# Vegetative Compatibility Groups of *Verticillium dahliae* from Ornamental Woody Plants

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## ABSTRACT

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Vegetative compatibility groups (VCGs) of 42 strains of *Verticillium dahliae* obtained primarily from ornamental woody plants were assessed through complementation tests of nitrate-nonutilizing (*nit*) mutants. Of these isolates, 30 were assigned to VCG1, two to VCG2, and four to VCG4. The VCGs of six strains were not determined because they either did not produce mutants or produced mutants that did not complement any of the test strains. The assignment of most of the woody-plant isolates from Illinois to VCG1 was in sharp contrast to the diverse range of host plants from which the strains were isolated. Data could indicate that an important inoculum source of the pathogen in urban areas is from established populations in nurseries that get distributed along with transplanting trees. NitM mutants appeared to be more stable than *nit1* mutants in maintaining mutant phenotypes. Employing multiple mutants of many isolates, complementation tests showed that the extent of prototrophic growth at the mycelium interface was characteristic of the mutants and may not necessarily reflect that of their parent strains. Thus, caution should be exercised in quantifying relationships among natural strains within VCGs solely based on the extent of the prototrophic growth of their complementing mutants. Also, restriction banding patterns of a polymerase chain reaction-amplified mitochondrial rDNA were used to identify strains of *V. dahliae* that could not be identified by morphology.

*Additional keywords*: molecular differentiation, shade trees, shrubs.

*Verticillium dahliae* Kleb. is an economically important vascular wilt pathogen of many agronomic, horticultural, and landscape plants. This pathogen has a wide host range consisting of at least 136 plant species with worldwide distributions (15,21,38,39). It causes Verticillium wilt of ornamental woody plants (16,20,32,40) and also is involved in urban tree decline (28). Verticillium wilt of woody plants is peculiar in that it occurs frequently in transplanted trees in human-dominated landscapes (32,41) but is rarely observed in undisturbed natural forests (40). Although a few cases of host-specific isolates have been reported (21), strains of *V. dahliae* generally do not show host specialization (38,39), which makes classification at the subspecies level difficult. Vegetative compatibility grouping, however, has proved useful for differentiating strains of *V. dahliae* (23,24,35,37,43).



Vegetative, or heterokaryon, compatibility occurs in many filamentous fungi (9,13,22,24,27,35,37) and has been widely used to study population dynamics of plant pathogenic fungi (8,10, 24,43) as well as nonpathogenic isolates (14). Vegetative compatibility group (VCG) identification is useful in determining races, formae speciales, pathogenicity groups (8,18,24,27,37), and genetic diversity of a population (1). Isolates forming a VCG are generally more genetically similar to one another than to strains in different VCGs (27,35).

Puhalla (35) first used VCGs to study *V. dahliae* via morphological mutants, and 16 VCGs were found among 86 isolates (37). Using nitrate-nonutilizing (*nit*) mutants, Joaquim and Rowe (23) found that many strains considered incompatible when microsclerotia color mutants were used to test vegetative compatibility were compatible when *nit* mutants were employed, and four VCGs were identified. A subsequent study lumped the strains in VCG3

established by Joaquim and Rowe (23) into VCG4 and created a VCG5 (43). VCG4 is the most predominant group in potato populations of *V. dahliae* in Ohio and nine other states (24). VCG4 is divided into subgroups 4A, 4B, and 4A/B based on differential vegetative compatibility with strains BB and S-39 and with tester strains of VCG3 (24). Pathogenicity tests showed that strains belonging to VCG4A were more virulent on potato than were strains belonging to VCGs 2 and 4B (24). A lack of VCG diversity in potato populations of *V. dahliae* also was found in two regions of California (43).

Vegetative compatibility of *V. dahliae* obtained from ornamental woody plants has not been extensively studied. Knowledge of vegetative compatibility of *V. dahliae* from these plants could enable us to identify potential inoculum sources of the pathogen in urban environments. The main objective of this investigation was to assess VCG diversity within a collection of isolates of *V. dahliae* obtained from ornamental woody plants over 30 yr (6).

