Pathogenicity, virulence, and molecular characterization of *Verticillium* species from spinach

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Fig. 1. A hybrid spinach seed production field in Washington. Note ten rows of the female parent line alternating with two rows of the male parent line.

OBJECTIVE

To characterize molecular diversity, pathogenicity, virulence, and host specificity among a diverse collection of isolates of *Verticillium* .

INTRODUCTION

Verticillium wilt, caused by *Verticillium dahliae*, is an economically important disease affecting > 200 plant species (3). Isolates of *V. dahliae* generally lack host specificity; however, there has been reported some degree of host specialization among isolates from a wide range of hosts (1). In 2005, *V. dahliae* was first reported to be pathogenic to spinach seed crops in the Pacific Northwest (U.S.) (Fig. 1) as symptoms develop only after the initiation of bolting (2). The morphological and molecular diversity has been examined among isolates of *Verticillium* recovered from seed from the U.S. spinach germplasm collection and from commercial spinach seed produced in different geographical areas. Isolates of *V. dahliae*, *V. tricorpus*, and *Gibellulopsis nigrescens* (formerly *V. nigrescens*) have been recovered from spinach seed (5) (Fig.2C). In addition, confirmation of the species has been done through the use of multiple molecular markers. Although the economic and biological importance of *V. dahliae* is known, little is known about the virulence of the species of *Verticillium* on spinach.

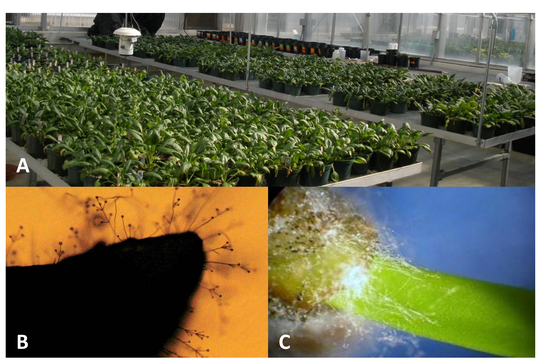

Fig. 2. A: Greenhouse inoculation test on spinach. B: Conidiophores of isolate Ls822 on spinach tissue. C: Microsclerotia and mycelium of *V. dahliae* on germinated spinach seed.

MATERIALS AND METHODS

Fungal Isolates. A carefully selected core collection of 40 isolates of *V. dahliae* and *Verticillium* species recovered from symptomatic spinach plants or spinach seed, as well as other hosts, representing different countries of origin, and vegetative compatibility groups (VCGs), were used in this study.

Molecular characterization. Isolates of *Verticillium* spp. were examined using primers ITS1 and ITS4 (6) for amplification and sequencing of the ribosomal internal transcribed spacer region. Alignment and phylogenetic relationships were determined by neighbor-joining using MEGA 4 software (4).

Virulence characterization. A commercial inbred spinach line, AC1, and lettuce line 'PI 251246' were used to assess virulence under greenhouse conditions (Fig. 2A) among the core group of isolates. Seed were sown in 200-cell seedling plug trays. Four-week-old plants were removed from the seedling trays, transplanted, and inoculated in a root-dip inoculation test (2). Four pots (replications)/isolate and three plants/pot were used.

Fig. 3. A: To the right, diseased spinach plants, and to the left, non-inoculated control plants. B: Verticillium wilt symptoms on spinach plant. C: To the left, a pot with non-inoculated plants and, to the right, two pots with inoculated lettuce plants. Arrows indicate leaves bearing symptoms of chlorosis and necrosis. D: Verticillium wilt symptoms on leaves of lettuce line PI 251246.

