Improved Medium for Selecting Nitrate-Nonutilizing (*nit*) Mutants of *Verticillium dahliae*

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ABSTRACT

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Nitrate-nonutilizing (*nit*) mutants are commonly used to determine vegetative compatibility between isolates of *Verticillium dahliae* by complementation (heterokaryon) testing. These mutants emerge spontaneously as chlorate-resistant sectors growing out of partially restricted, wild-type colonies on chlorate-amended media. The commonly used chlorate media are based on minimal medium (MMC) or cornmeal agar (CMC), amend-

ed with potassium chlorate. *nit* mutants recovered on these media constituted 10 to 36% (on MMC) and 25 to 45% (on CMC) of the apparently resistant sectors. An improved water agar chlorate medium (WAC) is described that is more effective for selecting chlorate-resistant *nit* mutants. WAC medium consists of agar (2%), glucose (0.02%), and potassium chlorate (2 to 5%). On WAC, growth of most *V. dahliae* isolates was strongly inhibited, and 66 to 100% (average >80%) of the chlorate-resistant sectors formed were *nit* mutants. Most mutants were characterized as *nit1*, and about 6% as *NitM*.

Identification of vegetative compatibility groups has proven to be an effective approach for studying genetic relationships in anamorphic populations of several fungi, including the important and widespread plant pathogen *Verticillium dahliae* (17,18,22,23). Nitrate-nonutilizing (*nit*) mutants are the most commonly used in complementation (heterokaryon) tests, because they arise spontaneously at a high frequency and can be easily recovered as chlorate-resistant sectors or clones (7). Usually, minimal medium amended with potassium chlorate (MMC), as described for *Fusarium oxysporum* (5,21), is suitable for other species and genera as well (10,13,14,20). This medium, along with chlorate cornmeal agar (CMC), has been commonly used for generating *nit* mutants from *V. dahliae* and *V. albo-atrum* (1,3,4,9,11,12,24-26). However, difficulties in isolating mutants from some strains (3,9,11,12,25,26) and the need to purify *nit* mutants from apparently chlorate-resistant sectors by isolating single germinating conidia or hyphal tips (4,9) have been reported. In an ongoing study of genetic diversity in populations of *V. dahliae* in Israel (16), we have encountered similar difficulties; the proportion of *nit* mutants recovered from resistant sectors developing on the common chlorate media is relatively small. Consequently, repeated inoculation on chlorate media and further purification steps have been necessary to obtain satisfactory numbers of *nit* mutants. The relatively low recovery of *nit* mutants among the sectors tested, possibly resulting from the difficulties involved in visually distinguishing resistant sectors from the wild-type parental colony on MMC and CMC, led us to look for a more effective chlorate medium. We hypothesized that a medium with a reduced nutrient level would be more restrictive to the radial growth of wild-type colonies, thereby allowing for the more conspicuous appearance of mutant sectors. The objective of this work was to formulate a chlorate medium that would improve the recovery of *nit* mutants from *V. dahliae*.

