Reassessment of Vegetative Compatibility Relationships Among Strains of *Verticillium dahliae* Using Nitrate-Nonutilizing Mutants

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ABSTRACT

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Twenty-two strains of *Verticillium dahliae*, originally assigned to 15 vegetative compatibility groups (VCGs) using microsclerotial color mutants, were tested for vegetative compatibility using complementary, auxotrophic nitrate-nonutilizing (*nit*) mutants. *Nit* mutants were generated from wild-type strains of *V. dahliae* by selecting chlorate-resistant sectors on cornmeal agar with dextrose amended with potassium chlorate (15–25 g/l). Complementation tests between *nit* mutants derived from these strains led to the identification of only four distinct VCGs. These results demonstrated that many strains considered to be incompatible when microsclerotial color mutants were used to test vegetative compatibility were compatible when *nit* mutants were employed. With the exception of strain PU, which was heterokaryon self-incompatible, all strains that were considered vegetatively compatible with the color mutant technique were also compatible when *nit* mutants were employed. Complementation tests, between *nit* mutants derived from 21 additional strains of *V. dahliae* from Ohio and tester strains representing the four VCGs, confirmed the existence of these four groups. Distribution of tested strains among the four VCGs were: three in VCG 1, 21 in VCG 2, two in VCG 3, and 15 in VCG 4. All strains within a VCG were strongly compatible with at least one of the selected tester strains but were not always completely incompatible with strains of other VCGs.

Verticillium dahliae Kleb. is an economically important fungal vascular-wilt pathogen of numerous plant species, including several major agricultural crops worldwide (30). Although a few strains of *V. dahliae* exhibit a high degree of host specificity (19,27), most strains have a wide host range. Because host specialization is lacking, differentiation among diverse strains of *V. dahliae* based on formae speciales has not been practical. Physiologic races based on host genetics have been defined in *V. dahliae* only in the case of the *Ve* gene in tomato (*Lycopersicon esculentum* Mill.) (17). Recent studies (34,36) have shown that vegetative compatibility analysis may serve as a useful tool to differentiate strains of *V. dahliae*.

Vegetative or heterokaryon compatibility has been widely documented among several plant-pathogenic fungi and has proven to be a powerful tool in examining genetic diversity among their natural populations (1,4–8,11,20,21,25,29,31–39). Fungal strains that anastomose and form heterokaryons with one another are considered to be vegetatively compatible and are assigned to a single vegetative compatibility group (VCG). Conversely, strains that are incapable of anastomosing with one another, and therefore fail to establish heterokaryons, are referred to as vegetatively incompatible.

Several criteria have been employed in assessing vegetative compatibility in fungi (1,4–8,14,31–42), and advantages and disadvantages of various methods have been discussed (3,5,14). Methods used with *V. dahliae* have included auxotrophic mutants (12,34,40–42) and morphological mutants either affecting the development of microsclerotial resting structures (40,41) or their pigmentation (microsclerotial color mutants) (34,36,42). These criteria have been employed in vegetative compatibility studies either singly or in combination with one another.

Puhalla (34) first demonstrated the existence of a vegetative compatibility system in *V. dahliae* similar to those described in other fungi. In a subsequent study, Puhalla and Hummel (36) identified 16 VCGs in *V. dahliae* among a worldwide collection from several hosts. Their conclusions were based on the use of microsclerotial color mutants induced by ultraviolet light. In wild-type strains of *V. dahliae*, microsclerotia are black. Heterokaryon formation was presumed to have occurred when a line of black microsclerotia developed at the mycelial interface between paired brown and albino color mutants. Results obtained in the earlier study of vegetative compatibility in *V. dahliae* indicated that groupings based on either morphological or nutritional markers correlated well (34). Although useful, both of these methods have inherent disadvantages. Induction of microsclerotial color mutants is laborious, requires a mutagen, and yields mutants at a very low frequency. Generation of auxotrophs also involves mutagenesis, and mutants are difficult to recover and usually grow poorly, or not at all, on unsupplemented media (5). In addition, certain auxotrophic or morphological color mutants are suspected to exert a pleiotropic effect on some vegetative compatibility reactions (4,40,41).

Auxotrophic, nitrate-nonutilizing (*nit*) mutants were first used by Puhalla to establish a vegetative compatibility system in *Fusarium oxysporum* (Schlecht.) emend. Snyd. & Hans. (35). *Nit* mutants can be readily recovered by selecting for chlorate resistance, a method that has been extensively employed in several fungi (5–9,11,18,23,28,29,35,39). Since these mutants are usually recovered at high frequencies, easily generated without a mutagen, and grow on unsupplemented media, this technique has been used widely in vegetative compatibility studies with *F. oxysporum* (2,6–8,11,20,21,25,31,35), *F. moniliforme* (Sheld.) emend. Snyd. & Hans. (23,39), *V. albo-atrum* Reinke & Berth. (5), and *Aspergillus flavus* Link (29).