Spinach seedlings were kept in a growth chamber and, after inoculation, were exposed to long photoperiods in a greenhouse, which induced bolting 11 days after inoculation. Severity of symptoms was assessed 27 days after inoculation. Briefly, each plant was rated on a 7 step scale, where: 0 = no symptoms, 1 = >0 to 10% of the plant showing chlorosis and/or necrosis, 2 = >10 to 25% chlorosis and/or necrosis, 3 = >25 to 50% chlorosis and/or necrosis, 4 = >50 to 75% chlorosis and/or necrosis, 5 = >75 to <100% chlorosis and/or necrosis, and 6 = dead plant (100% chlorosis and necrosis). For the assay on lettuce, disease severity was expressed as the percentage of symptomatic leaves (chlorotic and/or necrotic) per plant. Severity of symptoms was assessed 28 days after inoculation. At the end of both experiments, isolations were performed on symptomatic and asymptomatic plants (Fig. 2B). In addition to spinach and lettuce, greenhouse inoculation assays were conducted on cotton and tomato. All the experiments were conducted at least twice.

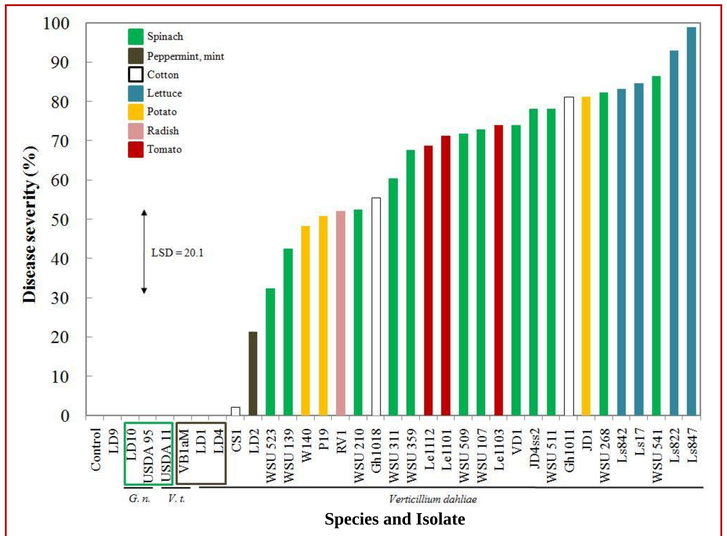

Fig. 4. Verticillium wilt severity on spinach line AC1 inoculated with isolates of *Verticillium* species

RESULTS AND DISCUSSION

Molecular characterization. A 429 bp sequence of the ITS region from the isolates examined was used to generate a neighbor-joining tree (Fig. 6). No variation was found between the 3 isolates of *V. tricorpus*; while, there were 3 nucleotide differences between the 2 isolates of *G. nigrescens* and 1 nucleotide difference among the *V. dahliae* isolates.

Virulence characterization. There was a wide range in disease severity among the isolates evaluated. On the spinach assay, 27 of 29 isolates of *V. dahliae* examined caused symptoms of Verticillium wilt on spinach, with significant differences in incidence and severity of Verticillium wilt among isolates ($P < 0.0001$ at $\alpha = 0.05$) (Fig. 3A, 3B, and 4). Disease incidence and severity ranged from 25.0 to 100.0% and 2.3 to 99.9%, respectively (Fig. 4). Isolates of *V. dahliae* in VCG 2B from spinach caused disease severity ratings ranging from 32.5 to 86.5% with a mean ± standard error of 74.5 ± 5.9%, and isolates in VCG 4B caused severity ratings from 42.5 to 78.1% with a mean ± standard error of 64.1 ± 6.1%. Isolates of *V. dahliae* LD1 and LD4 from peppermint and mint, respectively, as well as isolates of *V. tricorpus*, *V. albo-atrum,* and *G. nigrescens* were nonpathogenic under these conditions.

On the lettuce assay, of 31 isolates of *V. dahliae* examined, 30 were pathogenic on lettuce, with significant differences in disease severity and incidence among the isolates ($P < 0.0001$ at $\alpha = 0.05$) (Fig. 3C, 3D, and 5).

