Nitrate Nonutilizing Mutants of *Fusarium oxysporum* and Their Use in Vegetative Compatibility Tests

J. C. Correll, C. J. R. Klittich, and J. F. Leslie

Postdoctoral research associates and assistant professor, Department of Plant Pathology, Throckmorton Hall, Kansas State University, Manhattan 66506. Present address of first author: Department of Plant Pathology, University of California, Berkeley 94720. Address correspondence to J. F. Leslie.

Supported in part by Kansas Agricultural Experiment Station Hatch Project 547.
Contribution 87-315-J, Kansas Agricultural Experiment Station, Manhattan.
Accepted for publication 24 June 1987 (submitted for electronic processing).

ABSTRACT

Correll, J. C., Klittich, C. J. R., and Leslie, J. F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. Phytopathology 77:1640-1646.

Over 1,300 nitrate nonutilizing (*nit*) mutants were recovered from seven strains of *Fusarium oxysporum* cultured on two media, potato-dextrose agar or minimal agar, amended with 1.5% potassium chlorate. The mutants could be divided into three phenotypic classes by their growth on supplemented minimal agar medium. These classes presumably reflect mutations at a nitrate reductase structural locus (*nit1*), a nitrate-assimilation pathway-specific regulatory locus (*nit3*), and loci (at least five) that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity (NitM). *Nit* mutants in each phenotypic class were recovered from each of the seven strains of *F. oxysporum* tested. The majority (59–96%) of *nit* mutants recovered were *nit1* mutants. With all seven strains, *nit3* and NitM mutants were recovered at a much higher frequency from minimal agar medium amended with chlorate than from potato-dextrose agar amended with chlorate. For six of the seven strains, physiological complementation occurred (as a result of heterokaryon formation) between the different *nit* mutant phenotypes derived from the same parental strain. Complementation also was observed between some *nit* mutants with the same phenotype. No complementation was observed between any of the *nit* mutants recovered from a strain of *F. o.* f. sp. *melonis*; this strain has been designated "heterokaryon self-incompatible." The phenotypes of complementary *nit* mutants (arbitrarily designated *nitA* and *nitB*) recovered by other workers in three previous studies also were determined. Neither the *nitA* nor the *nitB* mutants corresponded to a particular mutant phenotype; all three phenotypes were found among the *nitA* and *nitB* mutants. With the exception of the heterokaryon self-incompatible strain, all *nit1* and *nit3* mutants from an individual strain readily complemented the NitM mutants derived from the same strain. The NitM mutants form heterokaryons rapidly and reliably and should be used as one of the *nit* mutant testers to identify the vegetative compatibility group to which isolates of *F. oxysporum* belong. Several procedures should expedite the screening of natural populations of *F. oxysporum* for vegetative compatibility.

Additional key words: nitrogen metabolism, VCG.

Fusarium oxysporum is an important vascular wilt pathogen of many agricultural crops (34,35). Strains of *F. oxysporum* are often highly host-specific. This specificity led Snyder and Hansen (45) to subdivide the species into formae speciales based on a strain's ability to cause disease on a particular host or group of hosts. Many formae speciales can be further subdivided into races on the basis of their virulence on differential host cultivars (3). Currently, there are over 122 formae speciales and races of *F. oxysporum* (3).

Although virulence has been an extremely useful characteristic for differentiating isolates of *F. oxysporum*, it is still only a single trait. Moreover, virulence has been shown to be influenced by a number of variables, including temperature (39,48), host age (22), and method of inoculation (27). Recently, Puhalla (41) has used another characteristic, vegetative (or heterokaryon) compatibility, as an alternative means of subdividing *F. oxysporum*.

Using a modification of a technique described by Cove (12), Puhalla (41) recovered nitrate nonutilizing (*nit*) mutants from 21 strains of *F. oxysporum* at high frequencies without the use of a mutagen. Some of the *nit* mutants recovered from the same parental strain were able to complement one another by forming a heterokaryon on a minimal agar medium (41) that contained sodium nitrate as the sole source of nitrogen; complementary *nit* mutants recovered from each strain were arbitrarily designated *nitA* and *nitB*. Heterokaryon formation between *nitA* and *nitB* mutants derived from the same parental strain was indicated by the development of dense aerial growth where mycelia of the two thin *nit* mutant colonies anastomosed; mycelia outside the anastomosed region remained thin. These complementary *nit* mutants have been used to force heterokaryons and thereby test isolates of *F. oxysporum* for vegetative compatibility with one another (4,8–10,16,17,23,24,41).