## MATERIALS AND METHODS

**Isolation of *V. dahliae* from naturally infected trees and shrubs.** *V. dahliae* was isolated from plant branches showing Verticillium wilt symptoms. Chips of discolored sap wood were placed on potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI) or on alcohol water agar (30) and incubated at room temperature (24–26 C) for up to 3 wk. Colonies developing from the diseased wood chips were transferred onto PDA from the hyphal tips. One isolate was retained from each diseased tree or shrub. Monoconidial cultures were prepared for all the isolates, and cultures were identified as *V. dahliae* by observing microsclerotia (19).

Additional isolates were supplied by colleagues from several states (Table 1). Original strain designations were retained if possible. After arriving, all cultures were single spored by streaking conidial suspension onto alcohol water agar. Colonies developing from single conidia, observed under a dissecting microscope, were transferred onto PDA. The isolates were identified as described. Cultures were stored either in silica gel at 5 C or in sterile glycerol (30% ) as a conidial suspension at −80 C.

**Identification of strains that lost the ability to form microsclerotia.** Some isolates had lost their ability to form pigmented resting structures (either microsclerotia or dark mycelium) in culture, and these isolates grew as puffy white mycelium with little conidia formation on PDA. Whether these strains were *V. albo-atrum* Reinke & Berthier or *V. dahliae* could not be determined morphologically. Identification was based on the restriction banding patterns of mitochondrial small-subunit rDNA (mtSrDNA), which was specifically amplified by polymerase chain reaction (PCR) with primers MS1 (5′-CAGCAGTCACGAATATTAGTCAATG-3′) and MS2 (5′-GCGGATTATCGAATTAAATAAC-3′) (46). Restriction banding patterns in this region of DNA were different between isolates of *V. albo-atrum* and *V. dahliae* (W. Chen, *unpublished data*)—the two most frequently encountered species of *Verticillium* in landscape woody plants (16,20,40,41). The procedures for DNA isolation, PCR conditions, and restriction enzyme analysis were similar to those described for *Pythium* species (5,7).

**Generation and characterization of *nit* mutants.** *Nit* mutants were generated by a technique of Cove (11), modified by Puhalla (36). Initially, four isolates were tested on three media—PDA, cornmeal agar (Difco), and a minimal medium, each containing 2% of $KClO_3$—for their efficiency in generating *nit* mutants. The composition of the minimal medium (MM) was described previously (9). Because cornmeal agar with chlorate (CMC) generated more mutants than did the other two media (*data not shown*), CMC was used for all other isolates. An agar plug from 2-wk-old cultures on PDA was placed in the center of a CMC plate (three plates per isolate) and incubated at 22 to 26 C for 2–3 wk. Previous studies (9,11,12) showed that the toxicity of chlorate is due to the conversion of chlorate to chlorite by nitrate reductase within wild-type mycelium. Wild-type mycelium showed restricted growth due to chlorite toxicity. Mutant cells that cannot metabolize chlorate or nitrate emerged from restricted wild-type colonies as fast-growing sectors. These fast-growing sectors were transferred onto MM containing nitrate as the only nitrogen source, and those sectors that grew as expansive colonies with thin mycelium growth, no aerial mycelium, and little or no conidial formation were considered *nit* mutants. All *nit* mutants exhibited wild-type growth on PDA. Monoconidial cultures were prepared from each *nit* mutant as described.

The *nit*-mutant phenotypes were determined by growing each mutant on a basal medium (MM without nitrogen) amended with one of four nitrogen sources: sodium nitrite (0.4 g/L), hypoxanthine (0.5 g/L), ammonium tartrate (0.8 g/L), or uric acid (0.2 g/L) (9,23). *Nit* mutants were first grown in MM, and then two agar plugs of each mutant containing mycelium were transferred onto basal medium containing the various nitrogen sources, with four agar plugs per plate so two mutants would be tested on one plate at the same time. The plates were incubated at 22–26 C, and colony growth was scored after 5–7 days. Mutants of tester strains from previously identified VCGs (23,43) also were tested the same way to ascertain their phenotypes prior to complementation study. Each *nit* mutant was tested twice (each time with two agar plugs) on each of the nitrogen sources. Assignment of *nit* mutants to biochemical phenotypes was based on nitrate metabolic pathways in *Aspergillus nidulans* (12), *Neurospora crassa* (29), and *Fusarium moniliforme* (26). *Nit* mutants incapable of using nitrate and hypoxanthine were considered NitM. *Nit* mutants capable of using hypoxanthine were considered *nit1*. *Nit1* presumably represents a mutation in a nitrate reductase structural locus, whereas NitM presumably represents a mutation in one of several loci coding for the assembly of a molybdenum-containing cofactor necessary for the activity of nitrate reductase and purine dehydrogenase (9,12,29). Distinguishing *nit1* and *nit3* mutants in *V. dahliae* was problematic because many of the *nit* mutants did not show growth on basal medium amended with nitrite in this study nor did they show growth in a previous report (43). Therefore, the mutants referred to as *nit1* are possibly *nit3* mutants. Because the objective of this study was to assess vegetative compatibility, and *nit1* (or *nit3*) was paired with a different phenotype (e.g., NitM), the inability to separate *nit3* from *nit1* does not compromise the objective.