The objectives of our investigation were twofold: 1) to evaluate the use of *nit* mutants in reassessing vegetative compatibility groupings in *V. dahliae* that previously had been defined by other methods, and 2) to examine vegetative compatibility relationships among some strains previously assigned to several VCGs (36) and among a small collection of strains from Ohio isolated from potato plants and soil.

MATERIALS AND METHODS

Isolation of *V. dahliae* from soil and naturally infected potato stems. Soil samples and basal stem sections of potato plants showing symptoms of Verticillium wilt were collected from fields in commercial potato production located in Columbiana, Portage, Sandusky, and Wayne counties, Ohio. An indirect method similar to that of Grogan et al (15) was used to assemble an isolate collection of *V. dahliae* from soil. Soil samples were air-dried for 4–5 wk at 22–26 C and then passed through a sieve with 2-mm openings to remove stones, clods, and large organic debris. A 10-g subsample of air-dried soil was washed with tap water through two stacked sieves with 125- and 38-μm openings. Residues trapped in each sieve were surface sterilized for 10 sec in 0.5% NaOCl, thoroughly rinsed with tap water, and then plated on sodium polygalacturonic acid agar medium (SPA) (26). Plates were incubated at 22–26 C for approximately 2 wk. Soil residues were then gently rubbed off from the agar surfaces under a stream of water, and each plate was inspected for colonies of *V. dahliae* using a stereo-dissecting microscope. Portions of individual colonies with associated microsclerotia were removed with a 5-mm-diameter cork borer. Colonies were placed in a blender containing approximately 200 ml of sterile distilled water and were comminuted at low speed for 3–5 min. The resultant mixture was collected on sterile Whatman No. 1 filter paper in a Büchner funnel, and liquid was removed by vacuum filtration. Collected microsclerotia were washed twice with sterile distilled water. Each filter pad was stored in a glass petri dish and left to dry for approximately 2 wk at 22–26 C to kill associated hyphal fragments and conidia. Microsclerotia were then individually transferred from filter pads to plates of SPA with a fine, sterile needle. Colonies of *V. dahliae* developing from these microsclerotia after 12–15 days at 22–26 C were subcultured onto potato-dextrose agar (PDA) by removing a 5-mm-diameter disk from the edge of each colony. Colonies, which were free of contaminants, were then stored at 5 C.

To isolate *V. dahliae* from potato stems, small pieces of vascular tissue were surface sterilized in 0.5% NaOCl for 30 sec, rinsed in sterile distilled water, and transferred to SPA plates. A 5-mm-diameter disk containing hyphal tips was removed from the edge of each colony of *V. dahliae* after 12–15 days at 22–26 C and subcultured onto PDA. Some SPA plates containing contaminated colonies were incubated longer until microsclerotia were visible. These microsclerotia were then processed by the technique described for soil isolates.

Monoconidial subcultures of all of the above isolates of *V. dahliae* were prepared and maintained in small culture vials containing a sterilized mixture of soil, perlite, and peat moss (1:1:1, v/v) for use throughout these studies. These were stored at 5 C until used. All strains collected in Ohio from potato plants or soil were designated by the letters P and S, respectively, followed by a number. Details of strains of *V. dahliae* previously assigned to VCGs by Puhalla and Hummel (36) are listed in Table 1.

Selection and characterization of *nit* mutants. Isolation of *nit* mutants was accomplished using a technique of Cove (9) for *A. nidulans* (Eidam) Winter as modified by Puhalla (35). Agar disks 5 mm in diameter were cut with a cork borer from the edge of wild-type colonies of *V. dahliae* growing on minimal agar medium (MM) (37). These were placed (four disks per plate) on cornmeal agar with dextrose (Difco Laboratories, Detroit, MI) amended with potassium chlorate (initially at concentrations of 15 g/L and later at 20 or 25 g/L) in 9-cm-diameter petri dishes. After incubation for 8–12 days at 22–26 C, chlorate-resistant sectors were subcultured onto MM in which the sole nitrogen source was nitrate. On this medium, most resistant sectors yielded colonies with an expansive but thin mycelial growth, indicative of their inability to utilize nitrate (5–9,18,20–25,28,39). All colonies originating from sectors with this type of growth response on MM were considered to be *nit* mutants. Wild-type strains grown on MM always showed characteristic wild-type colony morphology. All *nit* mutants exhibited wild-type growth on PDA. Monoconidial strains were then prepared from *nit* mutants and stored under the same conditions described for wild-type strains.

