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Large-scale Maize Seedling Infection with *Exserohilum turcicum* in the Greenhouse

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[Abstract] Northern corn leaf blight (NCLB) is a serious foliar disease of maize (*Zea mays*) worldwide and breeding for resistance is of primary importance for maize crop protection. Phenotyping for NCLB resistance is well established in the field, but such experiments depend on suitable environmental conditions and are seasonal. Here we describe a greenhouse seedling approach that is suitable for testing thousands of seedling plants in a single experiment with a duration of 37 days. Three scoring methods were used to quantify the disease severity: the area under the disease progress curve (AUDPC), the primary diseased leaf area of the inoculated leaves at 16 days post inoculation (PrimDLA at 16 dpi) and the incubation period (IP) that was determined as days from inoculation to symptom appearance. By testing a diverse panel of maize genotypes, a high correlation between the three different methods was observed (81.9% to 94.1%), indicating that each of scoring methods can be applied for disease quantification. Thus, the seedling assay developed served as a relatively simple and high-throughput method for phenotyping NCLB disease resistance under greenhouse condition.

Keywords: Northern corn leaf blight, Seedling assay, High-throughput, Disease quantification

[Background] Northern corn leaf blight (NCLB) is a ubiquitous foliar wilt disease that threatens maize production worldwide (Welz and Geiger, 2000). The disease is caused by the hemibiotrophic fungus *Exserohilum turcicum* (anamorph of *Setosphaeria turcica*), which favors a high-humidity and cool temperature environment. Under favorable conditions, fungal infection manifests itself as large and irregularly emerging lesions that destroy the entire foliage. Therefore, this disease decreases the active leaf area and the accumulation of photosynthesized products. Up to 50% grain yield loss was reported but the reduction largely depended on environmental parameters (*e.g.*, temperature, humidity), phases of maize development and hybrid susceptibility (Ullstrup, 1970; Pataky *et al*., 1998).

Precision phenotyping for NCLB disease resistance is critical for the determination of host resistance against *E. turcicum*. Testing for disease resistance in the field is well established, *e.g.,* by placing or distributing inoculums in the leaf whorl at the 4 to 6 leaf stage (or even older) plants (Dingerdissen *et al*., 1996; Lipps *et al*., 1997; Brown *et al*., 2001; Asea *et al*., 2009; Chung *et al*., 2010; Chung *et al*., 2011). Scoring for resistance can be conducted by determining the levels of susceptibility (1 to 9; 1, complete resistance, no symptoms; 9, 90-100% of leaf area infected), the primary diseased leaf area (PrimDLA) that was defined as the percentage of infected leaf area of the inoculated leaf, the diseased leaf area of the entire plant (DLA), the incubation period (IP) rated as the number of days post inoculation until first

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observing the wilting/lesion, the lesion number (LN) at 14 to 21 days post inoculation and finally the area under the disease progress curve (AUDPC). However, tests for resistance in the field are environmentally-dependent and time-consuming. Here we describe a simple greenhouse seedling assay by testing only the second leaf, thus being suitable for quantifying thousands of seedlings in a single experiment within 37 days.

Materials and Reagents

1. Pipette tips
2. General lab materials, including:
   Mesh (0.5 mm)
   Round Petri dish (9 cm)
   Inoculation needle
   Microspore glass
   Vessel
   Funnel
   50 ml Falcon tube, *etc*.
3. *E. turcicum* isolate Passau-1
4. Potato dextrose agar (PDA) (BD, Difco™, catalog number: 213400)
5. Tween 20 (Sigma-Aldrich, catalog number: V900548)
6. PDA medium (see Recipes)
7. Tween 20 solution (see Recipes)