Vegetative compatibility is mediated by multiple vegetative incompatibility loci (*vic*, or *het*, loci) in many fungi (1,2,5,14,28,38,43,44). In most cases, vegetative compatibility is homogenic; that is, two fungal isolates are vegetatively compatible only if the alleles at each of their corresponding *vic* loci are identical. In asexually reproducing fungi, vegetatively compatible strains are much more likely to be genetically similar than vegetatively incompatible strains. For example, strains of a fungus that are vegetatively compatible are quite similar with respect to such traits as colony size (8,14), antibiotic production (14), sanguinarine sensitivity (42), virulence (4,8,10,21), and isozyme patterns (4).

Puhalla (41) used *nit* mutants to test for vegetative compatibility among 21 strains of *F. oxysporum*; he found a correlation between vegetative compatibility group (VCG) and forma specialis, i.e., members of the same VCG belong to the same forma specialis. Subsequently, *nit* mutants have been used for vegetative compatibility tests to identify *F. o.* f. sp. *apii* race 2 from a population of *F. oxysporum* colonizing roots of celery (8), to differentiate races of *F. o.* f. sp. *pisi* (10), and to differentiate strains of *F. oxysporum* pathogenic to crucifers (4). *Nit* mutants also have been used to differentiate strains in the ubiquitous nonpathogenic portion of the *F. oxysporum* population (9). Thus, vegetative compatibility tests are proving to be a powerful tool for studying genetic diversity in *F. oxysporum*.

In this investigation, the frequency and physiological phenotype of *nit* mutants recovered from *F. oxysporum* were determined. *Nit* mutants in the different phenotypic classes were then evaluated for their usefulness in vegetative compatibility tests. The phenotypes of complementary *nit* mutants from 31 strains of *F. oxysporum* recovered by other investigators (8,9,41) also were determined.

MATERIALS AND METHODS

Strains. Seven strains of *F. oxysporum* were examined in detail (Table 1). Thirty-one strains of *F. oxysporum* from which complementary *nit* mutants were recovered in three previous studies (8,9,41) were also examined (Table 1). The seven strains examined in detail were each grown on complete medium (CM) for 5–7 days. Conidia from these seven strains were transferred to 3% water agar, and individual uninucleate microconidia were isolated using a Cailloux stage-mounted micromanipulator (Stoelting Co., Chicago, IL). A single germinating microconidium was then transferred to CM. All cultures were incubated at 25 C with a 12-hr dark/12-hr light cycle; cultures were placed 30–40 cm from the light source (one General Electric 20W cool-white fluorescent tube). The resulting colony was stored on sterile filter paper at 4 C (8). This parent culture was used as the starting inoculum for all subsequent tests.

Media. Some of the media used in this study have appeared in other publications (41,43), but for the sake of completeness, all are described below.

A basal medium was prepared as follows (per liter of distilled H_2O): sucrose, 30 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 10 mg; agar, 20 g; and trace element solution, 0.2 ml. The trace element solution contained (per 95 ml of distilled H_2O): citric acid, 5 g; $ZnSO_4 \cdot 7H_2O$, 5 g; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 1 g; $CuSO_4 \cdot 5H_2O$, 0.25 g; $MnSO_4 \cdot H_2O$, 50 mg; H_3BO_4, 50 mg; and $NaMoO_4 \cdot 2H_2O$, 50 mg.

CM was made by adding the following to 1 L of the basal medium: $NaNO_3$, 2 g; N-Z amine (Sheffield), 2.5 g; yeast extract (Difco), 1 g; and 10 ml of a vitamin solution. The vitamin solution contained (per liter of 50% ethanol): thiamine HCl, 100 mg; riboflavin, 30 mg; pyridoxine·HCl, 75 mg; D-pantothenate·Ca, 200 mg; *p*-aminobenzoic acid, 5 mg; nicotinamide, 75 mg; choline·Cl, 200 mg; folic acid, 5 mg; D-biotin, 5 mg; and *myo*-inositol, 4 g.