Phenotype identification of *nit* mutants was determined by their growth on a basal medium (BM) that was MM without nitrate. This medium was then amended with various nitrogen sources (6,9). *Nit* mutants first were grown for 5–7 days at 22–26 C on PDA or on BM amended with asparagine (1 g/L). Agar disks 5 mm in diameter were then removed from the edge of *nit* mutant colonies, and four each were placed on 9-cm-diameter plates of BM amended with one of the following nitrogen sources: sodium nitrate (2.0 g/L), sodium nitrite (0.4 g/L), hypoxanthine (0.5 g/L), ammonium-tartrate (0.8 g/L), or uric acid (0.2 g/L). Calcium carbonate (0.5 g/L) was added as a buffer to BM with ammonium tartrate because some *nit* mutants grew poorly on this medium, presumably due to a lowered pH resulting from the ammonium ion (10). All *nit* mutants were tested twice on each of the nitrogen sources. After 5–7 days of incubation at 22–26 C, *nit* mutants were examined for their growth response on each nitrogen source.

To be consistent with previously published information (5), *nit* mutants unable to utilize nitrate but able to use nitrite, ammonium, hypoxanthine, and uric acid were designated as Nit1 mutants. *Nit* mutants incapable of using nitrate and hypoxanthine but capable of utilizing the remaining three nitrogen sources were referred to as NitM. It was presumed that Nit1 mutants arose from a mutation at the structural locus of the gene for nitrate reductase, whereas the NitM phenotype originated from a mutation at one of several loci controlling the synthesis of a molybdenum-cofactor necessary for the activity of nitrate reductase and purine dehydrogenase (6).

Assignment of strains to VCGs. Two complementary *nit* mutant tester strains (Nit1 and NitM) were derived from the following strains, which previously had been assigned to separate VCGs by Puhalla and Hummel using color mutants (36): V-44 (VCG 1), BB (VCG 4), PG (VCG 5), MT (VCG 6), CF (VCG 8), CW (VCG 11), MC (VCG 14), TO (VCG 15), PCW (VCG 16). Nit1 and NitM mutants from strains PH (VCG 2) and 115 (VCG 3) were provided by J. E. Puhalla. To determine VCG relationships using the *nit* mutant system, these tester strains were paired with

TABLE 1. Host and geographic origin of wild-type strains of *Verticillium dahliae* previously assigned to vegetative compatibility groups (VCGs) by Puhalla and Hummel (36)

Strain designation	Host of origin	Geographical origin	Source[a]	VCG[b]
V-44	Cotton	USA (TX)	1	1
T9	Cotton	USA (CA)	2	1
138	Cotton	USA (MO)	2	1
PH	Pistachio	USA (CA)	1	2
WM	Cotton	USA (TX)	2	2
SS-4	Cotton	USA (CA)	3	2
115	Cotton	Syria	1	3
207	Potato	Australia	2	3
PU	Potato	United Kingdom	1	3
BB	Potato	USA (ID)	1	4
TA	Potato	USA (ID)	2	4
PG	Pepper	Greece	1	5
MT	Maple	Canada	2	6
CS-1	Cotton	Swaziland	1	7
CF	Cotton	France	1	8
CU	Catalpa	USA (IL)	1	10
CW	Cherry	USA (WA)	1	11
HY	Hops	USA (WA)	1	12
TC	Tomato	Canada	1	13
MC	Mum	USA (CA)	1	14
TO	Tomato	Canada	1	15
PCW	Pepper	USA (CA)	1	16

[a] Strains furnished courtesy of: 1 = J. E. Puhalla, California; 2 = P. C. Nicot, France; and 3 = J. E. DeVay, California.

[b] VCG = vegetative compatibility group (sensu Puhalla and Hummel [36]) determined by pairings of complementary microsclerotial color mutants, i.e., albino and brown. Strains were considered vegetatively compatible if their respective color mutants yielded a line of black microsclerotia at the mycelial interface when paired on potato-carrot-dextrose agar.

each other and with an additional 11 strains from the collection of Puhalla and Hummel (36) (Table 1).

Pairings of *nit* mutants in petri dish assays were conducted by placing a Nit1 or NitM mutant derived from one strain at the center of each plate containing MM. Two *nit* mutants, Nit1 and NitM derived from a tester strain representing a specific VCG, each were plated 1.0–1.5 cm apart on either side. All pairings were performed at least twice. Heterokaryon formation between complementary mutants was detected when prototrophic growth developed at the mycelial interface between the *nit* mutant positioned at the center of a plate and one or both *nit* mutants placed to either side (Fig. 1). Plates were scored for prototrophic growth 18–24 days following inoculation.

To determine whether strains of *V. dahliae* from Ohio belonged to any of the 16 VCGs identified by Puhalla and Hummel (36), interstrain pairings of *nit* mutants derived from an additional 21 strains selected from our potato plant and soil isolate collection were conducted with two complementary *nit* mutants of the 11 tester strains.

