoculation. In addition, the removal of plants into higher temperatures (25 C) 48 hr after inoculation was likely another reason for the critical expression of resistance to NLB, as was demonstrated by Thakur et al (20). It seems that, under these optimal conditions for penetration and symptom development of NLB, we were able to reliably detect resistant genotypes as early as 4–10 days after inoculation (Figs. 1 and 2). In addition, susceptibility was expressed clearly under the low light intensity (100 μmol·m^{-2}·s^{-1}) in the growth chamber and was similar to the data reported by Leath et al (12). This method solves the

problem of the long detection period for the *HtN* gene.

The comparison of the LTA method with the common drop application showed that our suggested method is precise and reliable for screening for resistance. Results showed that susceptible plants might "escape" as resistant by evaluation after 24 hr of wetness after drop application, even with the high concentration of 100,000 conidia per milliliter (Figs. 1 and 2A, D, and G). This concentration is two times higher than reported by Leath and Pedersen (10), and 10 times higher than the highest concentration used by Abadi et al to detect resistance against NLB (1). Susceptible plants could also appear resistant when they were kept only 24 hr in wetness after inoculation by the LTA method (Fig. 1B, E, and H). On the other hand, the rapid development of large symptoms (Fig. 1D–F), together with a high disease incidence (Fig. 2D–F), were the reasons that IL772a plants showed a clear susceptible reaction when they were kept for 48 hr in wetness after being inoculated either by droplet or LTA.

It was clearly demonstrated that high inoculum concentrations of 100,000 to 200,000 conidia per square centimeter followed by a long period of wetness (48 hr) avoid the masking of susceptibility to *E. turcicum*. Under these conditions, however, the resistant B73*HtN* and IL772a × B73*HtN* plants developed small lesions which remained restricted long after the experiment was terminated. Therefore, 48 hr of wetness might be used in both droplet and LTA methods for rapid detection of resistance to *E. turcicum*. The resistance of the tested plants to *E. turcicum* was restored when the same plants were reinoculated and kept in the greenhouse for another 60 days (Fig. 3). Despite the fact that the inoculation of mature plants is impractical for breeding programs and difficult to handle, their response to inoculation, as reported here, indicates that this method might be used regardless of the age of the plants, depending on the availability and adjustment of the appropriate equipment. And finally, the suggested procedure, which has been used in our breeding program since 1986, enables us to practically utilize the important *HtN* gene as a source for resistance to *E. turcicum*. In addition, this rapid detection of resistance provides a tool for screening several generations for resistance within one year.

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