Equipment

1. Pipettes
2. General greenhouse equipment, including jiffy pots (ø8 cm), tray and sieve tray (L/W: 50 cm/30 cm), *etc*.
3. Home-made iron frame cover with non-permeable plastic (L/W/H: 50/30/35 cm)
4. Sprayer (Semadeni, ø28 mm)
5. Autoclave
6. A home-made box (L/W/H: 54/30/25 cm, open at the bottom, 3 cm notches on each side) to shield the Blacklight Blue fluorescent tubes (Philips TL-D BLB, 15 W, peak at λ 356 nm) or any incubators that can fit the fluorescent tubes can be used alternatively
7. Sterile bench with UV light
8. Neubauer counting chamber (BRAND, catalog number: 717805)
9. Microscope (ZEISS, model: Axio Imager 2) or other light microscopes
10. Centrifuge (Eppendorf, model: 5810 R)

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Procedure

1. Preparation of seedling plants for infection

   This seedling assay was conducted under greenhouse conditions (16 h day, 20 °C/8 h night, 18 °C; light intensity, 160 μmol/m^2 sec^{-1} (400 W, MHL); relative humidity, *ca.* 60%) during the whole experiment. Three to four maize seeds were sown in each jiffy pot, and fifteen pots were placed in one tray (5 per row). After germination, up to 3 seedlings per pot (*ca.* 45 seedlings per tray) were kept. In general, the second leaves had fully emerged two weeks after sowing. Newly emerged leaves were completely removed by cutting every 2-3 days until the end of each experiment.

   Note: Removing the newly emerged leaves can keep seedling plants small and delay leaf senescence, which gives nice disease symptoms and the seedlings can be easily kept under space-limited high-humidity micro-conditions.

2. Propagation of *E. turcicum* isolate
   a. Inoculation with mycelia and culture on PDA medium plate (35 ml per plate, see Recipes) was carried out at the same day seed sowing (Figure 1A).
   b. Add 500 ml ddH_2O to 19.5 g of PDA powder and autoclave at 121 °C for 20 min. Pour approximate 35 ml of liquid media into round Petri dish plates in a sterile bench. Sterilize the PDA plates under UV light (30 min).

      Note: Less media will inhibit fungal growth after some time.
   c. Inoculation of PDA plates was conducted using an inoculation needle to pick up a small piece of *E. turcicum* mycelia that is generally kept at room temperature for long-term storage (up to one year). Fix the PDA plate using the permeable surgical tape.

      Note: The dehydration and contamination of conidia PDA plates were often observed several months later. Inoculation of PDA plates needs uncontaminated mycelia.
   d. Place the plates upside down and incubate at room temperature in the dark. When the conidia cover the complete medium plate (after about two weeks), incubate the plates under BLB UV light until harvest (10 h per day, 7 days). This may induce the fungal sporulation to produce more spores.
3. Harvest and preparation of spore suspension
   a. Freshly prepare 0.1% Tween 20 (v/v) (see Recipes) in sterile water. Pour 10 ml of 0.1% Tween 20 on the plate and scrape the surface with a glass slide to dislodge the spores from the hyphae.
   b. Collect and pour the spore suspension by filtering through one layer of a fine mesh (*ca.* 0.5 mm), which is placed in a funnel to remove most of the mycelia.
   c. Before counting, mix spore suspension thoroughly by inverting 2-3 times, since the spores settled at the bottom of the collection vessels.
   d. Determine the spore density by using a 0.1-mm thick Neubauer counting chamber. Adjust the concentration to 4.5 x 10^4 spores/ml for use. If the spore concentration of the suspension

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is lower than the concentration needed, concentrate the spores by centrifuging for 5 min (room temperature, 1,811 *x g*) and removing the extensive supernatant. In general, the spores from one PDA plate after 21 days culture would be enough for infecting 2-4 trays. The conidia PDA plates can be stored up to one year at room temperature, but the spore suspension should be freshly prepared for each infection experiment.