To preclude the possibility that paired strains were not anastomosing but simply cross-feeding extracellularly, pairings between *nit* mutants yielding prototrophic growth at the line of mycelial contact were retested by interposing a 47-mm × 0.2-μm polycarbonate membrane (Nucleopore Co., Pleasanton, CA) between the two complementary mutants. Prototrophic growth occurring between complementary mutants separated by a polycarbonate membrane would be indicative of cross-feeding, as the hyphae could not make physical contact to anastomose.

RESULTS

Isolation of *nit* mutants. Except for strain RI (VCG 9), all tested strains of *V. dahliae* spontaneously yielded fast-growing, chlorate-resistant sectors, although strain to strain variation in the frequency of sectoring was observed. Three types of resistant sectors were encountered; these emerged with a fanlike growth pattern from the perimeter of wild-type colonies. One type could be readily identified by dense mycelial growth in contrast to the sparse growth of the parent colony. A second type exhibited a thin mycelial growth in contrast to the dense mycelia of the wild-type colony. In the third and most frequently encountered type of sector, parental colony and sector both developed dense mycelia. Most of these types of chlorate-resistant sectors yielded thin or sparse colonies with an expansive growth pattern when subcultured onto MM. Some sectors failed to yield colonies with this growth type but instead exhibited characteristic wild-type colony morphology. These were probably *nit* mutants that reverted to the wild-type, sectors that were themselves heterokaryotic, or sectors that were chlorate-resistant but able to utilize nitrate (6,9,24,28).

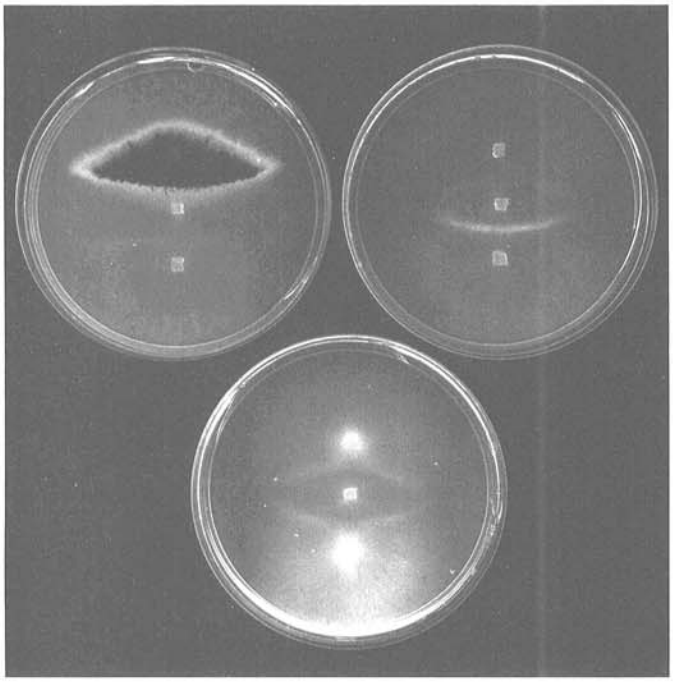

Fig. 1. Interstrain pairings of nitrate-nonutilizing (*nit*) mutants derived from strains of *Verticillium dahliae* previously assigned to vegetative compatibility groups (VCGs) by Puhalla and Hummel (36). Photo taken 16 days after inoculation of plates. In all pairings, Nit1 and NitM of tester strains are positioned at the top and bottom of plates, respectively. Plate on upper left shows NitM of strain 115 (VCG 3, inoculum block positioned in center of plate) yielding dense prototrophic growth with Nit1 of strain MC (VCG 14). Plate on upper right shows Nit1 of strain PH (VCG 2) in center of plate yielding slight prototrophic growth with NitM of MC. Bottom plate shows absence of prototrophic growth between Nit1 mutant of strain WM (VCG 2) in center of plate and NitM of V-44 (VCG 1).

Reassessment of vegetative compatibility groups. Two strains were considered vegetatively compatible if prototrophic growth at the zone of mycelial contact appeared in pairings between Nit1 and NitM mutants from each strain. Depending on the inherent ability of the strains to produce microsclerotia, prototrophic growth ranged from bands of completely black microsclerotia (e.g., strain 115 × MC) to white and fluffy mycelia (e.g., strain PCW × TO). Absence of prototrophic growth at the line of mycelial contact between Nit1 and NitM mutants from different strains was interpreted as a vegetatively incompatible reaction. In this study, wild-type strains were assigned to VCGs based on these responses of their respective *nit* mutants. A confounding factor, however, was that the extent of prototrophic growth at the mycelial interface between Nit1 and NitM mutants often varied among different strains. In some cases, growth was limited to a few small clumps of mycelia and/or microsclerotia along the line of mycelial contact. These limited reactions were scored as negative (0). In other cases, growth consisted of a very thin but solid line of mycelia and/or microsclerotia at the mycelial junction of both strains. These were considered weak, slow-growing heterokaryons and were scored as +/− (Fig. 1). Positive reactions (+) consisted of a thick band of mycelia and/or microslcerotia, which often overgrew the parental homokaryons (Fig. 1).

