Evaluation of Daphne Germplasm for Resistance to Daphne Sudden Death Syndrome Caused by the Soil-borne Pathogen *Thielaviopsis basicola*

David Noshad¹ and Andrew Riseman

UBC Botanical Garden and Centre for Plant Research, University of British Columbia, 6804 SW Marine Drive, Vancouver, BC, V6T 1Z4, Canada

Zamir Punja
Department of Biological Sciences, Simon Fraser University, Burnaby, BC, V5A 1S6, Canada

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Abstract. Many daphne cultivars are susceptible to fungal root pathogens and require frequent fungicide applications during production. To identify taxon differences to disease susceptibility, we evaluated 32 *Daphne* species and cultivars for resistance to the soil-borne pathogen, *Thielaviopsis basicola* (Berk. and Broome) Ferr., by both in vitro- and in vivo-based methods. Disease-free plant roots were inoculated with the pathogen through topical application of a spore suspension and observed weekly for disease development/progression. Significant variation for disease severity among the taxa evaluated was determined using a plant disease index. Plant reactions ranged from highly resistant, e.g., *D. tangutica* and *D. retusa*, to highly susceptible, e.g., *D. cneorum*. In addition, a high correlation was found between the in vitro and in vivo techniques for the seven selected species, indicating that they are comparable. However, the in vitro assay provided results in significantly less time than the in vivo assay.

The genus *Daphne* L. (Thymelaeaceae Juss.) is comprised of ≈95 species (Flora of China, in press) distributed through Africa, northern and southern Europe, the Middle East, Asia, and regions of Oceania. Of these species, several species have been commercialized because of their many desirable horticultural characteristics, including attractive foliage, plant habit, flower color, and most of all, pleasant fragrance. Specifically, *D. cneorum* L. (Rose daphne or garland flower) has become one of the most popular perennial flowering shrubs among ornamental plant growers. However, the genus has acquired a poor reputation because of poor long-term performance of this and other *Daphne* introductions. One of the major limitations to daphne’s survival in cultivation is Daphne Sudden Death Syndrome (DSDS), a disease incited by the fungal root pathogen *Thielaviopsis basicola* (Berk. & Br.) Ferraris (syn. *Chalara elegans* Nag Raj et Kendrick) (Noshad et al., 2006). This disease kills plants quickly, as the name suggests, following the first foliar symptoms. Observations on DSBS-infected plants indicate the following progression of symptoms: 1) brown to black necrotic lesions on the roots, 2) leaf chlorosis leading to abscission, 3) whole plant stunting, and 4) stem collapse and plant death (Noshad et al., 2006).

*Thielaviopsis basicola* is a fungus common in both cultivated and noncultivated soils (Adams and Papavizas, 1969; King and Presley, 1942; Nag Raj and Kendrick, 1975; Yearwood, 1981) and is generally considered a facultative parasite (Anderson and Welacky, 1988; Bottacin et al., 1994; Papavizas, 1968; Reddy and Patrick, 1989). *Thielaviopsis basicola* has the ability to parasitize a wide range of important agricultural hosts, including cotton (*Gossypium hirsutum* L.), bean (*Phaseolus vulgaris* L.), carrot (*Daucus carota* L.), pansy (*Viola tricolor* var. *hortensis* DC.), peanut (*Arachis hypogaea* L.), and tobacco (*Nicotiana tabacum* L.) (Hood and Shew, 1996, 1997a; Smith, 1960; Snyder et al., 1959). After infection, the fungus causes both black root rot diseases and seedling damping-off. In Canada, this pathogen causes black root rot on carrot (*Daucus carota* (Punja et al., 1992) and tobacco (*Nicotiana tabacum*) (Schipper, 1970; Gayed, 1972; Stover, 1950; Stover, 1956) while also inducing disease on ornamental species such as poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzsch) Graham) and petunia (*Petunia x hybrida* Vilm.) (Mims et al., 2000; Punja et al., 1999). Identification of this pathogen is straightforward because the fungus produces two distinctive spore types, chlamydospores (aleuriospores) and phialospores (endoconidia). Chlamydospores are characterized by their thick pigmented walls divided into distinct compartments or segments with each able to germinate (Wheeler and Stipanovic, 1979; Wick and Moore, 1983). Phialospores, on the other hand, are hyaline, nondivided, and have a rectangular shape (Arseniuk and Czembror, 1999; Shew and Meyer, 1992; Hood and Shew, 1997b; Jones, 1991; Tabachnik et al., 1979).