4. Infection of maize seedlings by spray
   a. Inoculate *E. turcicum* to maize seedlings at 21 days after sowing when the second leaf fully emerges and becomes deep green. This date can be adjusted according to the growth status of seedling plants. Spray four trays using a total of 4 ml of spore suspension.
   b. After infection, water the tray extensively from the bottom to promote transpiration. A very high humidity micro-environment is achieved by placing non-permeable plastic hoods on top of each tray (Figure 1A).
      Note: The high humidity micro-environment can promote E. turcicum infection.

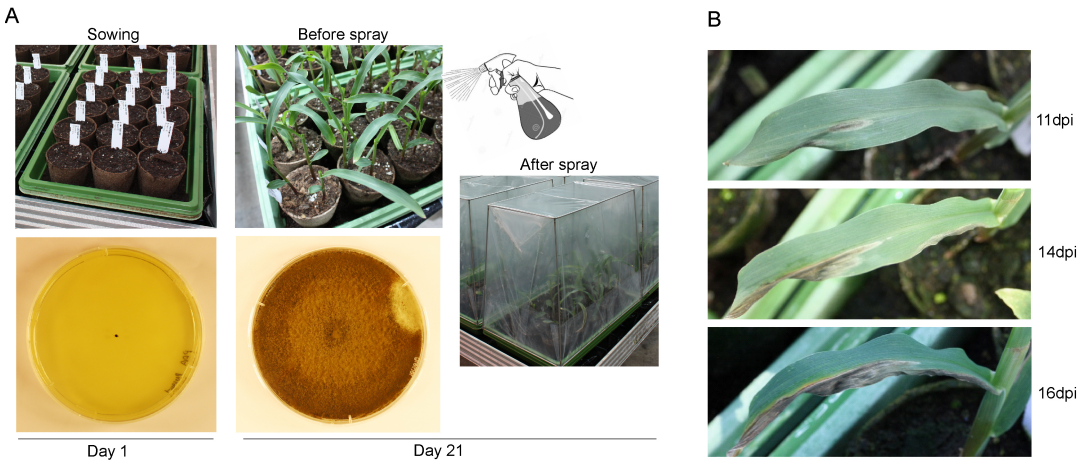

Figure 1. The seedling assay for testing NCLB disease resistance in the greenhouse. A. The pipeline for making inoculation. Parallel sowing and culture start of *E. turcicum* on a PDA medium plate performed 21 days before inoculation. The first and second leaves were subjected for inoculation by spraying spore suspension (4.5 x 10^4 spores/ml, 4 ml for 4 trays). The micro-environment with higher humidity was achieved by watering the tray extensively to promote transpiration and by covering the trays with a non-permeable cover until the end of each experiment. B. Symptoms of *E. turcicum* infected seedling. dpi, days post inoculation.

5. Scoring symptoms
   a. Scoring individual seedling for disease symptoms was conducted between 11 and 25 days post inoculation in an interval of 1 to 2 days (Figure 1B). The period for scoring can be adjusted according to the levels of susceptibility/resistance.
   b. Perform scoring for disease symptoms manually by visualization. Three disease parameters AUDPC, PrimDLA at 16 dpi and IP are used for disease quantification. IP and PrimDLA are rated individually, while AUDPC is calculated using the mean of all test plants in each

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genotype. In case of plants that were uninfected at 25 dpi, the respective IP was rated as 25 days. In general, 10 to 15 plants of each genotype are tested in each experiment. The IP, PrimDLA and AUDPC are calculated as follows:

$$IP = \frac{d_1 + d_2 + \cdots d_n}{n}$$

where, d_1 = days of symptom appearance post inoculation in plant d_1, n = number of total inoculated plants.

$$PrimDLA = \frac{p_1 + p_2 + \cdots p_n}{n}$$

where, p_1 = percentage of diseased leaf area of the second leaf in plant p_1, n = number of total inoculated plants.

$$AUDPC_{PrimDLA} = \sum_{i=1}^{n-1} \frac{(y_i + y_{i+1})(t_{i+1} - t_i)}{2}$$

where, y_i = PrimDLA at day i, $t_{i+1} - t_i$ = day interval between two ratings, n = number of ratings (Chung *et al.*, 2011).
Note: The use of less plants may lead to large variation and less reliability because of the quantitative nature of NCLB resistance.