All pairing combinations that were positive for heterokaryon formation were tested for cross-feeding by interposing a polycarbonate membrane between the paired strains. In no case did prototrophic growth form where mutant pairs were separated by a membrane. This confirmed that physical contact, presumably followed by anastomosis and heterokaryon formation between complementary *nit* mutants, was necessary for prototrophic growth to occur.

Either a Nit1 and/or NitM mutant was obtained from each of the 22 strains that had been previously assigned to 15 VCGs by Puhalla and Hummel (36). Pairings between these *nit* mutants and both Nit1 and NitM mutants of strains representing 11 different VCGs, revealed that many strains judged incompatible by Puhalla and Hummel using color mutants were considered compatible when *nit* mutants were used (Fig. 2). For example, pairings between *nit* mutants derived from tester strains 115 (VCG 3), PG (VCG 5), CF (VCG 8), and MC (VCG 14) yielded extensive prototrophic growth at the mycelial interface (e.g., 115 × MC, Fig. 1), indicating that these strains are all in the same VCG. Similarly, strain CS-1 (VCG 7) produced moderately strong heterokaryons with strains 115 and PG; CW (VCG 11) and HY (VCG 12) paired well with MT (VCG 6); TC (VCG 13) paired with PH (VCG 2); and TO (VCG 15) paired with PCW (VCG 16). In addition to strong heterokaryons, a whole array of weak, slow-growing heterokaryons were observed between strains previously assigned to several VCGs. For instance, most strains in VCG 2 yielded weak heterokaryons with 115 (VCG 3) and with all other tester strains (PG, CS-1, CF, and MC) that were compatible with tester strain 115. Interstrain pairings involving *nit* mutants of MT (VCG 6), HY (VCG 12), and PCW (VCG 16) yielded weak heterokaryons with BB (VCG 4), whereas CW (VCG 11) strongly complemented BB.

With the exception of strain PU, all strains that were considered compatible via the color mutant technique also were found to be compatible when *nit* mutants were employed. For instance, strains 138 and T9 assigned to VCG 1 by Puhalla and Hummel (36) also were strongly compatible with V-44 (VCG 1). Similarly, strains WM and SS-4 assigned to VCG 2 also were compatible with tester strain PH, representing VCG 2. In addition, strain 115 (VCG 3) was compatible with 207 (VCG 3), and BB (VCG 4) was compatible with TA (VCG 4). Interestingly, strain PU, previously assigned to VCG 3, was incompatible with 115 (VCG 3) as well as with all other tester strains. Since pairings in all combinations between 10 Nit1 and four NitM mutants derived from strain PU failed to yield prototrophic growth, this strain was considered to be heterokaryon self-incompatible (5,6,20,23).

Pairings involving strains of *V. dahliae* from Ohio with tester strains representing 11 VCGs described by Puhalla and Hummel (36) confirmed these data (Fig. 3). For instance, strains that were compatible with 115 (P-10, P-19, P-20, S-80, S-92, S-113, S-221, S-250, and P-355) also were capable of complementing strongly with PG (VCG 5), CF (VCG 8), and MC (VCG 14), and all were weakly compatible with PH (VCG 2). At least two strains (S-65 and S-204) yielded stronger heterokaryons with PH (VCG 2) than with 115 (VCG 3). A second group of strains, comprising S-55, P-103, P-107, S-119, and S-228, was compatible with tester strain BB (VCG 4). In addition, these strains and all those that reacted weakly with BB (S-39, S-54, S-94, and S-247) were compatible with CW (VCG 11). Most strains strongly compatible with BB (S-55, P-103, P-107, S-119, and S-228) were also strongly

VCG	Tester Strain	V-44(1)	T9(1)	138(1)	PH(2)	WM(2)	SS-4(2)	115(3)	207(3)	PU(3)	BB(4)	TA(4)	PG(5)	MT(6)	CS.1(7)	CF(8)	CU(10)	CW(11)	HV(12)	TC(13)	MC(14)	TO(15)	PCW(16)
1	V44	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	PH	0	0	0	+	+	+	+/−	+/−	0	0	0	0	0	+/−	+/−	0	0	0	+	+/−	0	0
3	115	0	0	0	+/−	+/−	0	+	+	0	0	0	+	0	+	+	0	0	0	+/−	+	0	0
4	BB	0	0	0	0	0	0	0	0	0	+	+	0	+/−	0	0	0	+	+/−	0	0	+	+/−
5	PG	0	0		0			+		0	0	0	+		+	+	0		0	+/−	+	0	
6	MT	0	0	0	0	0	0	0	0	0	+/−	+/−		+	0	0	0	+	+	0	0		
8	CF	0	0	0	0	+/−		+	+	0	0	0	+	0	+/−	+	0	0			+	0	0
11	CW	0	0		0	0		0		0	+		0	+	0	0	0	+			0	+/−	
14	MC	0	0	0	+/−	+/−		+	+	0	+/−		+		+/−	+	0	0			+	0	0
15	TO	0	0		0			0	0	0	+		0		0	0	0				0	+	+
16	PCW	0	0	0	0	0		0		0	+/−		0		0	0	0	0			0	+	+