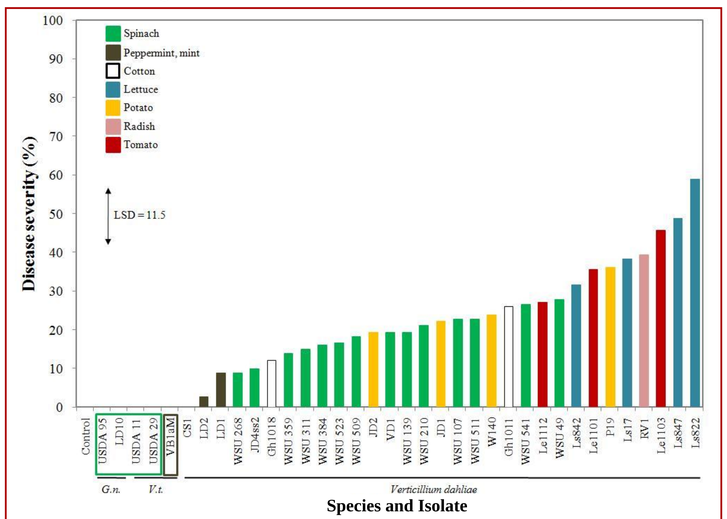

Fig. 5. Verticillium wilt severity on lettuce line 'PI 251246' inoculated with isolates of *Verticillium*.

In in the assay on lettuce, disease incidence and severity ranged from 41.7 to 100.0% and 2.8 to58.9%, respectively (Fig. 5). Isolates of *V. dahliae* in VCG 2B from spinach caused disease severity ratings on lettuce line P1 251246 from 8.9 to 26.7% with a mean ± standard error of 17.7 ± 2.2%; and isolates in VCG 4B from spinach caused ratings from 13.9 to 27.8% with a mean ± standard error of 19.5 ± 2.1%. An isolate of *V. dahliae* (CS1) from cotton was nonpathogenic in the assay on lettuce. Isolates of *V. dahliae* of VCG 2A from lettuce caused symptoms on lettuce line P1 251246 ranging from 31.7 to 58.9% severity.

In all assays, *V. dahliae* was recovered only from petiole sections sampled from symptomatic plants inoculated with *V. dahliae*. *V. tricorpus*, *V. albo-atrum,* and *G. nigrescens* were not recovered from petioles or stem sections of asymptomatic plants inoculated with the respective isolates.

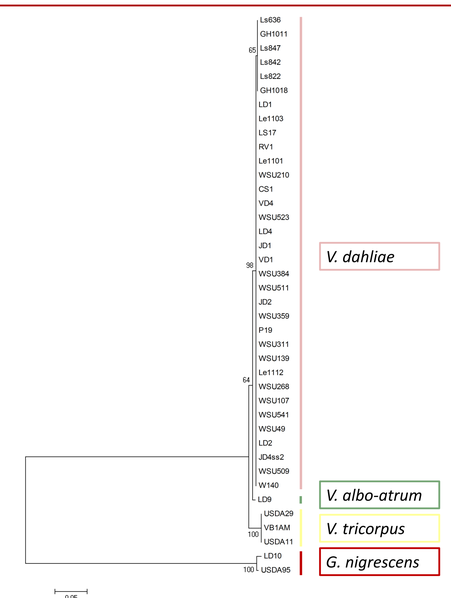

Fig. 6. Neighbor-joining tree derived from sequences of the ITS rDNA region. Bootstrap values (1000 replicates) are indicated adjacent to the nodes.

Results indicated that ITS-PCR can be a valuable diagnostic tool for each of the *Verticillium* species examined. Additionally, results suggested that there is differential pathogenicity among species; and that there are virulence differences among isolates of *V. dahliae* from different hosts. Together, these results indicate that *Verticillium* and related species associated with spinach display genetic diversity as well as substantial variability in virulence and pathogenicity to spinach. The identification of rotation crops that can influence infection of spinach and vice versa, and developing a detailed understanding of the molecular diversity of individual isolates and their specific virulence, may help in the development of management strategies of this important pathogen.

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