Field and Greenhouse Procedures to Evaluate Onions for Botrytis Leaf Blight Resistance

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Abstract. Greenhouse and field methods were developed to screen *Allium* spp. for resistance to botrytis leaf blight (causal agent *Botrytis squamosa* Walker). In greenhouse evaluations, plants were sprayed with laboratory-grown mycelial fragment inoculum and were incubated at 20°C in a chamber with an atomizing fogger. For field inoculations, a portable fog system with windbreaks was erected around experimental plots, and the plants were sprayed with the inoculum on evenings when windless, temperate (18 to 22°C) conditions were forecasted. The most effective mycelial fragment inoculum was <21 days old and had ≈ 45 to 50 colony-forming units/ μ l, resulting in an absorbance at 450 nm of 0.2 to 0.3. Rubbing the wax cuticle from leaves was essential to disease development in greenhouse but not in field experiments. Evaluations of eight *Allium* species, including 55 *A. cepa* L. accessions, were in agreement with previous studies.

Botrytis leaf blight (BLB) is one of the most damaging diseases of bulbing onion in temperate climates. The disease cycle, epidemiology, and pathogenesis of this organism have been described by Lorbeer (1992). Left uncontrolled, the disease severely reduces yields by blighting the leaves. Most of the extensive fungicide applications to commercial onion fields in the northeastern United States and Canada are for BLB control (Hoffmann et al., 1996). As the pressure to reduce fungicide usage in commercial agriculture mounts, growers will rely increasingly on plant resistance as part of their disease control programs.

Previous reports described laboratory evaluations of onion seedlings or leaf sections (Currah and Maude, 1984) or field and greenhouse experiments with natural and artificial infection (Bergquist and Lorbeer, 1971). BLB symptom development in laboratory screens using seedlings or excised leaf segments is different than that on mature plants in the field (Currah and Maude, 1984), raising concerns about the value of these methods for predicting field performance. However, field experiments relying on natural infection are difficult to execute because natural inoculum rarely

infects experimental plots uniformly and without interference from other pest problems. In our study, we describe effective field and greenhouse screening methods using a mycelial fragment inoculum easily prepared from local *B. squamosa* isolates and systems for maintaining leaf wetness, a condition essential to BLB development (Shoemaker and Lorbeer, 1977). These methods result in normal BLB development on susceptible plants.

Materials and Methods

Plant materials. Seed of *A. cepa* L. cv. Spartan Banner '80 was donated by Asgrow Seed Co., 'Norstar' was donated by American Takii, and *A. fistulosum* L. cv. Shimotae was donated by Ontario Foods. A total of 84 accessions representing eight species within *Allium* section *cepa* and *A. roylei* Stearn were provided by the U.S. Dept. of Agriculture, Agricultural Research Service, Western and Northeast Regional Plant Introduction Stations.

Greenhouse inoculations. A fog chamber (2.4 m long $\times$ 1.4 m wide $\times$ 1.1 m high) was constructed within a greenhouse to maintain temperature and leaf wetness conditions. End walls and the floor were constructed of 1.3-cm-thick transite; the ceiling was 1-cm-thick Plexiglas, and side walls were sliding double-pane glass doors. Chamber cooling was provided by two 0.9 $\times$ 0.6-m condenser coils, one mounted on the upper portion of each end wall. Coolant was provided to the coils by a refrigeration compressor (capacity 1.3 $\times$ 10⁷ J). Chamber heating was provided by two 200-W electric strip heaters (Chromalox, Pittsburgh) mounted beneath the condenser coils. The refrigeration compressor and the heaters ran continuously during experiments; when the

chamber temperature fell to <19°C, a thermostat-actuated solenoid diverted coolant from the condensers. The solenoid restored coolant to the condensers when chamber temperature was >21°C. Thus, chamber temperature was maintained at 20 $\pm$ 1°C. Fail-safe thermostats deactivated the entire chamber if the temperature was >27°C or dropped to <13°C. Six 2.4-m fluorescent lights supplemented natural lighting for 12 h daily (total light intensity at canopy height $\approx$ 300 μ mol·m⁻²·s⁻¹). Continuous leaf wetness, the critical feature for infection and disease development (Shoemaker and Lorbeer, 1977), was maintained by a pair of atomizing mist foggers (model 707SM; Hermidifier Co., Lancaster, Pa.). The foggers were controlled by a programmable repeating timer (model 30M8001; Tork, Mt Vernon, N.Y.) set to a repeating cycle of 2 min on : 8 min off or 3 min on : 7 min off. Small ($\approx$ 0.1 cm in diameter) droplets of moisture were continually present on leaf surfaces under these conditions, and chamber relative humidity varied from 94% (foggers off) to 100% (foggers on). Leaf surfaces became dry when the foggers were activated for shorter times, and activating the foggers for longer times formed large droplets on the leaves, washing inoculum from them.