MATERIALS AND METHODS

Isolation of *Verticillium* from plants and soil. *V. dahliae* Kleb. was isolated from cotton (*Gossypium hirsutum* L.), potato (*Solanum tuberosum* L.), eggplant (*Solanum melongena* L.), *Chrysanthemum* spp., other host plants, and soil. Plants showing symptoms of *Verticillium* wilt were collected from fields and greenhouses. Pieces of vascular tissue excised from basal stem segments were surface-sterilized with 0.5% NaOCl for 1 min, rinsed in sterile distilled water, and placed on either Czapek-Dox agar (CDA; Biolife, Milan, Italy) amended with streptomycin or penicillin sulfate (100 mg/liter), or *Verticillium*-selective medium (2). Plates were incubated at 24°C for 5 to 7 days until colonies with verticillately branched conidiophores developed around the stem tissue. The soil dilution method was used to isolate *Verticillium* from naturally infested soil. Each fresh sample of soil from the field or plant rhizosphere was mixed thoroughly, and then triplicate 5-g subsamples were suspended in 250 ml of sterile distilled water and shaken for 20 min. Soil suspension (0.3 ml per plate, 10 plates per subsample) was spread on alcohol agar (19) and incubated for 10 to 12 days at room temperature. Colonies of *V. dahliae* with black microsclerotia could be distinguished against a white background. Conidia from colonies were streaked on CDA and incubated overnight at 24°C. Germinated conidia were individually transferred to fresh CDA and maintained at 24°C. Out of 200 monoconidial isolates used, about 37% were obtained from cotton or cotton field soil, 47% from potato or potato field soil, and the remainder from other plant species. Subcultures of all isolates were stored on CDA at 5 to 7°C, and some were also stored on sterilized, dried Whatman no. 54 filter paper (Whatman International, Ltd., Maidstone, England) (6).

Generation and characterization of *nit* mutants. Three potassium chlorate-containing media in 9-cm-diameter petri plates were used to generate *nit* mutants: cornmeal agar with 0.02% glucose (CMC) (4), minimal medium (MMC) (5,21), and water agar (2%) with 0.02% glucose (WAC). The media were amended with 20 to 50 g/liter of potassium chlorate. Mycelial agar plugs of *V. dahliae* isolates were removed and placed on the chlorate media, either in the center of the agar surface or at three to six separate points, and the plates were incubated at 24°C. Chlorate-resistant

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sectors that grew out of the developing colonies, evident after 10 to 28 days, were transferred to CDA plates (5 cm in diameter). Sectors that grew on CDA as expansive colonies with a thin, non-aerial mycelium were considered *nit* mutants. The CDA medium amended with NaNO₂ (0.5 g/liter) or hypoxanthine (0.2 g/liter) was used for partial phenotyping of the *nit* mutants (5). Mutants that grew sparsely on hypoxanthine were classified as NitM, whereas mutants that grew profusely on hypoxanthine were classified as *nit1*.

Recovery of *nit* mutants from CMC. To determine the effectiveness of CMC in generating *nit* mutants from various isolates, four monoconidial subcultures were prepared from each of 50 isolates and used as replicates in four experiments (one subculture per experiment). Mycelial plugs from the *V. dahliae* subcultures were placed in the center of CMC plates containing 2.0 or 2.5% chlorate, three to five plates per isolate, and incubated for 2 to 4 weeks at 24°C. Radial growth of wild-type colonies was only partially retarded on this medium, although growth was appressed with little aerial mycelium and fewer microsclerotia. Chlorate-resistant sectors appeared as slightly faster-growing mycelium at the colony perimeter, or as patches of more dense, aerial growth scattered about the colony. A transfer loop was used to remove conidia from such sectors or patches and streak them on CDA. The resultant growth was often a mosaic of both wild-type and thin mycelium, requiring repeated purification steps. Only colonies that grew on CDA as expansive, thin mycelium were considered *nit* mutants.

Recovery of *nit* mutants from three chlorate media. In addition to CMC, MMC and WAC were used at two potassium chlorate concentrations (20 and 50 g/liter). Three *V. dahliae* isolates, one from eggplant and two from cotton, were used. Mycelial plugs of each isolate were inoculated at three points in each plate, three plates for every medium-isolate combination. Chlorate-resistant sectors or patches were subcultured on CDA after 10 to 21 days of incubation, and the proportion of *nit* mutants was determined 5 days later.

Recovery of *nit* mutants from WAC. WAC is a very simple medium consisting of agar, glucose, and potassium chlorate (20 or 50 g/liter). The generation of *nit* and NitM mutants was investigated using 200 different isolates in four experiments. Two types of colony growth and sector formation were observed on this medium, depending on the isolates tested and their chlorate sensitivity. Growth of most wild-type isolates was limited, reaching 3 to 4 mm around the inoculum plug after 10 to 14 days of incubation.