Data analysis

Our previous work demonstrated that a seedling assay can be used to determine the presence of the quantitative NCLB resistance gene *Htn1* in maize, as well as the wheat broad-spectrum fungal disease resistance gene *Lr34* in transgenic maize lines (Hurni *et al*., 2015; Sucher *et al*., 2017). To test if the seedling assay can be used in diverse maize germplasm, we tested this method in a panel of maize lines (Table S1). This panel included six maize breeding lines that were kindly provided by KWS (Einbeck, Germany), and 127 exotic and historic maize lines that were collected before the 1990s from dozens of countries (IPK Genebank, Gatersleben, Germany). Interestingly, a continuous range of NCLB disease resistance/susceptibility was observed (Figures 2A-2C). The susceptible recurrent parental line RP1 was strongly infected, while near-isogenic line containing the introgressed resistance gene *Htn1* was highly resistant (Figures 2A-2C). While no visible disease symptoms were detected in ten genotypes, most accessions were infected with visible disease symptoms (AUDPC > 0) and 59% of genotypes were highly susceptible (PrimDLA_16 dpi ≥ 40%). Very importantly, three disease parameters AUDPC, PrimDLA_16 dpi and IP were highly correlated

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(R^2, 81.9 to 94.1) (Figures 2D-2F). Thus, each of the three parameters can be used for quantifying NCLB disease. For example, if PrimDLA is determined at 16 dpi for disease quantification, the data can be obtained after only 37 days.

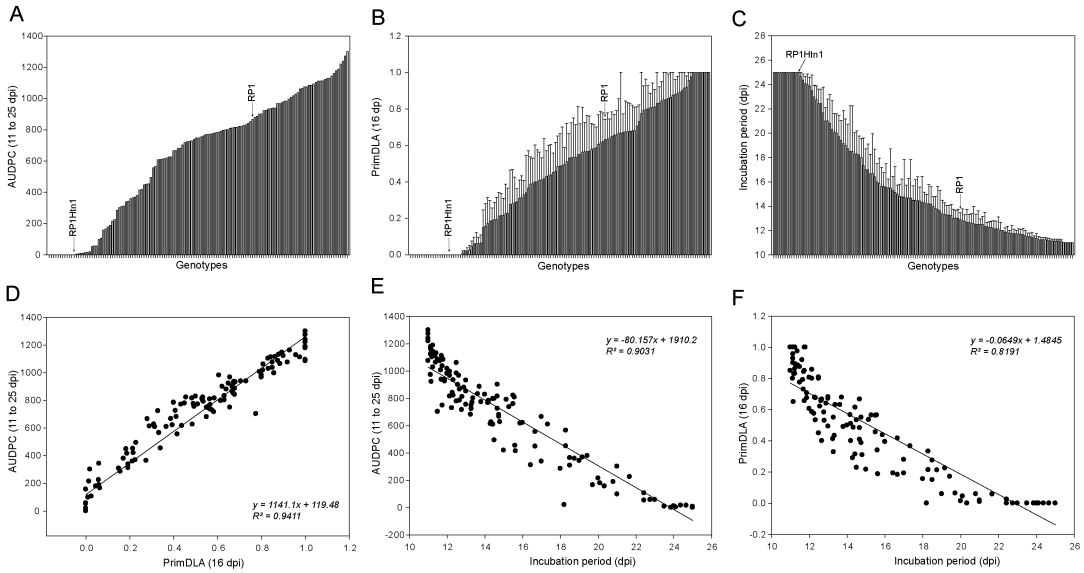

Figure 2. NCLB disease severity and correlation among disease parameters. Six genotypes from KWS Einbeck and 127 exotic maize genotypes from the IPK Genebank were tested in the seedling assay (Table S1). A. The area under the disease progress curve between 11 and 25 dpi; B. The primary diseased leaf (PrimDLA) area at 16 dpi; C. The incubation period that was rated as days from inoculation until appearance of disease symptoms; D. Correlation between AUDPC and PrimDLA at 16 dpi; E. Correlation between AUDPC and IP; F. Correlation between IP and PrimDLA at 16 dpi. Error bars indicate ± standard error (SE). dpi, days after inoculation.