Fig. 2. Pairings between nitrate-nonutilizing (*nit*) mutants derived from strains of *Verticillium dahliae* previously assigned to vegetative compatibility groups (VCGs) by Puhalla and Hummel using microsclerotial color mutants (36). Nit1 or NitM mutants of each strain listed on the horizontal axis were paired with Nit1 and NitM mutants of tester strains on the vertical axis. Numbers in vertical column and in parentheses following strain designations in horizontal column refer to VCGs as assigned by Puhalla and Hummel (36). 0 = prototrophic growth absent or inconspicuous between *nit* mutants of strains (horizontal axis) and Nit1 and NitM mutants of tester strains (vertical axis); +/− = slight prototrophic growth at the mycelial interface between *nit* mutants of strains (horizontal axis) and Nit1 and/or NitM mutants of tester strains (vertical axis); + = *nit* mutants of strains (horizontal axis) yielded a dense prototrophic growth at the mycelial interface with Nit1 and/or NitM mutants of tester strains (vertical axis); empty square = not tested.

VCG	Tester Strain	P-10	P-19	P-20	S-39	S-54	S-55	S-65	S-80	S-92	S-94	P-103	P-107	S-113	S-119	S-180	S-204	S-221	S-228	S-247	S-250	P-355
1	V44	0	0	+/−	0	0	0	0	+/−	0	0	0	0	0	0	0	0	+/−	0	0	0	0
2	PH	+/−	+/−	+/−	0	0	0	+	+/−	+/−	0	0	0	+/−	0	0	+	+/−	0	0	+/−	+/−
3	115	+	+	+/−	0	0	0	+/−	+	+	0	0	0	+	0	+/−	+/−	+	0	0	+	+
4	BB	0	0	+/−	+/−	+/−	+	0	0	0	+/−	+	+	0	+	+	0	0	+	+/−	0	0
5	PG	+	+	+	0	0	0	+/−	+	+	0	0	0	+	0	0	+/−	+	0	0	+	+
6	MT	0		0	+		+/−	0		0	+	+/−			+/−	+		0		+	0	
8	CF	+	+	+	0	0	0	+/−	+	+	0	0	0	+	0	0	+/−	+	0	0	+	+
11	CW	0	0	0	+	+	+	0	0	0	+	+	+	0	+	+	0	0	+	+	0	0
14	MC	+	+	+	0	0	0	+/−	+	+	0	0	0	+	0	0	+/−	+	0	0	+	+
15	TO	0	0	0	0	0	+	0	0	0	0	+		0	+/−	+/−	0	0	+	0	0	0
16	PCW	0	0	0	0	0	+/−	0	0	0	0	+/−	+/−	0	+/−	0	0	0	+/−	0	0	0

Fig. 3. Pairings between nitrate-nonutilizing (*nit*) mutants derived from strains of *Verticillium dahliae* obtained from potato stems and soil in Ohio and tester strains representing 11 vegetative compatibility groups (VCGs) identified by Puhalla and Hummel using microsclerotial color mutants (36). A Nit1 or NitM mutant of each strain listed on the horizontal axis was paired with Nit1 and NitM mutants of tester strains on vertical axis. 0 = prototrophic growth absent or inconspicuous between *nit* mutants of strains (horizontal axis) and Nit1 and NitM mutants of tester strains (vertical axis); +/− = slight prototrophic growth at the mycelial interface between *nit* mutants of strains (horizontal axis) and Nit1 and/or NitM of tester strains (vertical axis); + = *nit* mutants of strains (horizontal axis) yielded a dense prototrophic growth at the mycelial interface with Nit1 and/or NitM mutants of tester strains (vertical axis); empty square = not tested.

or weakly compatible with TO (VCG 15), and all were weakly compatible with PCW. Those that yielded weak, slow-growing heterokaryons with BB (S-39, S-54, S-94, and S-247) were totally incompatible with PCW.

Because most VCGs identified by Puhalla and Hummel (36) were found to be invalid using *nit* mutants, a revised VCG system for *V. dahliae* was devised (Table 2). All strains within a VCG are strongly compatible with at least one of the tester strains but not always absolutely incompatible with strains belonging to other VCGs.