Complementation (heterokaryon) tests. Complementation between phenotypically distinct *nit* mutants was tested on CDA in 5-cm petri plates. Three mutants were inoculated per plate, 1 to 1.5 cm apart in a triangular pattern, and incubated at 24°C. Complementation was evident by the formation of a dense, wild-type mycelium where two mutants had met and formed a stable heterokaryon, usually 14 to 28 days after inoculation.

RESULTS

Recovery of *nit* mutants from CMC. An average of 1.6 sectors per colony was obtained, and about 35% of the sectors tested

were *nit* mutants (Table 1). Among the *nit* mutants, 8.4% were classified as NitM, whereas the majority were *nit1*. Some *nit* mutants grew very slowly on CDA amended with nitrite, or developed leaky, rather than expansive, thin mycelium on this medium. Consequently, they were also regarded as *nit1* mutants, whereas no *nit3* mutants could be recognized. All tested *nit* mutants exhibited wild-type growth on potato dextrose agar (PDA).

Recovery of *nit* mutants from three chlorate media. Neither sectoring nor the proportion of *nit* mutants were consistently increased by the higher chlorate concentration (Table 2), but further purification of *nit* mutants from mixed sectors was easier, because growth of parental colonies was more inhibited. Although fewer sectors were induced on WAC than on CMC or MMC, the proportion of *nit* mutants recovered from these sectors and their actual number were significantly higher (Table 2). These results suggested that WAC should be more effective than CMC or MMC in generating *nit* mutants, hence, this potential was further evaluated.

Recovery of *nit* mutants from WAC. From the restricted colony, resistant sectors appeared as a thin, fast-growing mycelium gradually surrounding the inoculum to form an asymmetric, oval-shaped colony. When conidia from such sectors were streaked onto CDA, they almost always gave rise to typical, sparsely growing *nit* mutant colonies. With most isolates, *nit* mutants were, thus, recovered from 70 to 100% of the inoculum plugs. Some isolates that were less sensitive to the chlorate concentration used grew on WAC initially as a thin colony whose radial growth was only partially reduced. On this background, resistant sectors appeared later as dense, asymmetric mycelial patches around the inoculum. Conidial transfers from such patches gave rise to mixed wild-type and mutant growth on CDA. In about 10% of the isolates tested, chlorate-resistant nitrate-utilizing (*crn*) sectors appeared frequently,

TABLE 2. Recovery of nitrate nonutilizing (*nit*) mutants from three isolates of *Verticillium dahliae* on three chlorate-containing media

Medium ^a	KClO ₃ (%)	Isolate	No. of sectors ^b	<i>nit</i> mutants (%)
CMC	2	ep8	12	25
		cot30	16	38
		cot66	17	35
		Mean	15.0	32.7
	5	ep8	12	25
		cot30	11	36
		cot66	20	45
		Mean	14.3	35.3
MMC	2	ep8	8	25
		cot30	10	10
		cot66	19	32
		Mean	12.3	22.3
	5	ep8	14	36
		cot30	18	22
		cot66	11	36
		Mean	14.3	31.3
WAC	2	ep8	12	100
		cot30	10	100
		cot66	11	64
		Mean	11.0	88.0
	5	ep8	9	100
		cot30	11	91
		cot66	12	83
		Mean	10.7	91.3

Analysis of variance

Source	df	F	P	F	P
Medium (M)	2	1.67	0.228	50.54	0.0001
KClO ₃ (C)	1	0.04	0.850	0.80	0.388
M × C	2	0.23	0.795	0.13	0.879

^a CMC = chlorate cornmeal agar, MMC = chlorate minimal medium, and WAC = chlorate water agar.

^b Number of sectors per nine inoculations.