Minimal medium (MM) was made by adding 2 g of $NaNO_3$ to 1 L of the basal medium.

Nit mutants were generated on minimal agar medium with chlorate (MMC) and potato-dextrose agar medium with chlorate (PDC). MMC was prepared by adding the following to 1 L of the basal medium: L-asparagine, 1.6 g; $NaNO_3$, 2 g; and $KClO_3$, 15 g. PDC was prepared by adding the following to 1 L of distilled H_2O: potato-dextrose broth (Difco), 24 g; agar, 20 g; and $KClO_3$, 15 g.

Genetic terminology. The genetic terminology used for a fungus with no known teleomorph is inherently difficult because analysis of meiotic products to determine allelism is not possible. Therefore, the mutants recovered in this study have been named in accordance with published information and the suggestions of Yoder et al (49). Two of the phenotypic classes identified in this study result from mutations in a single locus in *Aspergillus nidulans, Neurospora crassa*, and *F. moniliforme* (26,32). We have no evidence to indicate that the corresponding mutants in *F. oxysporum* result from mutations at more than a single locus. Consequently, we have used genotype symbols (*nit1* and *nit3*) to denote these mutants. It is likely, however, that the mutants in a

TABLE 1. Forma specialis, host and geographic origin, vegetative compatibility group (VCG), and classes of complementary *nit* mutants from strains of *Fusarium oxysporum*

Strain[a]	Forma specialis	Host, geographic origin	VCG[b]	Classes of complementary *nit* mutants[c]		
				nit1	*nit3*	NitM
O-1	*apii* (race 2)	Celery, California	0010	A	B	···
C42*	*apii* (race 2)	Celery, California	0010	A	B	C
O-2	*apii* (race 1)	Celery, France	0011	···	A	B
O-3	*apii* (race 1)	Celery, New York	0012	A	···	B
O-4	*dianthi*	Carnation, California	0020	A	···	B
O-6	*medicaginis*	Alfalfa, California	0040	A	B	···
O-734	*chrysanthemi*	Chrysanthemum, Florida	0050	A	···	B
O-844	*chrysanthemi*	Chrysanthemum, California	0051	A	···	B
O-923	*tracheiphilum*	Cowpea, Mississippi	0060	A	···	B
O-928	*pisi* (race 2)	Pea, Washington	0070	B	···	A
O-959	*medicaginis*	Alfalfa, North Carolina	0041	···	A	B
O-1078*	*lycopersici* (race 2)	Tomato, Florida	0030	A	···	B
O-1087	*niveum*	Watermelon, Florida	0080	···	A	B
O-1090	*radicis-lycopersici*	Tomato, Ontario	0090	A	···	B
O-1123	*conglutinans*	Cabbage, Minnesota	0100	A	B	···
O-1139*	*vasinfectum*	Cotton, Mississippi	0110	A	B	···
O-1222*	*cubense* (race 1)	Banana, Australia	0120	A	B	···
E660A*[d]	*melonis* (race 1)	Melon, Maryland	0134	···	···	···
T62*[d]	*melonis* (race 1)	Melon, Israel	013?	···	···	···
NP-G16*	···	Celery, California	2001	···	A	B
NP-J11	···	Celery, California	2002	A	···	B
NP-F1	···	Celery, California	2003	A	···	B
NP-H21	···	Celery, California	2004	A	···	B
NP-A1	···	Celery, California	2005	A	···	B
NP-H24	···	Celery, California	2006	···	A	B
NP-A19	···	Celery, California	2007	A	B	···
NP-B20	···	Celery, California	2008	A	···	B
NP-B38	···	Celery, California	2009	A	B	···
NP-B48	···	Celery, California	2010	A	B	···
NP-H18	···	Celery, California	2011	A	···	B
NP-I15	···	Celery, California	2012	A	B	···
NP-J8	···	Celery, California	2013	A	···	B
NP-J16	···	Celery, California	2014	A	B	···

[a] Strain numbers preceded by O were described by Puhalla (41) and those preceded by NP or C were described by Correll et al (8,9); NP isolates were recovered from asymptomatic root tissue and were nonpathogenic on celery. * = Strains examined in detail.