Botrytis squamosa isolate MD-16 (collected from a commercial onion field in Orange County, N.Y.) was maintained on solidified potato dextrose agar (Bacto potato dextrose agar; Difco Laboratories, Detroit) under ambient laboratory conditions of light and temperature (20 $\pm$ 2°C, diffuse natural daylight supplemented by 8 h fluorescent room lights daily, $\approx$ 10 μ mol·m⁻²·s⁻¹). To prepare inoculum, plugs of the fungus were cultured, without shaking, in 250-ml flasks containing 500 ml liquid potato dextrose broth medium (Bacto potato dextrose broth; Difco Laboratories) for 10 to 25 days before inoculation. On the day of inoculation, the liquid was decanted from flasks, and the mycelial mat was rinsed three times with distilled water to remove staling products. Mycelial mats from two or three containers (dry weight 350 to 450 mg/container) were combined with 250 ml distilled water and homogenized for 1 min. The homogenate was strained through two layers of cheesecloth, after which the filtrate again was homogenized and strained as previously described. Distilled water was added to the filtrate such that inoculum absorbance at 450 nm (A_{450}) was 0.2 to 0.3. One drop of Tween 20 was added per 200 ml final volume.

Mature *A. cepa* cv. Spartan Banner '80 plants (or other genotypes as noted) with leaf diameters >1.2 cm were prepared for inoculation by gently wiping leaves with dry non-absorbent cotton (Absorbent Cotton Co., Valley Park, Mich.) to remove the wax cuticle from the entire leaf. The interior of the fog chamber was sprayed with water, and the foggers were activated several hours before inoculation. Plants were inoculated using an automotive paint sprayer (model 15513; Sears Roebuck and Co., Chicago) operating at 207 kPa, covering the leaf surfaces with fine droplets. The sprayer was shaken frequently during

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inoculation to keep mycelial fragments suspended. Immediately after inoculation, plants were placed in the chamber in a completely randomized design with at least three replicate plants per genotype or treatment. The number of plants per experiment was limited by the chamber size.

Plants were incubated in the fog chamber for 5 to 7 days, by which time, susceptible plants developed numerous lesions and some blighted leaves. Lesions were coalesced and could not be counted on severely blighted leaves. Three representative leaves with distinct lesions were selected from each of three or more replicate plants, and lesions were counted on a 15-cm-long section excised from the central portion of each leaf (see Fig. 1). Lesions per square centimeter/leaf area (lesion abundance) was calculated for each plant as lesion count $\div$ (15 cm $\times$ midsection width), averaged over the three leaf sections. Genotype or treatment means were compared by Student's *t* test, the Tukey–Kramer HSD test, or Dunnett's comparison with a control using JMP version 3 statistical software for the Macintosh (SAS Institute, Cary, N.C.).

Field inoculations. Seeds for the field inoculations were sown in flats 5 May 1994 and were grown under natural light at 20C in a greenhouse. Seedlings were transplanted to the field 27 June, 5 July, and 11 July in a randomized complete-block design with three replicate 7.3 $\times$ 7.3-m² blocks. Each block contained 104 one-row plots, 0.9 m long, with rows 0.6 m apart. Each plot contained $\leq$ 20 seedlings of an accession or the susceptible or resistant control (*A. cepa* cv. Spartan Banner '80, eight plots per block, and *A. fistulosum* cv. Shimotae, four plots per block, respectively). Pest control and fertility management followed standard commercial practices, except that no fungicides were applied.

The three blocks were inoculated separately, and a portable fogging system (see Fig. 2) was erected within each block before inoculation. A total of 49 ultra-low-volume (orifice diameter 0.030 cm, capacity 3.10 liters·h⁻¹ at 276 kPa) fog nozzles (Baumac FN-1018-12; Hummert International, Earth City, Mo.) were mounted atop polyvinyl chloride pipe risers 0.6 m high, with 1.2 m between risers, uniformly covering a 7.3 $\times$ 7.3-m² area. This area was surrounded by a 1.2-m-high windbreak constructed from snow fence sandwiched between two layers of 15- μ m-thick polyethylene sheeting. Water flow to the nozzles was regulated by an in-line solenoid valve controlled by a programmable repeating timer (Tork) set to a repeating cycle of 2 min on : 8 min off or 3 min on : 7 min off.