**Stability of *nit* mutants.** This experiment was initiated because some of the *nit* mutants reverted to wild-type growth on MM. The *nit* mutants were grown in MM in duplicate petri plates for 20 days. Any presence of prototrophic growth on sector(s) of the mutants was noted, and the percentage of NitM and *nit1* mutants that had sectors that reverted to wild type were calculated. The experiment was repeated once.

**Assignment of strains to VCGs.** Every *nit1* mutant was paired with every NitM mutant generated in this study and with NitM tester strains of previously identified VCGs (23,43). NitM mutants of tester strains T9, 115, V44, WM, and S-39 were generated by Joaquim and Rowe (23) and were provided by R. C. Rowe. *Nit* mutants of strains CF, MC, CW, and MT were produced by Strausbaugh et al (43) and supplied by A. R. Weinhold. The tester strains from Joaquim and Rowe (23) (except the unviable culture of V-44 received) showed typical phenotypes of NitM and were extensively paired with mutants generated in this study. Some of the mutants from the other study (43) either reverted to wild type or could not metabolize uric acid and were not extensively tested in this study. Therefore, and hereafter, all VCG designations mentioned are *sensu* Joaquim and Rowe (23) unless otherwise specified. At first pairing, agar plugs (3 × 3 mm) with mycelium of five *nit1* mutants were placed on the edge of a 3-cm-diameter circle, and a NitM mutant was placed in the center of the circle. In the second pairing, five NitM mutants formed a circle, and a *nit1* mutant was placed in the center. Sometimes up to three circles were arranged on one plate (Fig. 1A). Every *nit1* mutant was paired with every NitM mutant at least twice. Two pairs of mutants (9-6*nit1* × 1990-1NitM and V-46*nit1* × T9NitM) produced inconsistent results in the first two pairings. A third pairing was conducted, and positive reactions were observed. Plates were incubated at 22–26 C and observed for prototrophic growth every 5 days for a total of 25 days. Comple-

mentation, a result of heterokaryon formation between *nit* mutants was evidenced by the prototrophic growth resulting in dense aerial mycelium and/or profuse sporulation and microsclerotia formation at the mycelial interface. Strains whose *nit* mutants formed prototrophic growth in complementation tests were considered vegetatively compatible and were grouped in one VCG.

The possibility that the prototrophic growth was due to syntrophism (cross-feeding) (45), rather than anastomosis, was tested. Ten positive pairings were randomly selected and tested with sterile polycarbonate membranes (1.5 × 4 cm, 0.25 μm pore size) vertically inserted in the center of MM plates before agar was solidified. The pairing mutants were placed on the plates so they were separated by the membrane (Fig. 1B).

## RESULTS

**Identification of *V. dahliae*.** Most of the strains used in the study could be easily identified to species by observing verticillate conidiophores and formation of microsclerotia as described previously (19). However, some strains lost their ability to form pigmented structures in culture and, therefore, could not be identified to species by morphology. Identification of these strains was based on restriction banding patterns of a PCR-amplified DNA region from the mt-SrDNA. The amplified DNA product was about 600 bp long and uniform in length among all the isolates of *V. dahliae* and *V. albo-atrum* examined. However, restriction banding patterns differentiated the two species (Fig. 2). Strains 1972-2 and 1990-9 (Fig. 2, lanes 12 and 13, respectively) had lost their ability to form microsclerotia, but the amplified mt-SrDNA from these two strains had the same banding patterns as the other morphologically identified strains of *V. dahliae*. All isolates used in this study, including the tester strains from earlier studies, showed the same banding patterns. The strains that showed the restriction banding pattern of *V. albo-atrum* were excluded from this study.