DISCUSSION

Chlorate was employed successfully to isolate *nit* mutants from most wild-type strains of *V. dahliae*, although frequency of sectoring varied among strains. Klittich et al (22) showed that sectoring frequency is under nuclear genetic control in *F. moniliforme* and is mediated by a polygenic system that functions additively. Movement of transposable genetic elements induced by high concentrations of chlorate was proposed as the most probable mechanism for sectoring in this fungus (23).

The subdivision of *V. dahliae* into absolutely incompatible VCGs has been controversial (16,17,40,41). Differences in methodology in assessing vegetative compatibility applied to different sets of strains have been a complicating factor (16,40,41). In the present study, auxotrophic *nit* mutants were employed to assess vegetative compatibility in 22 strains of *V. dahliae* that had been previously assigned to 15 VCGs by pairings of microsclerotial color mutants (36).

Our results indicate that different methods of analyzing vegetative compatibility can lead to conflicting results. For instance, strains PG, MC, CF, and 115 were incompatible with one another when using microsclerotial color mutants to test for vegetative compatibility, but they were all clearly compatible when *nit* mutants were used (Fig. 2). Consistent with these results were additional pairings between *nit* mutants derived from Ohio strains compatible with 115. Nine strains from Ohio were all clearly compatible with strains PG, CF, and MC. As a consequence, these strains must be assigned to the same VCG. Similarly, strains TO and PCW were clearly compatible with one another and therefore belong to the same VCG. The same conclusion arose from other pairing combinations, such as those involving strains MT, HY, and CW or strains PH and TC, although the heterokaryotic reactions were less intense.

Variable results with vegetative compatibility analysis based on different methods have been reported previously (5). Of six strains of *V. albo-atrum* from hops (*Humulus lupulus* L.) that were considered vegetatively compatible in pairings of complementary nutritional auxotrophs (4), four were clearly incompatible with the other two when *nit* mutants were employed (5). Recently, Heale (17), in a review of heterokaryon incompatibility in *Verticillium*, suggested that pairing brown and albino microsclerotial color mutants to assess compatibility in *V. dahliae* requires many hyphal fusions to produce a visible heterokaryotic reaction, i.e., a line of black microsclerotia. He also stated that the use of paired nutritional auxotrophs on MM was a more sensitive method to detect rare hyphal fusions between highly incompatible strains, which most likely would go undetected under the color mutant criterion. As a consequence, strains assigned to separate VCGs using color mutants may not necessarily be totally incompatible but may fail to produce a sufficient number of anastomosing bridges to yield a visible heterokaryotic reaction. Since the disagreement between Puhalla and Hummel's results and those presented here is confined to strains that they considered incompatible, our results provide evidence in support of Heale's hypothesis.

An additional complicating factor in assessing vegetative compatibility in this study was that the extent of prototrophic growth in interstrain pairings often varied among strains and individual *nit* mutants. Prototrophic growth among strongly compatible strains could be detected 7–10 days after inoculation as a line of dense mycelia and/or microsclerotia. This band of prototrophic growth was usually at least 1.0–2.5 cm in width after 18–24 days. In contrast, weak heterokaryons were usually noticeable only 10–15 days after incubation, were not expansive, and consisted of submerged mycelia and/or microsclerotia with little or no aerial mycelia. The physical appearance of mycelia of these slow-growing heterokaryons was similar to that described for other fungi (6,11,39).

In several interstrain pairings that yielded weak, slow-growing heterokaryons, replicated tests did not always agree. In addition, there were cases where the amount of prototrophic growth seemed to be dependent upon strain and *nit* mutant phenotype. Sometimes a Nit1 of a strain would react weakly with a NitM of a tester strain, but a NitM of the same strain would fail to yield any visible reaction with a Nit1 of the same tester strain. In other instances, a NitM of one strain would yield moderate to strong prototrophic growth with a Nit1 and/or NitM of a tester strain but a Nit1 mutant of the same strain would react only weakly with a NitM of the tester strain. Nit1 and NitM mutants derived from strain S-180 are examples of this. A NitM of S-180 reacted strongly with Nit1 of strain BB, whereas a Nit1 of S-180 yielded only a weak heterokaryotic reaction with NitM of BB.

On the basis of data collected in pairings between *nit* mutants derived from strains originally assigned to VCGs by Puhalla and Hummel (36), and in pairings between *nit* mutants of these strains and *nit* mutants from Ohio strains, we have rearranged the vegetative compatibility groupings in *V. dahliae* (Table 2). Among 43 strains of *V. dahliae* from several hosts, only four distinct VCGs were identified. Prototrophic growth between complementary *nit* mutants of strains within a VCG ranged from very weak to very strong. All strains assigned to a particular VCG were required to complement strongly with at least one of two tester strains (Table 2). Strains V-44, 138, and T9 remain in VCG 1

TABLE 2. Reclassification of vegetative compatibility groupings (VCGs) of strains of *Verticillium dahliae* based on compatibility reactions of nitrate-nonutilizing mutants