Little is known about the factors (e.g., cultural conditions, host plant genetics) that affect DSBS development beyond anecdotal observations and practices. For example, during nursery production of daphne cultivars, many producers apply prophylactic fungicide treatments to help ensure crop health. Although this is a relatively common practice, no published literature was found that directly addresses the efficacy of this practice to control DSBS. In addition, even if effective at controlling DSBS, reliance on fungicides is unsustainable and undesirable. One alternative to this practice is the development of disease-resistant daphne cultivars. This strategy is typically more desirable because it can be highly effective in reducing disease, is environmentally benign, and usually entails little or no additional expense to producers (Crute and Pink, 1996; Dahlberg and Bandypadhyay, 2001; Díaz-Pérez, 1995; Reeleeder, 1999). However, it typically requires a long time horizon to achieve. To date, native host plant resistance to *T. basicola* has been identified during germplasm screens as part of various crop improvement or breeding programs. For example, *Nicotiana glauca* Graham was identified as resistant to *T. basicola* and subsequently incorporated into a tobacco (*N. tabacum*) breeding program (Trojak-Goluch, 2005). In another germplasm screen, *Gossypium arboreum* L. PI 1415 was found to be resistant to *T. basicola* and subsequently incorporated into a diploid cotton-breeding program (Berbec and Berbec, 1976; Shankara and Owling, 1999; Wheeler et al., 1998). Based on these reports, evaluating *Daphne* germplasm for resistance to DSBS, through controlled screens, is a reasonable approach to identify host plant resistance to this pathogen. Therefore, the objectives of this study were 1) to develop an efficient method for evaluating resistance of *Daphne* spp. to *T. basicola*; 2) to compare in vivo and in vitro methods for their efficiency in identifying resistance of selected *Daphne* taxa; 3) to develop a useful disease progression index (DPI) for use in taxa evaluations; and 4) to rank *Daphne* germplasm for resistance to *T. basicola*.

Materials and Methods

Plant material. Thirty-two species and cultivars of *Daphne* were collected and maintained at the UBC Botanical Garden and Center for Plant Research, Vancouver, BC, Canada. All 32 taxa were included in an in vivo challenge while a subset of seven

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¹To whom reprint requests should be addressed; e-mail dnoshad@interchange.ubc.ca.
species were used in an in vitro challenge (Table 1). Container-grown stock plants were used to supply tissue for both in vitro tissue culture establishment and traditional vegetative propagation. Rooted plants were produced in July and August from terminal cuttings (50 to 100 mm in length) with the flower buds and lower leaves removed. Cuttings were made with a single shallow cut and soaked in an antifungal solution (Physon 20; Maril Products) for 60 s. The cuttings were allowed to dry momentarily before being dipped in 0.4% IBA powder (Stim Root #2; Plant Products Co. Ltd., Brampton, Ontario, Canada) and then placed in 6-cm pots filled with a course rooting medium [10 parts propagation grade perlite, 8 parts peat, 6 parts granite grit #2, 1 part pumice (double screened to remove fine particles), dolomite lime 65AG at 900 g m⁻³, and Micromax (Scotts, Georgetown, Ontario, Canada) trace elements at 400 g m⁻³]. The cuttings were placed under intermittent mist with bottom heat set at 22 °C. Rooted cuttings were transferred to a polyhouse in October where they were allowed to go dormant but kept frost-free. They were repotted in May into 12-cm pots filled with a well-drained medium [8 parts peat, 8 parts Surface MVP, 6 parts granite grit #2, 4 parts screened and pasteurized soil, 1 part pumice, dolomite lime 65AG at 670 g m⁻³, Micromax micronutrients at 540 g m⁻³, Osmocote 18-6-12 (Scotts, Georgetown, Ontario, Canada) at 2150 g m⁻³, and Psi Matrix (TerraLink Horticulture, Abbotsford, BC, Canada) wetting agent]. All stock plants were grown under shadeclot during the summer months and moved to a heated polyhouse during the winter months to prevent frost damage. Fertilization regimen included yearly top-dressing with Osmocote 18-6-12 at 5 g/1-gal pot. Fungicides were not used during stock production because we had very little disease pressure and we did not want to risk crosscontamination affecting the in vivo assay.

Pathogen culture and suspension preparation. A single aggressive pathogenic isolate of T. basicola was cultured from diseased daphne plants and used throughout this study (Noshad et al., 2006). A suspension of endoconidia was prepared by gently washing the surface of 3-week-old colonies with deionized water and vortexing the wash solution for 30 s. The resulting suspension was filtered twice through four layers of cheesecloth to remove agar, hyphae, and chlamydoconidia. The spore suspension was calibrated with a hemocytometer and adjusted with deionized water to obtain a final concentration of endoconidia of 1 x 10⁶ mL⁻¹ before inoculation.