**Generation and characterization of *nit* mutants.** A total of 112 *nit* mutants were generated, and their phenotypes were characterized. Forty-seven could not catabolize hypoxanthine and were considered NitM. Four mutants that did not metabolize hypoxanthine and uric acid were considered nitrogen-nonutilizing mutants (Table 1) but were grouped into NitM for pairing

TABLE 1. Isolates of *Verticillium dahliae* used in this study, their host and geographic origins, year of isolation, sources, vegetative compatibility group (VCG), and the number of NitM mutants and total number of nitrate-nonutilizing (*nit*) mutants generated and used in complementation study

| Isolate | Host origin | Geographic origin | Year of isolation | Source[a] | VCG | *nit* mutants[b] | |
|---|---|---|---|---|---|---|---|
| | | | | | | NitM | Total |
| 9-4 | Sugar maple | Illinois | 1983 | 1 | 2 | 0 | 6 |
| 9-5 | Tartarian maple | Illinois | 1958 | 1 | 1 | 0 | 2 |
| 9-6 | Yellowwood | Illinois | 1972 | 1 | 1 | 1 | 4 |
| 9-8 | Magnolia | Illinois | 1985 | 1 | 1 | 7 | 8 |
| 9-10 | Green ash | Illinois | 1985 | 1 | 1 | 2[c] | 2 |
| 9-11 | Sugar maple | Illinois | 1987 | 1 | 1 | 2[c] | 2 |
| 9-14 | Green ash | Illinois | 1988 | 1 | 1 | 0 | 2 |
| 1972-1 | Little leaf linden | Illinois | 1972[d] | 6 | 1 | 0 | 1 |
| 1972-2 | Golden ram tree | Illinois | 1972[d] | 6 | 1 | 0 | 4 |
| 1972-4 | Smoke tree | Illinois | 1972[d] | 6 | nd[e] | 0 | 0 |
| 1972-5 | Sugar maple | Illinois | 1972[d] | 6 | nd | 1 | 1 |
| 1989-1 | Sugar maple | New Jersey | Unknown | 6 | 1 | 0 | 4 |
| 1989-2 | Sugar maple | New Jersey | Unknown | 6 | 1 | 0 | 1 |
| 1990-1 | Japanese maple | Illinois | 1990 | 5 | 1 | 7 | 7 |
| 1990-2 | Sugar maple | Illinois | 1990 | 5 | 1 | 2 | 2 |
| 1990-3 | Japanese maple | Illinois | 1990 | 5 | 1 | 0 | 1 |
| 1990-4 | Unknown | Massachusetts | Unknown | 4 | 2 | 0 | 4 |
| 1990-5 | Mum | Massachusetts | Unknown | 4 | 1 | 2 | 2 |
| 1990-6 | Sugar maple | Massachusetts | Unknown | 4 | 4 | 1 | 5 |
| 1990-8 | Sugar maple | Ohio | Unknown | 3 | 1 | 0 | 5 |
| 1990-9 | Black gum | Ohio | Unknown | 3 | 1 | 3 | 3 |
| 1990-10 | Pin oak | Ohio | Unknown | 3 | 1 | 2 | 3 |
| 1990-12 | Smoke tree | Ohio | Unknown | 3 | 4 | 0 | 2 |
| 1990-13 | Viburnum | Ohio | Unknown | 3 | nd | 7 | 7 |
| 1991-1 | Sumac | Illinois | 1991 | 5 | 1 | 3[c] | 3 |
| 1991-2 | Viburnum | Illinois | 1991 | 5 | 1 | 0 | 1 |
| V-49 | Pin oak | Illinois | 1960 | 2 | 1 | 1 | 1 |
| V-63 | Redbud | Illinois | 1960 | 2 | 1 | 0 | 1 |
| V-64 | Amur maple | Illinois | 1958 | 2 | 1 | 3[c] | 4 |
| V-66 | Unknown | Illinois | Unknown | 2 | 1 | 0 | 2 |
| V-69 | Magnolia | Illinois | 1959 | 2 | 4 | 0 | 2 |
| V-77 | Sugar maple | Indiana | 1960 | 2 | 4 | 1 | 4 |
| V-79 | Azalea | Illinois | 1964 | 2 | 1 | 0 | 3 |
| V-81 | Viburnum | Illinois | 1964 | 2 | 1 | 0 | 2 |
| V-82 | Barberry | Illinois | 1964 | 2 | nd | 3 | 3 |
| V-83 | Viburnum | Illinois | 1964 | 2 | 1 | 2 | 2 |
| V-92 | Sugar maple | Indiana | 1967 | 2 | 1 | 0 | 1 |
| V-98 | Purple smoke tree | Illinois | 1968 | 2 | 1 | 0 | 3 |
| V-99 | Black gum | Illinois | 1969 | 2 | 1 | 0 | 1 |
| V-101 | Japanese maple | Illinois | 1970 | 2 | 1 | 1 | 1 |
| V-105 | Sugar maple | Illinois | Unknown | 2 | nd | 0 | 0 |
| HR015 | Horseradish | Illinois | 1989 | 7 | nd | 0 | 0 |

