A Rapid Screening Approach to Identify Resistance to Basil Downy Mildew (Peronospora belbahrii)

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Abstract. Sweet basil (Ocimum basilicum L.) is among the most widely popular and economically important culinary herbs. Worldwide production of sweet basil has been threatened by a newly emerging disease, downy mildew (Peronospora belbahrii). Although tolerance and resistance have been identified in other Ocimum species, the traditional sweet basils all have been reported to be highly susceptible. There is an urgent need for evaluation of basil germplasm to identify sources of host resistance to P. belbahrii within Ocimum spp. and especially among O. basilicum species. In searching for genetic resistance, we developed a rapid approach to screen and evaluate downy mildew response at the cotyledon and true leaf growth stages under controlled environmental conditions. To confirm the reliability and reproducibility of this screening method, an experiment was conducted in which three basil species (Ocimum basilicum, sensitive; O. xcitriodorum, tolerant; and O. americanum, resistant to basil downy mildew) were evaluated for response to downy mildew inoculations at three growth stages. Disease incidence (DI) at the cotyledon growth stage was equal to or greater than true leaf growth stages for all species indicating that cotyledon response to downy mildew inoculations is a viable marker for predicting true leaf stage resistance. This approach was then used to screen 36 USDA-NPGS O. basilicum accessions at cotyledon and first true leaf growth stages to identify promising downy mildew-resistant breeding lines. Thirty accessions were susceptible at both growth stages (DI = 1.0). Four accessions exhibited little or no sporulation at either growth stage (DI less than 0.06), three of which showed other symptoms including chlorosis and necrosis. One accession, PI 652053, demonstrated no signs or symptoms but differed greatly from other accessions in regard to leaf morphology and habit. Results show that a resistant, mature plant can be identified at the cotyledon growth stage, providing a robust, low-input approach to identify promising downy mildew-resistant breeding lines. Thirty accession, PI 652053, demonstrated no signs or symptoms but differed greatly from other accessions in regard to leaf morphology and habit. Results show that a resistant, mature plant can be identified at the cotyledon growth stage, providing a robust, low-input approach to identify promising downy mildew-resistant breeding lines. Thirty accessions were evaluated for response to downy mildew inoculations at three growth stages.
reach full maturity (Putievsky and Galambosi, 1999) and is chilling-sensitive, which limits the number of field evaluations possible in temperate, northern regions of the United States. Evaluating seedlings for downy mildew resistance at the first leaf (cotyledon) growth stage is highly desirable because it allows for the rapid screening of many genotypes and conservation of resources necessary when screening mature plants. However, this approach is only effective when it is demonstrated that mature plants maintain or increase disease tolerance relative to younger growth stages (Wang et al., 2000). The relationship between cotyledon and true leaf growth stages in response to downy mildew has been explored in different species with contradictory results (Coelho et al., 2009; Kim et al., 1989; Leckie et al., 1996; Monteiro et al., 2005; Silue et al., 1996). To be an effective approach to screening for BDM resistance in basil, the interaction between growth stage and disease response must first be evaluated and understood. The objective of this study was to determine whether screening basil seedlings for resistance to downy mildew at the cotyledon growth stage is a reliable approach for identification of resistant genotypes at mature plant growth stages.

Materials and Methods

In this study, three basil species were evaluated for their response to BDM infection at the cotyledon and true leaf stages. *Ocimum basilicum* ‘DiGenova’ (DG, sensitive to BDM), *O. x citriodorum* ‘Sweet Dani Lemon Basil’ (SD, tolerant to BDM), and *O. americanum* ‘Spice’ (SPI, resistant to BDM) were selected based on their varying resistance to downy mildew in field evaluations (Wyenandt et al., 2010) (Table 1). An additional 36 *O. basilicum* accessions, representing a wide geographic distribution, were obtained from the U.S. Department of Agriculture (USDA) National Plant Germplasm System (NPGS) and evaluated for response to *P. belbahrii* infection at the cotyledon and first true leaf set growth stages. Seed for all treatments were sown in soilless media, Fafard Growing Mix 2 (Sun Gro Horticulture, Agawam, MA), and placed under intermittent mist in Rutgers University greenhouses until emergence of cotyledons from the soil surface. Seedlings were thinned to one plant per cell in a 72-cell (first experiment) or 128-cell (second experiment) flat.