[b] Code follows that of Puhalla (41). First three numbers refer to the forma specialis to which the strain belongs, and the fourth refers to the VCG within the forma specialis. Nonpathogenic isolates are designated by code numbers above 2000.

[c] Phenotypic classes of complementary *nit* mutants (*nitA* and *nitB*) recovered in three previous studies (8,9,41). *nit1* = Nitrate reductase structural locus, *nit3* = nitrate-assimilation pathway-specific regulatory locus, NitM = one of five loci coding for molybdenum-containing cofactor.

[d] Strains of *F. o.* f. sp. *melonis* were supplied by D. J. Jacobson, University of California, Berkeley. Jacobson and Gordon (24) had previously observed that no complementation had occurred between *nit* mutants recovered on PDC from strain T62.

third phenotypic class result from mutations at one of several loci; therefore, we have given this class of mutants a phenotype designation (NitM). To simplify terminology within the genus, designations of *nit* mutants recovered from *F. oxysporum* correspond with those previously assigned in *F. moniliforme* (26).

Generation of nitrate nonutilizing mutants. A mycelial transfer (2-mm^3 CM block) of each strain was put in the center of a petri dish (6 cm in diameter) containing either PDC or MMC. The plates were incubated under the conditions described above and examined periodically for the appearance of fast-growing sectors from the initially restricted colony. Growth of wild-type strains is restricted on chlorate (41), presumably because cblorate is reduced by nitrate reductase to highly toxic chlorite. Nitrate nonutilizing mutants are unable to reduce chlorate to chlorite and hence are chlorate-resistant. All sectors were transferred to MM, and those that grew as thin expansive colonies with no aerial mycelium were considered *nit* mutants. All *nit* mutants were resistant to chlorate and showed wild-type growth on CM.

***Nit* mutant phenotypes.** The physiological phenotypes of *nit* mutants recovered from *F. oxysporum* were interpreted on the basis of information on nitrate reduction in other fungi (Table 2). The *nit* mutants were assigned to different phenotypic classes on the basis of their growth on media containing one of five different nitrogen sources: 1) nitrate medium = MM as described above, 2) nitrite medium = basal medium plus 0.5 g/L $NaNO_2$, 3) hypoxanthine medium = basal medium plus 0.2 g/L hypoxanthine, 4) ammonium medium = basal medium plus 1 g/L ammonium tartrate, and 5) uric acid medium = basal medium plus 0.2 g/L uric acid. To determine the physiological phenotype, a mycelial transfer (2-mm^3 MM or CM block) of the *nit* mutant was put on each of the five media. The plates were incubated as described above, and the colony morphology was scored relative to the wild-type parent after 4 days (Fig. 1, Table 2).

Some *nit* mutants were tested for nitrite excretion. Each colony was grown on urea medium (basal medium plus 0.4 g/L urea) in a 9-cm petri dish for 4 days. The plate was then flooded (approximately 10 ml) with a solution of 3 M $NaNO_3$ (255 g/L). After 8–24 hr, the nitrate solution was poured off and the presence of nitrite was determined by adding 1 ml of a sulfanilamide solution (75 ml distilled H_2O, 25 ml concentrated HCl, 1 g sulfanilamide) and 1 ml of a color indicator (100 ml distilled H_2O, 20 mg *N*-1-naphthyl-ethylenediamine·2HCl) to the plate. The presence of nitrite is indicated by bright purple color, relative to the wild-type colony (12).