[a] 1 = D. Neely, Ill. Nat. His. Surv.; 2 = E. Himelick, Ill. Nat. His. Surv.; 3 = L. Schreiber, U.S. Natl. Arbor. Stn., Del., Ohio; 4 = F. Holmes, Univ. Mass.; 5 = W. Chen; 6 = Ill. Nat. His. Surv. Cult. Collec.; and 7 = D. Eastburn, Univ. Ill.

[b] The number of the *nit1* (or *nit3*) mutants was the difference between the number of total and NitM mutants.

[c] Includes a nitrogen-nonutilizing mutant.

[d] Year of isolation for these four isolates is possibly the last transfer date.

[e] Not determined.

purposes. The other 61 *nit* mutants that did not utilize nitrate but were capable of using hypoxanthine were considered *nit1* (possibly *nit3*). Frequently, only one type of mutant was obtained from a given wild strain. For instance, all seven *nit* mutants obtained from strain 1990-13 showed the NitM phenotype (Table 1). The three media containing chlorate did not show any difference in generating mutant phenotypes. For instance, of the seven mutants of isolate 1990-1, two were from CMC, two from MM amended with chlorate, and three from PDA amended with chlorate. All seven mutants showed the NitM phenotype. *Nit* mutants were not obtained from three strains, 1972-4, V105, and HR015 (Table 1), despite repeated attempts with increasing concentrations of $KClO_3$. These three strains showed expansive colony growth on CMC and covered the whole plates in 20–25 days. They appeared to be chlorate resistant but nitrate utilizing.

**Stability of *nit* mutants.** When grown on MM for more than 20 days, some *nit* mutants showed prototrophic growth of aerial, white, puffy mycelium in one or more restricted sectors, which was considered an indicator of instability in the *nit* mutants. NitM mutants were generally more stable than *nit1* (or *nit3*) mutants. In the first test, 52 of the 61 *nit1* mutants had sectors showing wild-type colony morphology, whereas two of 51 NitM mutants showed reversal of wild-type growth. In the second test, 80% of *nit1* mutants and 3% of the NitM mutants had sectors that reverted to wild-type colony morphology.

**Complementation test and assignment of VCGs.** Multiple mutants from single parent strains always gave one type of reaction (either positive or negative) with a given tester strain. However, various degrees of reaction (the extent of prototrophic growth or microsclerotia formation) were observed. For instance, when paired with a 9-8 NitM mutant, a *nit1* mutant of 1990-8 showed moderate aerial mycelium growth. Another *nit1* mutant from the same parent strain showed strong profuse mycelium growth and microsclerotia formation at the mycelium interface when paired with the same 9-8 NitM mutant. Both of the *nit1* mutants from 1990-8 were capable of utilizing all nitrogen sources except nitrate and clearly exhibited the *nit1* phenotype. The extent of prototrophic growth in the complementation tests appeared to depend on the *nit* mutants, not necessarily their parent strains.