Recipes

1. PDA medium
   Add 500 ml ddH_2O to 19.5 g of PDA powder and autoclave at 121 °C for 20 min
2. Tween 20 solution
   Add 200 µl Tween 20 to 100 ml of sterile water

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References

1. Asea, G., Vivek, B. S., Bigirwa, G., Lipps, P. E. and Pratt, R. C. (2009). Validation of consensus quantitative trait loci associated with resistance to multiple foliar pathogens of maize. *Phytopathology* 99(5): 540-547.
2. Brown, A. F., Juvik, J. A. and Pataky, J. K. (2001). Quantitative trait loci in sweet corn associated with partial resistance to Stewart's wilt, northern corn leaf blight, and common rust. *Phytopathology* 91(3): 293-300.
3. Chung, C. L., Jamann, T., Longfellow, J. and Nelson, R. (2010). Characterization and fine-mapping of a resistance locus for northern leaf blight in maize bin 8.06. *Theor Appl Genet* 121(2): 205-227.
4. Chung, C. L., Poland, J., Kump, K., Benson, J., Longfellow, J., Walsh, E., Balint-Kurti, P. and Nelson, R. (2011). Targeted discovery of quantitative trait loci for resistance to northern leaf blight and other diseases of maize. *Theor Appl Genet* 123(2): 307-326.
5. Dingerdissen, A. L., Geiger, H. H., Lee, M., Schechert, A., Welz, H. G. (1996). Interval mapping of genes for quantitative resistance of maize to *Setosphaeria turcica*, cause of northern leaf blight, in a tropical environment. *Mol Breed* 2(2): 143-156.
6. Hurni, S., Scheuermann, D., Krattinger, S. G., Kessel, B., Wicker, T., Herren, G., Fitze, M. N., Breen, J., Presterl, T., Ouzunova, M. and Keller, B. (2015). The maize disease resistance gene *Htn1* against northern corn leaf blight encodes a wall-associated receptor-like kinase. *Proc Natl Acad Sci U S A* 112(28): 8780-8785.
7. Lipps, P. E., Pratt, R. C., Hakiza, J. J. (1997). Interaction of *Ht* and partial resistance to *Exserohilum turcicum* in maize. *Plant Dis* 81(3): 277-282.
8. Pataky, J. K., Raid, R. N., Du, T. L. and Schueneman, T. J. (1998). Disease severity and yield of sweet corn hybrids with resistance to northern leaf blight. *Plant Dis* 82(1): 57-63.
9. Sucher, J., Boni, R., Yang, P., Rogowsky, P., Buchner, H., Kastner, C., Kumlehn, J., Krattinger, S. G. and Keller, B. (2017). The durable wheat disease resistance gene *Lr34* confers common rust and northern corn leaf blight resistance in maize. *Plant Biotechnol J* 15(4): 489-496.
10. Ullstrup, A. J. (1970). A comparison of monogenic and polygenic resistance to *Helminthosporium turcicum* in corn. *Phytopathology* 60(11): 1597-1599.

bio-protocol www.bio-protocol.org/e2567 Vol 7, Iss 19, Oct 05, 2017
DOI:10.21769/BioProtoc.2567

11. Welz, H. G. and Geiger, H. H. (2000). Genes for resistance to northern corn leaf blight in diverse maize populations. *Plant Breeding* 119: 1-14.