Field experiment inoculum preparation was similar to that for greenhouse experiments. Inoculum was prepared from 20 flasks of 16- to 22-day-old mycelial fragment suspension with inoculum density adjusted to $A_{450} = 0.3$. Blocks were inoculated on the evenings of 24 Aug. and 9 and 20 Sept. 1994. Still, temperate (18 to 22C) conditions favorable for BLB infection and disease development were forecasted for these evenings. The fog system was activated for $\geq$ 12 h after inoculation. The

inoculated plot was examined several times daily for the next 7 days to determine leaf wetness. When leaf surfaces were wet and the weather forecast called for further rain, fog, or dew, the fog system was deactivated. Seven days after inoculation, three plants were selected randomly from within each plot, and lesions per square centimeter were counted as described for greenhouse inoculations. Counts from the three inoculated blocks were averaged. *Allium* spp. accessions were considered resistant if their average lesions per square centimeter count was $<$ 30% of that of the susceptible control or if the lesions formed

were superficial (not expanding and lacking a water-soaked margin).

Results

Greenhouse inoculations. Typical BLB lesions and blighting developed on susceptible plants in the fog chamber within 5 to 7 days of inoculation (Fig. 1). Disease symptoms were identical to those seen in commercial fields, with parchment-colored, collapsed central lesions surrounded by grayish water-soaked halos. Lesions expanded, eventually coalescing to blight leaves. Older (outermost)

Fig. 1. Leaf sections (150 mm long) of (top) susceptible *A. cepa* cv. Spartan Banner '80 (with numerous oval lesions) and (bottom) resistant *A. fistulosum* cv. Shimotae (unaffected) inoculated with mycelial fragments of *Botrytis squamosa* and incubated in the greenhouse fog chamber. Symptoms shown are typical of those that develop 5 to 7 days after inoculation.

Fig. 2. (A) Polyethylene windbreak (1.2 m tall) surrounding the fogging system, both of which are moved from one replicate block to another for inoculation and disease development. (B) Moisture is provided by timer-controlled, low-volume fogger nozzles on 0.6-m-tall risers distributed throughout the block. The polyethylene windbreak helps maintain moisture on the leaves.

leaves are more susceptible to the disease (Shoemaker and Lorbeer, 1977), and the oldest leaves of plants in these experiments also showed the most blighting.

Inoculum with $A_{450} = 0.2$ to 0.3 was adequate for greenhouse inoculations, resulting in one to three lesions/cm² on leaves of the susceptible control *A. cepa* cv. Spartan Banner '80. More dilute inoculum was not as effective (Table 1). To relate A_{450} to inoculum potential, 200 μ l full-strength inoculum and a series of 10-fold dilutions were plated onto potato dextrose agar medium and incubated 2 days; colonies were counted on a 9-cm² area. Colonies on plates with full-strength inoculum ($A_{450}=0.36$) were too close to each other to be counted, and there were few colonies on plates with 100-fold or 1000-fold dilutions. There were 943 ± 188 colonies on plates with the 10-fold inoculum, giving 47 ± 9 colony-forming units/ μ l in the undiluted inoculum, which produced 1.4 lesions/cm² on leaves of inoculated plants.

Plants sprayed with inoculum cultured for 14 days developed many more lesions than those sprayed with 25-, 36- or 50-day-old inoculum having a similar A_{450} (2.24 vs. ≤ 0.25 lesions/cm², $n = 6$, $P \leq 0.05$, Tukey-Kramer HSD test). In seven experiments, inoculum <21 days old formed more lesions on leaves of 'Spartan Banner '80' than older inoculum did (1.56 vs. 0.30 lesions/cm², $P \leq 0.05$, t test).

Removal of the wax cuticle by wiping leaves with cotton was essential to successful inoculation of greenhouse-grown plants and was in agreement with Sutton et al. (1984). Plants wiped with cotton before inoculation developed 3.71 lesions/cm², but nonrubbed plants in the same experiment developed only 0.65 lesions/cm² ($n = 15$, $P \leq 0.05$, t test).

Allium cepa accessions in greenhouse evaluations were susceptible to BLB, but *A. fistulosum* and *A. roylei* were highly resistant (Table 2), confirming previous reports (Bergquist and Lorbeer, 1971; Currah and Maude, 1984; de Vries et al., 1992).