Three VCGs were identified after complementation tests with all possible combinations. Four isolates were assigned to VCG4 (Table 1) based on the positive complementation reactions of their *nit1* mutants with NitM mutants of tester strains BB and S-39. One of the four isolates (1990-6) complemented strongly with tester BB but weakly with tester S-39 and could belong to the subgroup VCG4A (24). Two isolates (9-4 and 1990-4) were assigned to VCG2 based on the prototrophic growth between their *nit1* mutants and NitM mutants of tester strains WM and 115. The assignment of strains to VCG1 was more complicated. The tester mutants of previously identified VCG1 were T9NitM

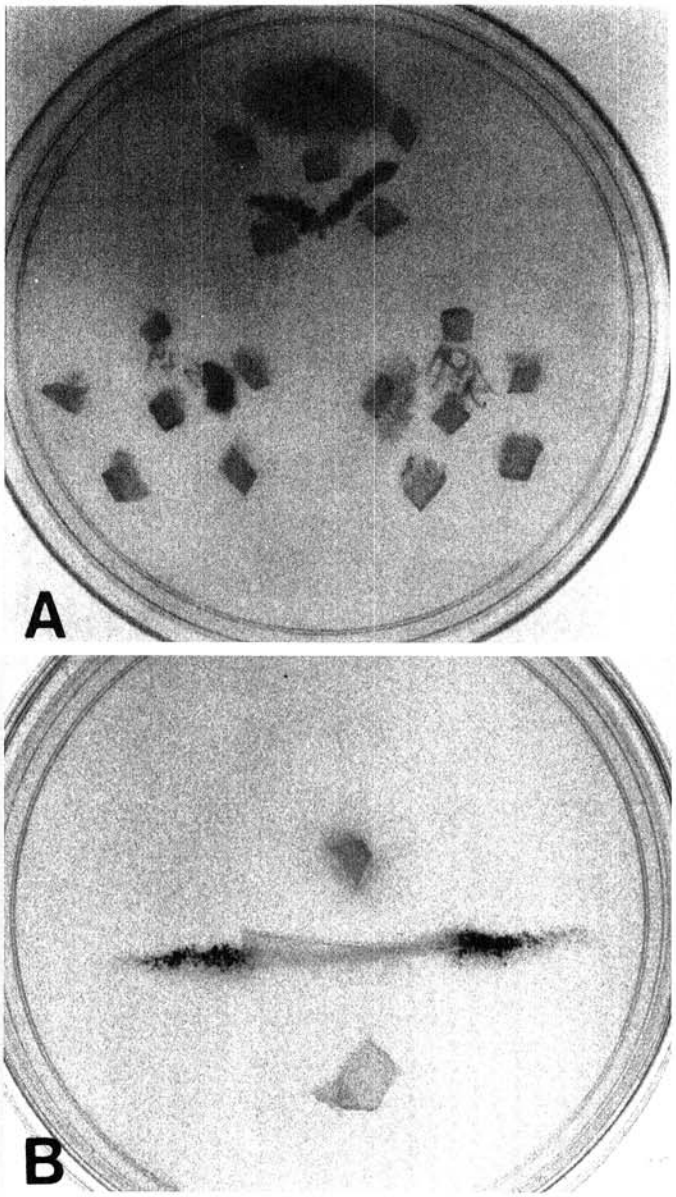


**Fig.1 A,** Nitrate-nonutilizing (*nit*) mutant complementation test on minimal medium with five *nit1* mutants forming a circle surrounding a NitM mutant, with three circles arranged in one plate. **B,** A membrane positioned between complementing mutants blocked mycelium contact and prevented prototrophic growth and microsclerotia formation. Microsclerotia formed outside the membrane, where contact between the mycelia occurred.