In vivo challenge. The conidial suspension (5 mL) was topically applied to healthy roots of 2-year-old nursery-grown plants. Production containers were modified to contain a clear panel behind a lightproof “door” to allow for direct observation of the infection process without further disturbance to the root system (Fig. 1A). All procedures were the same for control plants except for the application of distilled water instead of the spore suspension. To allow for uniform conditions after inoculation, plants were transferred to a greenhouse and grown under natural light at 25 ± 1 °C and a relative humidity between 70% and 80%.

In vitro challenge. Seven of the 32 taxa were selected for inclusion in an in vitro challenge (Fig. 2). Clean cultures of these taxa were established and axillary shoot proliferation obtained from nodal explants cultured on either MS (Murashige and Skoog, 1962) or Woody Plant Medium (McCown and Lloyd, 1983) supplemented with various concentrations of plant growth regulators [i.e., indole 3-butyric acid (IBA),

### Table 1. Daphne taxa used in the Thielaviopsis basicola resistance bioassay with region of nativity or origin and mean plant disease index (PDI) values 8 weeks after inoculation.

| Taxa | Nativity/origin | Mean PDI (sd) |
|------|-----------------|---------------|
| D. alpina L. | Italy | 12.5 (0.92) * |
| D. arbuscula Celak. | Czech Republic | 43.3 (1.74) abcd |
| D. bholua Buch-Ham. ex | Nepal | 12.5 (0.82) ab |
| D. Xburkwoodii 'Carol Mackie' | Horticultural origin | 29.2 (1.32) cdefgh |
| D. caucasica Pall. | Russia | 9.2 (1.02) h |
| D. circassica L. | Russia | 46.6 (1.66) abcde |
| D. cneorum L. | Czech Republic | 64.2 (1.89) a |
| D. collina Smith | Turkey | 51.7 (1.31) abcd |
| D. Esechmannii | Horticultural origin | 23.3 (1.17) delghi |
| D. genkwa Siebold & Zucc. | China | 43.3 (1.31) abcd |
| D. genkwa (Hackenberg group) | Horticultural origin | 15.0 (2.22) dfgh |
| D. girdium L. | Spain | 27.5 (1.22) abde |
| D. giralidi Nitsche | W. China | 15.8 (1.18) fghi |
| D. jacinthea Sibth. & Sm. | Greece | 13.3 (1.09) fghi |
| D. kosanii Stoj. | Bulgaria | 20.8 (1.3) fghi |
| D. laureola L. | N. Africa | 23.3 (1.34) delghi |
| D. ‘Lawrence Crocker’ | China-Yunnan | 40.8 (1.80) abcd |
| D. longilobata Turril. | Horticultural origin | 30.8 (1.06) abcd |
| D. Xmantensiana | Horticultural origin | 57.5 (1.87) abde |
| D. mezereum L. | Russia | 10.0 (1.02) hi |
| D. mezereum (alba) | Horticultural origin | 15.0 (1.32) ab |
| D. Xnapolitana | Horticultural origin | 39.2 (1.46) abcddefghi |
| D. odora Auct. | China | 31.7 (1.21) abcd |
| D. oleoides Schreber | Turkey | 17.5 (1.19) fghi |
| D. pontica L. | Russia | 60.8 (1.54) ab |
| D. retusa Hemsli. | China | 0.0 (0) fghi |
| D. Xrollsdorfii ‘Arnold Cihlarz’ | Horticultural origin | 25.8 (1.54) abcd |
| D. rossetti Gab. | Horticultural origin | 15.8 (1.06) fghi |
| D. Xschmannii | Horticultural origin | 34.2 (1.40) bcdefgi |
| D. tangutica Maxim. | China | 0.0 (0) fghi |
| D. transcaucasica Pobed. | Turkey | 19.2 (1.30) abcd |
| D. ‘Wilhelm Shacht’ | Horticultural origin | 19.2 (1.18) abcd |

*Mean values followed by a common letter are not different (P ≤ 0.05) by Tukey’s honest significant differences test.

![Fig. 1. Bioassay containers used for Daphne taxa challenged with Thielaviopsis basicola; (A) in vivo assay pots modified with viewing panel for direct observation of diseased roots; (B) in vitro assay with conidial suspension injected next to a root segment while still embedded in the culture medium.](image-url)
The most susceptible to *T. basicola* infection (PDI = 72.2), whereas *D. tangutica* and *D. retusa* were the most resistant (PDI = 3.3 and 3.58) (Fig. 2). For the five remaining taxa, PDI ranged between these extremes and with varying levels of chlorosis, leaf abscession, and stunting observed (Fig. 2).