Basil downy mildew inoculum (sporangia) was obtained from a 2011 field trial at the Rutgers Agricultural Research and Extension Center in Bridgeton, NJ, and used to inoculate susceptible *O. basilicum* ‘DiGenova’ stock plants. Identification of *P. belbahrii* as the causal agent for disease signs and symptoms was verified by polymerase chain reaction using specific primer sets previously described (Belbahri et al., 2005). Stock inoculum was maintained on susceptible host plants in an isolated room within the Rutgers University research greenhouse. Sporangia solutions for the experiments in this study were prepared by agitating freshly sporulating leaves of stock plants in distilled water for 5 min. This solution was filtered through a 40-μm nylon mesh cell strainer (Thermo Fisher Scientific, Bridgewater, NJ) and centrifuged at 3000 g for 10 min. The supernatant was discarded and the pellet resuspended in a known volume of distilled water. A Reichert Bright-Line Haemocytometer (Hausser Scientific, Horsham, PA) was used to adjust the final solution concentration as needed for each experiment.

Two chambers were constructed and installed in a single greenhouse room to provide the necessary conditions for *P. belbahrii* infection of inoculated materials. A dew chamber was constructed in which 100% relative humidity and leaf wetness were maintained by two Tiron 707U Series atomizing humidifiers (Tiron Air Purification Systems, Sanford, NC) on opposite ends of the enclosure (Fig. 1). A mist chamber, consisting of a partial enclosure with an opening at the base, was constructed and overhead irrigation was applied every 2 h for 5 min during the light cycle only. Temperature settings were set to 20/24 °C corresponding to a 12-h/12-h light/dark cycle in which supplemental light was provided only when photosynthetically active radiation fell below 300 μE·m⁻²·s⁻¹.

**Expt. 1.** In the first experiment, DG, SD, and SPI seedlings were evaluated for presence of sporulation at the cotyledon, first, and second true leaf set growth stages. Every basil species–growth stage combination formed an independent treatment consisting of 12 plants per experimental unit in a randomized complete block design. Seven- to eight-day-old plants (cotyledon growth stage) were inoculated by pipetting 20-μL droplets of 5 × 10⁴ sporangia/mL onto the adaxial surface of each cotyledon (40 μL/plant). Fourteen-d-old first true leaf pair growth stage and 21-d-old (second true leaf pair growth stage) plants were inoculated by saturating leaves with a 1 × 10⁴ sporangia/mL solution (≈2 mL per seedling) using a handheld sprayer. Inoculated seedlings were immediately transferred to the dew chamber for 48 h, then removed and placed in the mist chamber to allow for periods of leaf dryness and emergence of sporangia-phores from the leaf surface. A control was administered using distilled water and maintained in separated chambers to account for potential seedborne infection. Seedlings were evaluated for disease susceptibility 7 and 15 d post-inoculation (DPI). This experiment was repeated three times and data were analyzed according to the Kruskal-Wallis test (SAS Version 9.4, SAS Institute Inc., Cary, NC).

**Expt. 2.** In a second experiment, the cotyledon and first true leaf pair growth stages were screened on the same plants of 36 USDA-GRIN *O. basilicum* accessions. Treatments consisted of 16 plants per accession growth stage combination and DG, which was included as a susceptible control or negative control. In the first experiment, DG and SD plants inoculated at Day 7 often failed to produce true leaves as a result of the intensity of infection and environmental conditions in the dew and mist chambers. To allow seedlings to form true leaves, cotyledons of 10-d-old seedlings were drop inoculated with 20-μL droplets of 5 × 10⁴ sporangia/mL as described in Expt. 1 and scored 10 DPI. Seedlings were removed from the mist chamber and grown out to the first true leaf pair growth stage. Plants were then spray-inoculated with a 1 × 10⁴ sporangia/mL solution (≈2 mL per seedling) and placed in the dew chamber. After 48 h, seedlings were moved to the mist bench and true leaves evaluated for disease response as per the first experiment. Temperature, light, and overhead irrigation settings were identical to the previous experiment.

To allow for simple and rapid scoring of many seedlings for response to downy mildew, a binary scale was used in which 0 = resistant (sporulation was not visible on abaxial leaf surface) or 1 = susceptible (sporulation was visible on abaxial leaf surface). Leaves exhibiting chlorosis in the absence of observable sporulation were scored using a microscopic staining method previously described (Koroch et al., 2013). Plants in which sporangiospores were observed excising stomates were considered susceptible for either cotyledon or true leaf growth stages. In both experiments, scoring generated a proportion of infected seedlings or disease incidence (DI) for each treatment group (Table 2).

**Results and Discussion**

Variation in response to inoculation was observed among the three basil species evaluated in the first experiment (Table 2). True leaves and cotyledons of all plants were scored 7 and 15 DPI representing early and a later stage of disease progress. The SPI displayed no visible sporulation across experiments, whereas all three growth stages of DG were completely infected 15 DPI with the exception of one seedling that became infected 17 DPI. Susceptibility of all growth stages in DG among repeated experiments provided evidence that appropriate environmental conditions and pathogen virulence were present. In comparison, SD demonstrated

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Table 1. Commercial basil varieties evaluated in Expt. 1.