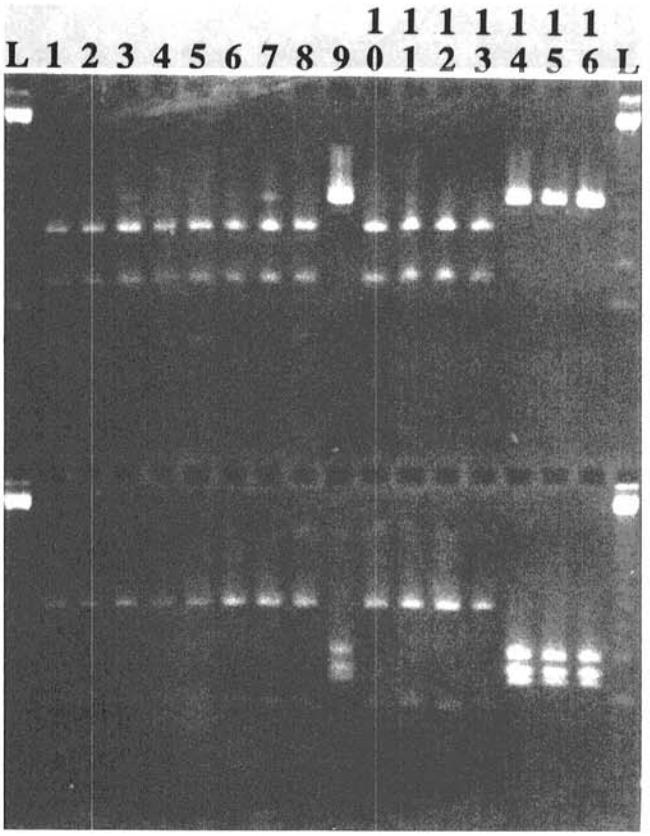


**Fig. 2.** Banding patterns of restriction enzyme-digested mitochondrial small subunit rDNA, which was amplified by polymerase chain reaction. The enzymes used were *Mbo*I (top panel) and *Taq*I (bottom panel). Lane L: BRL 123-bp DNA ladder; lanes 1–8, 10, and 11: morphologically identified isolates of *Verticillium dahliae*; lanes 9 and 12–14: isolates that lost their ability to form pigmented resting structures in culture; lanes 15 and 16: isolates of *V. albo-atrum*.

and T9-N from studies of Joaquim and Rowe (23) and Strausbaugh et al (43), respectively. *Nit1* mutants of 21 isolates produced prototrophic growth when paired with the tester mutants, and these 21 isolates were assigned to VCG1. Nine other isolates (9-10, 9-11, 1990-1, 1990-2, 1990-5, 1990-9, V-49, V-84, and V-101), also assigned to VCG1, were not paired with the VCG1 tester strains because they did not yield *nit1* mutants. The NitM mutants of these nine isolates produced prototrophic growth when paired with *nit1* mutants that complemented the VCG1 tester strains. The compatibility among these nine strains was not determined. A total of 30 isolates was assigned to VCG1. There were six strains whose VCGs were not determined. Three of the six strains did not produce any *nit* mutants (Table 1). The other three strains did not show complementation with any of the other mutants used in the study. Each of the three strains produced only one mutant phenotype, and their self-compatibility could not be assessed.

No evidence was found for cross-feeding in the 10 randomly selected pairings after testing with polycarbonate membrane. Prototrophic growth occurred only outside the membrane where mycelia made contact (Fig. 1B).

## DISCUSSION

Forty-two strains of *V. dahliae* obtained from ornamental woody plants were assessed for their vegetative compatibility. A sample of 42 isolates is relatively small compared to sample sizes from agronomic or horticultural crops. It does represent a considerable effort for isolates from urban woody plants. Data in this study suggest that there is limited VCG diversity in the population of *V. dahliae* from ornamental woody plants in Illinois. The sample sizes from other states were too small to make an accurate assessment. There was an uneven distribution of isolates among the three VCGs. Thirty of the strains were assigned to VCG1, two to VCG2, and four to VCG4; the VCGs of six strains were not determined. The assignment of most woody-plant isolates to VCG1 was surprising because the isolates were obtained from such a diverse array of plant species. Heterokaryon and a subsequent parasexual cycle are the only known means of gene exchange within this fungus. Isolates within a VCG are more genetically similar than those in different VCGs (27,35). The actual genetic relatedness of *V. dahliae* within VCGs remains to be confirmed by independent techniques. The data could indicate that less diversified populations of *V. dahliae* were established in nurseries and were distributed along with soil and trees that, although infected, showed no symptoms.

This study differs from previous VCG studies of *V. dahliae* in two aspects. First, it addresses a population of *V. dahliae* primarily from landscape woody plants. Previous studies included some isolates from ornamental woody plants in Illinois (37). Because the original strain designations were neither retained nor given, it is uncertain that those strains also were included in this study. Second, multiple mutants from wild strains were employed in complementation tests. This approach revealed that the mutants of the same parent strain may not always give the same extent of prototrophic growth when paired with the same tester mutant. Although the results were confounded by the inability to differentiate *nit1* from *nit3* mutants, some *nit* mutants that clearly exhibited the *nit1* phenotype also showed variation in prototrophic growth when paired with the same NitM tester strain. Early studies observed a similar phenomenon and a dependence of complementation reactions on *nit*-mutant phenotypes (23,24, 3). Thus, the extent of prototrophic growth is a characteristic of the mutants and may not necessarily reflect that of their parent strains. Consequently, measurement of quantitative relationships of wild strains within VCGs solely based on the extent of prototrophic growth of their mutants may be inappropriate. Complementation between *nit* mutants of *V. dahliae* is complex; not all strains within a VCG complement one another, and some weak complementation reactions in inter-VCG pairings also were observed (23,24,42,43). The genetics that control incompatibility in *V. dahliae* are not well understood (17,44,45), and the lack of a sexual life cycle has hampered genetic studies of this fungus (35,45). The lack of understanding of the mechanisms that control incompatibility prevents us from interpreting the complex patterns of complementation reactions observed.