Reassigned VCG	Tester strain[a]	Strain designations and original VCG assignments by Puhalla and Hummel (36) based on microsclerotial color mutants	Ohio potato plant and soil strains
1	V-44, T9	V-44, 138, T9 (VCG 1)	
2	PH	PH, WM, SS-4 (VCG 2), TC (VCG 13)	S-65, S-204
	115	115, 207 (VCG 3), PG (VCG 5), CS-1 (VCG 7), CF (VCG 8), MC (VCG 14)	P-10, P-19, P-20, S-80, S-92, S-113, S-221, S-250, P-355
3	PCW	TO (VCG 15), PCW (VCG 16)	
4	BB	BB, TA (VCG 4)	S-55, P-103, P-107, S-119, S-228
	MT	MT (VCG 6), CW (VCG 11), HY (VCG 12)	S-39, S-54, S-94, S-247, S-180

[a] Strains within each VCG were grouped under the tester strain with which they yielded the most dense prototrophic growth.

(Table 2). Strains PH, WM, and SS-4 remain in VCG 2. In addition, strains 115, 207, PG, CS-1, CF, TC, MC, and all Ohio strains compatible with any of these were also assigned to VCG 2. To preserve the original VCG numbers as much as possible, all strains that reacted strongly with BB or MT were assigned to VCG 4.

Further subclassification of strains into additional VCGs is more problematic. Strain PCW is weakly compatible with strains that are compatible with BB but incompatible with strains that are compatible with MT. Therefore, strain PCW was assigned to VCG 3. Other difficulties may arise, such as those posed by strain TO, which is compatible with BB but incompatible with most strains that are compatible with MT. Since the behavior of strain TO resembles that of PCW, TO also was assigned to VCG 3. Because the only *nit* mutant recovered from strain CU failed to complement any of the tester strains, it is unclear whether this strain is heterokaryon self-incompatible or represents a new VCG.

Several factors that alone or in combination may lead to formation of slow-growing heterokaryons are nuclear ratio imbalance (39), influence of cultural conditions (e.g., temperature) on heterokaryon formation (41), and limited anastomosis brought about by either a relatively slow radial growth rate of strains or allele differences at a single vic or het locus (loci that control heterokaryon compatibility). The mechanism of heterokaryon incompatibility in *V. dahliae* is unknown and may be complex, involving nuclear as well as cytoplasmic genes (40). Typas (40) has suggested that, at least in some strains, cytoplasmic killing reactions identical to those described in other fungi (1,13,32,38) may be responsible for some incompatibility reactions. The rate at which lethal reactions proceed may be dependent on strain combinations. If cytoplasmic denaturation of anastomosed cells in *V. dahliae* is not rapid enough to halt the process because of some degree of compatibility, slow-growing heterokaryons could conceivably arise from the translocation of complementation products from anastomosed cells to other adjacent cells. Prototrophic growth consisting of dense mycelia and/or microsclerotia between complementary *nit* mutants is presumably the product of anastomosis and heterokaryosis. Microsclerotia are either produced alone or concomitantly with mycelia in interstrain combinations in *V. dahliae*. Since genetic control of these structures is at least partially cytoplasmic (41), it is conceivable that certain weak reactions we observed may be due to heteroplasmons rather than to heterokaryons.

In our study, strain assignment to a specific VCG was difficult in some cases. This was due to the lack of a quantitative method to assess heterokaryon formation in pairings of *nit* mutants, coupled with strain and *nit* mutant variation in ability to produce strong heterokaryons and the apparent lack of absolute incompatibility between some VCGs. Strains in VCGs 1, 2, and 4 exhibit a high degree of incompatibility with each other, and in general, assigning strains to these VCGs should not be difficult. On several occasions, however, small scattered clumps of mycelia and/or microsclerotia were observed in pairings between certain *nit* mutants of strains of VCGs 1 and 2 and of VCGs 2 and 4.

Tester strains PH and 115 (VCG 2) are weakly compatible with one another. However, several *nit* mutants from other strains were capable of complementing strongly with both (T. R. Joaquim and R. C. Rowe, *unpublished*). Similar results were observed with the tester strains of VCG 4. Considering these results, caution must be exercised in selecting tester strains. We suggest that, in addition to selecting and using sets of complementary *nit* mutants (Nit1 and NitM) from tester strains that yield strong heterokaryons, several tester strains should be used in assessing vegetative compatibility relationships. The number of testers used should depend on the heterogeneity of reactions produced between members in each VCG.

Data presented in this study show that VCG diversity in *V. dahliae* may not be as extensive as originally believed, that overlap between groups occasionally occurs, and that assignment of strains to VCGs is markedly influenced by the method selected for analyzing vegetative compatibility. Nevertheless, vegetative compatibility analysis is a useful tool for subspecific differentiation among some strain groups of *V. dahliae* and may provide information useful in ecological or epidemiological studies.

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