<table>
<thead>
<tr>
<th>Plant ID</th>
<th>Scientific name</th>
<th>Cultivar</th>
<th>Source</th>
<th>Field disease evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DK</td>
<td><em>Ocimum basilicum</em></td>
<td>DiGenova</td>
<td>Stokes</td>
<td>Highly susceptible</td>
</tr>
<tr>
<td>SD</td>
<td><em>Ocimum x citriodorum</em></td>
<td>Sweet Dani Lemon Basil</td>
<td>Johnny’s Selected Seeds</td>
<td>Tolerant</td>
</tr>
<tr>
<td>SPI</td>
<td><em>Ocimum americanum</em></td>
<td>Spice</td>
<td>Richters Herbs</td>
<td>Resistant</td>
</tr>
</tbody>
</table>
Table 2. Variation of response by basils to basil downy mildew at cotyledon stage, first, and second true leaf pair at 7 and 15 d post-inoculation (DPI), Expt. 2.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Growth stage</th>
<th>Disease incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 DPI</td>
<td>15 DPI</td>
</tr>
<tr>
<td>DG</td>
<td>Cotyledon</td>
<td>0.89 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>First true leaf pair</td>
<td>0.72 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Second true leaf pair</td>
<td>0.81 ± 0.05</td>
</tr>
<tr>
<td>SD</td>
<td>Cotyledon</td>
<td>0.69 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>First true leaf pair</td>
<td>0.53 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>Second true leaf pair</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>SPI</td>
<td>Cotyledon</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>First true leaf pair</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>Second true leaf pair</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

*D5 = O. basilicum ‘DiGenova’; SD = O. xcitriodorum ‘Sweet Dani Lemon Basil’; SPI = O. americanum ‘Spice’. No significant difference among growth stages across all cultivars according to the Kruskal-Wallis test (df = 2, χ² = 0.318; P = 0.853).

*a among three repeated experiments.

An important characteristic of *P. belbahrii* is the capacity to persist either in or on seed (Farahani-Kofoet et al., 2012; Garibaldi et al., 2004a) providing the opportunity for dissemination and early infection through contaminated seed. Basil genotypes susceptible at early growth stages such as SD have the potential to act as a carrier and distributor of the pathogen as seed or young seedlings (cotyledon and first true leaf growth stages). Evaluation based solely on mature plants ignores the potential for downy mildew susceptibility as transplants.

In the second experiment of this study, 30 of 36 *O. basilicum* USDA-NGPS accessions exhibited sporulation on all plants (DI = 1.0) evaluated at cotyledon and true leaf growth stages (Table 3) and were considered susceptible. No sporulation was observed at both growth stages for three accessions and a fourth in which one plant was susceptible (DI = 0.06) (Table 3). The USDA lines PI 172996, 172997, and 172998 were considered potential sources of resistance; however, true leaves exhibited chlorosis and necrosis, suggesting a hypersensitive response. Despite this putative defense response in preventing sporulation, disease symptoms of the leaf limit the breeding potential for fresh and culinary basils. In contrast, PI 652053 displayed no signs or symptoms of basil downy mildew, but the leaf morphology, habit, and aroma of this accession differ significantly from that of other *O. basilicum* species and suggest the need for taxonomical re-evaluation. This accession can be considered a potential source of resistance; however, its value as downy mildew-resistant breeding material needs to be determined by its capacity to cross-pollinate with a sweet basil (*O. basilicum*). In PI 296391 and PI 652054, decreased disease incidence occurred at the first true leaf pair (Table 3), demonstrating the same trend that was observed in SD.

Results of this study demonstrate that evaluation of basil cotyledons for resistance to downy mildew under controlled conditions is effective in predicting mature plant resistance. *P. belbahrii* is an obligate biotroph requiring live host tissue to survive. Thus, controlled conditions are necessary to provide an environment which ensures consistent disease pressure while maintaining physiological function and growth in the host. This study describes a two-chamber system to optimize screening conditions and which allows for accurate, reliable identification of promising genotypes. The cotyledon growth stage can be reached 7 to 10 d after sowing and requires a fraction of the space occupied by mature plants. In the second experiment of this study, a total of 608 seedlings, consisting of 36 USDA accessions and a control (DG), were simultaneously evaluated. An experiment of this magnitude would require extensive labor and materials if conducted as a field study.

Greenhouse screening allows for year-round evaluation and avoids the costly evaluation of susceptible genotypes in the field. Screening at the cotyledon growth stage will...
alleviate the demand on time and resources required to screen many genotypes such as in segregating breeding populations (i.e., F2 or backcross). This approach will greatly accelerate basil downy mildew breeding programs by providing an accurate, low-cost, and rapid assessment of susceptibility or resistance to BDM.

Literature Cited