Strains of *V. dahliae* varied widely in their sectoring frequency. Some strains readily produced *nit* mutants upon selection on chlorate-containing medium, others produced a few mutants, and still others produced none despite repeated attempts. Klittich et al (25) showed that the sectoring frequency was controlled by nuclear polygenes with additive effect for *Fusarium moniliforme*. Transposable elements also were proposed as a mechanism for the sectoring frequency (25). Transposable elements would become more active in the presence of chlorate ions, and the strains with more transposable elements would sector more frequently. In this study, frequently, only one mutant phenotype was generated from a given strain. Cove (11) discovered that the nitrogen source can affect the phenotype frequency of generated *nit* mutants in *Aspergillus nidulans*. In the initial trials of this study, three media containing chlorate did not show a difference in generating *nit*-mutant phenotypes.

Some *nit* mutants have a tendency to revert to the wild type (43). *Nit1* mutants of *V. dahliae* had a much higher frequency of sectors reverting to wild type than did NitM mutants. This feature also could be related to the wild strains, because frequently only one mutant phenotype was generated from wild strains. As a result, it has been a good practice for T. R. Joaquim and R. C. Rowe to distribute NitM mutants as tester strains. Four *nit* mutants in this study showed the phenotype that is incapable of utilizing hypoxanthine and uric acid. This phenotype is believed to result from mutation at the major nitrogen regulatory locus (9,12) and was not observed in earlier studies of *V. dahliae* (23,24,43). In one of the studies (43), however, uric acid was not tested as a substrate in determining mutant phenotypes.

Another interesting observation of this study is that Verticillium wilt of ornamental woody plants is caused mainly by *V. dahliae*. *V. albo-atrum* is infrequently isolated from woody plants, although there is much confusion in the earlier literature concerning the two species (15,17). Isolates of *V. dahliae* accounted for more than 90% of the *Verticillium* collection accumulated over the past 30 yr at the Illinois Natural History Survey. The collection included all disease specimens received from around Illinois, and, apparently, no attempts were made to differentiate the two species at the time of isolation (16,20). This observation supports the statement by Sinclair et al that "*V. dahliae* is the species that most commonly attacks trees and shrubs throughout the United States and southern Canada" (40).

Molecular techniques were used to identify isolates that could not be identified by morphology. Five strains lost their ability to form pigmented resting structures on PDA and produced only hyaline mycelium. This phenomenon was reported in early studies of *Verticillium* spp., including *V. dahliae* (4,34). Stable hyaline variants of *Verticillium* spp. could be produced through mutagenic treatments, and the production of pigmented structures seemed to be at least partially under cytoplasmic control (45). Those strains cannot be identified based on morphology. To identify those strains, techniques independent of morphology are necessary. Restriction banding patterns of PCR-amplified portions of mt-SrDNA differentiated the two species (Fig. 2). Variations at this region of DNA could be used to separate other *Verticillium* spp. *V. tricorpus* I. Isaac showed restriction banding patterns of the same DNA region that are different from those of either *V. dahliae* or *V. albo-atrum* (W. Chen, *unpublished data*). Other techniques also are available for distinguishing *Verticillium* spp. (2,3,31). Intraspecific genetic variations within *V. dahliae* were shown by restriction fragment length polymorphisms (33). The subspecies grouping based on these polymorphisms did not correlate with host plants or geographic origins.

Future research will be directed toward pathogenicity tests of the various VCGs on landscape woody plants, development of molecular and biochemical markers relating to VCGs, and genetic relatedness of strains within VCGs. Vegetative compatibility grouping is a very useful tool and can provide valuable information

concerning genetic diversity of pathogen populations; it will continue to aid future studies on the epidemiology and ecology of Verticillium wilt.

## LITERATURE CITED


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