TABLE 1. Recovery of nitrate nonutilizing (*nit*) mutants from 50 isolates^a of *Verticillium dahliae* on chlorate cornmeal agar (CMC)

Replication ^a	No. of sectors	<i>nit</i> mutants		NitM	
		No.	% total sectors	No.	% <i>nit</i> mutants
1	523	200	38	21	10.5
2	578	168	29	14	8.3
3	594	219	37	14	6.4
4	530	200	38	17	8.5
Total/mean (±SD)	2,225	787	35.5 ± 4.3	66	8.4 ± 1.6

^a Each isolate was tested four times using a different monoconidial subculture (replication) in each of four experiments (total = 200 subcultures).

making repeated inoculations necessary to obtain *nit* mutants. Of the 200 isolates tested (Table 3), more than 80% of the sectors yielded *nit* mutants, but the average frequency of NitM mutants was lower (5.5% versus 8.4%) than that obtained on CMC (Table 1). Several hundred isolates were later treated on this medium, and all of them gave rise to *nit* mutants.

Stability of *nit* mutants. Most *nit* mutants were stable when grown on CDA. However, 12% of the NitM and 22% of the *nit1* mutants developed one or more sectors of prototrophic growth after 20 to 30 days.

Complementation tests. For all tested strains (except two self-incompatible ones), formation of stable heterokaryons and biochemical complementation were observed between mutants of different phenotypes (*nit1* and NitM) derived from the same parental strain. Complementation also occurred in about 80% of the pairings between different NitM mutants.

DISCUSSION

Cove (7) found that chlorate-resistant mutants of *Aspergillus nidulans* are usually also *nit* mutants, growing as a thin, expansive mycelium on nitrate minimal medium. Puhalla (21) illustrated the use of complementary *nit* mutants to test for heterokaryosis in studies of vegetative compatibility in *F. oxysporum*. The major advantages of using *nit* mutants are their ready recovery without mutagenesis from chlorate-containing media, and their ability to grow expansively (but sparsely) on unsupplemented minimal media. Chlorate-resistant mutants, unable to metabolize nitrate (and chlorate), emerge from the restricted parental wild-type colony as faster-growing sectors.

Recovery of *nit* mutants from *Verticillium* using this method has been more difficult than from other fungal species, because chlorate does not greatly restrict the growth of many strains (4,9,26) that also show considerable variation in growth and sectoring patterns when grown on the commonly used media (4,9,11). Chlorate media used by various researchers have been based on minimal medium (1,3,4,9,24–26), cornmeal agar (1,3,4,11,12,26), PDA (3,26), and supplemented minimal medium (26), with chlorate concentrations ranging from 1.5 to 3%. Although growth of aerial mycelium is usually suppressed on such media, radial growth is only partially reduced, and irregular colony margins may be mixed or confused with apparently resistant sectors. Conidial or mycelial transfers from such sectors frequently give rise to wild-type or mixed growth instead of the *nit* phenotype, as also often occurs with transfers from aerial hyphae that grow as patches on top of the parental colony (1,3,4,9,11). Additional transfers are necessary, in such cases, to purify *nit* mutants from other components of the mixed growth (4,9). Neither sectoring nor the proportion of *nit* mutants were consistently increased by raising the chlorate concentration in the media from 2 to 5%, although further purification of *nit* mutants required fewer subculturing steps.

The 200 *V. dahliae* isolates included in this study varied considerably in sectoring frequency. About 10% of the isolates formed very few sectors, and repeated inoculations on chlorate media (sometimes as many as 25 to 30) were required to generate *nit* mutants from them. Similar difficulties have been encountered by others (3,9,11,12,25,26). The proportion of *nit* mutants recovered from sectors formed on MMC was 10 to 36% in our study and 34% (1) and 57% (9) in other studies, whereas the corresponding values for CMC were 25 to 45% and 40% (1), in agreement with another report that recovery was slightly better on CMC than on other media (3). Overall, these proportions are low, indicating a substantial waste of effort and materials, especially when studying large populations of *Verticillium*.