Field inoculations. A total of 79 accessions of seven *Allium* spp. within section *cepa* were evaluated in the field in 1994. All three *A. fistulosum* accessions evaluated were resistant to BLB according to the evaluation criteria previously described, as were five of the seven *A. altaicum* Pall. accessions, six of the seven *A. galanthum* Kar. and Kir. accessions, and three of the four *A. pskemense* B. Fedtsch. accessions. The single *A. oschaninii* O. Fedtsch. accession, both *A. vavilovii* M. Pop. et Vved.

Table 1. Effect of *Botrytis squamosa* mycelial fragment inoculum A_{450} (absorbance at 450 nm) on leaf lesion abundance of *A. cepa* cv. Spartan Banner '80.

Inoculum A_{450}	Plants inoculated	Leaves evaluated	Lesions/cm ²
0.00	3	9	0.00 a ^c
0.14	3	9	0.21 a
0.28	9	27	2.59 b

^aMean separation in rows by the Tukey-Kramer HSD test at $P \leq 0.05$.

Table 2. Susceptibility and resistance of *Allium* spp. to botrytis leaf blight of onion in greenhouse evaluations.

Entry	Plants inoculated	Leaves evaluated	Lesions/cm ²
<i>A. cepa</i> cv. Spartan Banner '80	6	18	1.56
<i>A. vavilovii</i> PI 406677	3	9	2.86 ^{ns}
<i>A. pskemense</i> PI 280549	3	9	0.56 ^{ns}
<i>A. galanthum</i> PI 280666	3	9	0.42 ^{ns}
<i>A. roylei</i> C502	9	27	0.23 [*]
<i>A. fistulosum</i> cv. Shimotae	6	18	0.00 [*]

^{ns}, ^{*}Nonsignificant or significant from the control (*A. cepa* cv. Spartan Banner '80) at $P \leq 0.05$, according to Dunnett's test.

accessions, and 53 of the 55 *A. cepa* accessions were not resistant. Of two resistant *A. cepa* accessions identified in the field, only one (PI 273212 from Poland) also was resistant in subsequent greenhouse evaluations. This accession developed 20% of the lesions of the susceptible control in the field (0.04 vs. 0.21 lesions/cm²), and these lesions did not expand to blight the leaves. In three subsequent greenhouse inoculations, PI 273212 developed significantly fewer lesions than 'Spartan Banner '80' (0.16 vs. 0.63 lesions/cm², $P \leq 0.05$, t test). Furthermore, lesions on PI 273212 leaves were smaller than those on leaves of 'Spartan Banner '80' (average area 0.90 vs. 4.50 mm² per lesion, $P \leq 0.001$, t test).

Discussion

Botrytis squamosa naturally infects onion leaves by means of conidia. However, this fungus sporulates erratically in culture and thus wild-type isolates do not reliably produce sufficient conidia for inoculation. Bergquist and Lorbeer (1973) developed a highly sporulating strain of *B. squamosa* by exposure to nitrosoguanidine followed by backcrossing to a wild type. Another highly sporulating *B. squamosa* strain was developed through several years of selection in the laboratory (de Vries et al., 1992). However, using specially selected laboratory strains to predict resistance to the disease in the field is questionable because they may represent only a small sample of the pathogen's total genetic diversity. These strains also may lose virulence after extended laboratory culture. However, large amounts of mycelial fragment inoculum are easily produced from wild-type *B. squamosa* isolates, which more accurately represent pathogen diversity. The field and greenhouse inoculations with mycelial fragments resulted in disease development and symptoms identical to those commonly seen in commercial fields (Lorbeer, 1992). Our determinations of the susceptibility and resistance of *Allium* spp. using mycelial fragment inoculum are in agreement with previous reports, in which plants were inoculated with conidia (Bergquist and Lorbeer, 1971; Currah and Maude, 1984; de Vries et al., 1992).

Resistance to BLB in *Allium* spp. has been known for some time (Bergquist and Lorbeer, 1971), but the sterility of *Allium* interspecific hybrids prevents the transfer of this and other useful traits to *A. cepa* (McCollum, 1971,

1982; Saini and Davis, 1967; van Raamsdonk et al., 1992). However, fertile interspecific hybrids between *A. roylei* and *A. cepa* have been reported (de Vries et al., 1992). We know of no previous reports identifying *A. cepa* germplasm resistant to BLB. *Allium cepa* PI 273212 is a readily useful source of BLB resistance, but the response of this accession to other strains of *B. squamosa* is unknown, as is the inheritance and durability of resistance.

Used in combination, these field and greenhouse evaluation methods make possible the simultaneous evaluation of many onion genotypes for resistance to BLB in the field; confirmation of the resistance of selected types in the greenhouse; and selection for resistance among large, segregating populations in a breeding program.

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