**Assay comparison.** For the seven taxa included in both assays, strong similarities were present for overall taxa performance, whereas a significant difference in the timing of disease progression was present. A high correlation coefficient (R = 0.87) was calculated between these systems for PDI values, indicating both systems were comparable in evaluating disease susceptibility among these seven taxa. In addition, the rank order of taxa based on DPI values for the two methods were identical, further supporting these methods as equal in evaluating disease susceptibility. However, the in vitro system produced results in significantly less time. On average, 3 fewer weeks were required to reach the same level of disease progression as compared with the in vivo system.

**Discussion**

The identification and incorporation of host–plant resistance into susceptible plants is an often sought-after goal for many breeding programs. It has been successfully achieved using both conventional breeding as well as biotechnological methods. However, despite significant differences between these two approaches, both rely on a robust germplasm screen to differentiate germplasm performance after pathogen exposure. A screen incorporated into a traditional breeding program often involves the evaluation of progeny derived from hybridizations between resistant and susceptible parents or the evaluation of related taxa if resistance was not present in the most advanced gene pool (Agrios 1997; Darynol and Natsuaki, 2005; Iglesias and Pico, 2000; Punja, 2001). This approach has been successfully used to transfer *T. basicola* resistance between related species. In independent breeding programs, *T. basicola*-resistant tobacco genotypes were developed based on germplasm screens of related Nicotiana species (Bai et al., 1996; Palakarcheva, 1995; Trojan, 2005). Specifically, the use of a robust germplasm screen allowed researchers to identify *T. basicola* resistance in *Nicotiana debneyi* (Bai et al., 1996; Wilkinson and Ruffy, 1991) and to further conclude it was conditioned by a single dominant gene. Once transferred to a susceptible genetic background, this gene conferred the same degree of resistance as found in the original *N. debneyi* accession (Bai et al., 1996; Keller et al., 1999; Legg and Litton, 1981; Palakarcheva, 1995). In a *Gossypium* germplasm screen designed to identify *T. basicola* resistance, significant variation was observed among taxa with the strongest resistance identified in *Gossypium arboreum*. This resistance was then successfully transferred to commercial cotton cultivars (Rothrock, 1992; Walker et al., 1999;

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Wheeler et al., 1999). Development of a robust T. basi cola germplasm screen is valuable in not only identifying taxon-specific variation for pathogen resistance, but also for further evaluation of the plant’s genetic structure. Among the 32 Daphne taxa evaluated, significant differences were present for resistance to T. basi cola under both in vivo and in vitro challenges. Of the 32 taxa, D. tangutica and D. retusa displayed the greatest resistance and remained symptom-free during the in vivo challenges while displaying only mild symptoms in the in vitro challenges. At the other extreme, Daphne cneorum was clearly the most susceptible taxon in both screens and became fully diseased followed by plant death in the shortest amount of time. The observed range in disease resistance among Daphne taxa indicates our challenge was effective and identifies D. tangutica and D. retusa as potential sources for resistance.

The inoculation and screening methods were proven robust in terms of the isolate’s pathogenicity, disease characterization, consistency over time, and in its ability to differentiate taxa. Based on the one isolate used, overall pathogenecity was adequate in allowing assessment of disease incidence and severity (i.e., root and foliar symptoms) on all taxa. Also, the concentration of conidia used was sufficient to cause disease but not to overwhelm the defense mechanism(s) and prevent taxon differences from being displayed. Finally, both screening methods produced comparable and consistent results over an 11-month period despite the in vivo challenge being conducted in an outdoor polyhouse exposed to seasonal fluctuations. These observations validate our screen methodology and support its continued use in identifying genetic variation among Daphne species for resistance to DSDS.

The results of the in vitro and in vivo experiments indicate a strong correlation between these two assay methods. However, there were differences in disease progression rates between them. Typically, the in vitro challenge produced a comparable level of disease as the in vivo challenge but in 2 to 3 weeks’ less time. Differences in disease development rates between plants produced from tissue culture and traditional propagation have been reported and may be based on anatomical (e.g., root structure), biological (e.g., adaptation mechanisms with other organisms), or physiological (e.g., difference in biochemical compounds) differences (Diaz-Pérez, 1995). Further research into these differences may reveal important information on the pathogen infection process and the various factors that influence both successful infection and successful defense.

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