The WAC developed during this study offers an improved, inexpensive alternative to the media commonly used to generate *nit* mutants from *V. dahliae*. The proportion of *nit* mutants recovered from sectors formed on this medium ranged from 64 to 100%,

averaging over 80%. Thus, WAC is about twice as effective as CMC, and the number of sectors required for recovery of a given number of *nit* mutants is proportionally smaller. Furthermore, since growth of most wild-type isolates is very restricted on WAC, five or six plugs can be inoculated on one 9-cm petri plate (as compared with one plug per plate of CMC or MMC), which was sufficient for the recovery of at least one *nit* mutant (usually three to five) per plate after 10 to 14 days of incubation. This difference translates into substantial savings on plates, medium, and incubation space. Incubation period can also be shortened on this medium, since *nit* mutants were recovered from most isolates after 10 to 14 days.

nit mutants may originate with mutations at several loci involved in nitrate reduction (8,15). Most *nit* mutants can usually be divided into three phenotypic classes (*nit1*, *nit3*, and NitM), distinguishable by their growth response with nitrate, nitrite, and hypoxanthine as the nitrogen sources (5). In our work, *V. dahliae* was routinely grown on CDA (in which the nitrogen source is nitrate), and CDA amended with nitrite or hypoxanthine was used for partial phenotyping of the *nit* mutants. The availability of nitrate (in addition to the amendments) does not interfere with phenotypic identification, and distinction between *nit1* and NitM mutants of *V. dahliae* is more reliable on this set of media than on media used for *Fusarium* phenotyping (5). Only two phenotypes were identified among the approximately 3,000 mutants of *V. dahliae* generated in this and subsequent studies. Most mutants were classified as *nit1*, while only about 7% were characterized as NitM. In comparison with the previously reported values of 28 to 54% NitM (3,9,12,24,25), this proportion is small. The difference may be related to culture age and storage conditions; we have noticed that old cultures of *V. dahliae*, especially those stored on an organic medium such as PDA, often give rise to higher proportions of NitM mutants than do freshly isolated strains maintained on CDA. Here, we worked primarily with fresh isolates. No *nit3* mutants were identified in this study, although this phenotype could readily be identified among *nit* mutants of *V. nigrescens* using the same recovery and phenotyping procedures (N. Korolev, unpublished data). Several researchers have reported an inability to identify *nit3* among their mutants (3,12,24,25), while two others (1,9) reported the occurrence of *nit3* in 11.6 and 5% of the mutants recovered. The inability of many mutants to grow on the nitrite-amended media used to distinguish between *nit1* and *nit3* phenotypes has been suggested as an explanation for the failure to identify *nit3* mutants (3,25). Similarly, some of our mutants that exhibited very slow or leaky growth on nitrite-amended CDA were classified as *nit1*, because they did not develop a thin, expansive mycelium on this medium.

In addition to the 200 isolates documented here, the new WAC medium has been used with about 300 additional isolates of *V. dahliae*, and *nit* mutants were obtained from all of them. The WAC medium has proven suitable for generating *nit* mutants from other *Verticillium* species as well (N. Korolev, unpublished data) and should be useful in applications involving vegetative compatibility determinations amongst large populations of *V. dahliae* and other species.

TABLE 3. Recovery of nitrate nonutilizing (*nit*) mutants from 200 isolates of *Verticillium dahliae* on chlorate water agar (WAC)

Experiment ^a	No. of sectors	<i>nit</i> mutants		NitM	
		No.	% total sectors	No.	% <i>nit</i> mutants
1	161	140	87	3	2.1
2	168	147	88	15	10.2
3	159	125	79	10	8.0
4	115	109	95	2	1.8
Total/mean (±SD)	603	521	87.0 ± 6.6	30	5.5 ± 4.2

^a Fifty isolates per experiment.

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