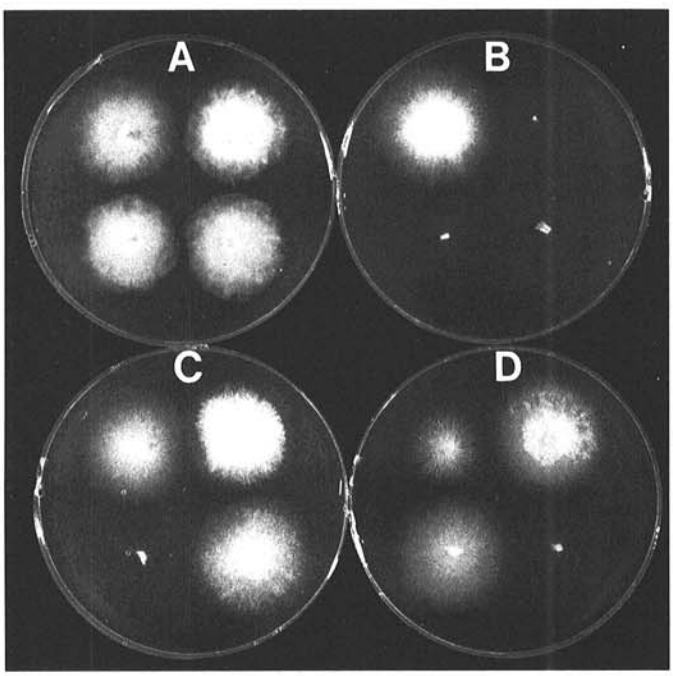

Fig. 1. Growth of wild-type parental strain (NP-G16) of *Fusarium oxysporum* and three nitrate nonutilizing (*nit*) mutant phenotypes from NP-G16 on media with one of four different nitrogen sources. The arrangement of colonies on each plate is: upper left, wild-type strain NP-G16; upper right, *nit1* mutant; lower left, *nit3* mutant; lower right, NitM mutant. **A,** Ammonium medium. **B,** Nitrate medium (although difficult to see, all four colony diameters on this plate are similar); note thin growth of the three mutants. **C,** Nitrite medium; note thin growth of *nit3*. **D,** Hypoxanthine medium; note thin growth of NitM.

Complementation tests. Vegetatively compatible *nit* mutants may complement one another by forming a heterokaryon on MM (41), i.e., dense aerial growth develops where mycelia of the two *nit* mutant colonies come in contact, anastomose, and form a heterokaryon. Pairings were made by placing mycelia from each *nit* mutant 1–3 cm apart on MM. Pairings were incubated as described above for 7–14 days and then scored for complementation. All of the *nit* mutants recovered from the same parent were paired with at least one *nit1*, one *nit3*, and one NitM mutant from that parent. Also, some *nit* mutants of the same phenotype were paired. *Nit* mutants recovered from the heterokaryon self-incompatible strain T62 were tested for complementation more rigorously. Every *nit* mutant recovered from this strain was paired with four NitM mutants (two from PDC and two from MMC) as well as a *nit1* and a *nit3* mutant from strain T62.

A *nit1* (or *nit3*) mutant and a NitM mutant from a strain in each VCG listed in Table 1 were paired in all combinations to confirm that each VCG was unique.

RESULTS

***Nit* mutant isolation.** Spontaneous chlorate-resistant sectors were readily recovered from all seven strains of *F. oxysporum* when cultured on either MMC or PDC. Chlorate-resistant sectors were recovered at a mean frequency between 0.92 and 1.65 sectors per colony on PDC and between 0.33 and 0.96 sectors per colony on MMC (Table 3). The majority (78–98%) of the chlorate-resistant sectors recovered were unable to utilize nitrate as a sole nitrogen source and consequently grew as thin expansive colonies with no aerial mycelium on MM (Fig. 1); these sectors were designated *nit* mutants. A few sectors were recovered from each strain that were chlorate-resistant but able to utilize nitrate.

***Nit* mutant phenotype identification.** The nitrate reduction pathway has been well characterized in both *N. crassa* (32,33) and *A. nidulans* (12,13). These two fungi require only two enzymes to reduce nitrate to ammonium: nitrate reductase, which converts nitrate to nitrite, and nitrite reductase, which reduces nitrite to ammonium (Fig. 2). Numerous genes, however, control the nitrate

TABLE 2. Identification of nitrate nonutilizing (*nit*) mutants from *Fusarium oxysporum* by growth on different nitrogen sources

Mutation[a]	Mutant designation	Growth on nitrogen sources[b]					Nitrite excretion[c]
		Nitrate	Nitrite	Ammonium	Hypoxanthine	Uric acid	
None	Wild-type	+	+	+	+	+	Slight
Nitrate reductase structural locus	*nit1*	−	+	+	+	+	NT
Nitrite reductase structural locus	?[d]	−	−	+	+	+	Yes
Pathway-specific regulatory locus	*nit3*	−	−	+	+	+	No
Molybdenum cofactor loci	NitM	−	+	+	−	+	NT
Major nitrogen regulatory locus	?[d]	−	−	+	−	−	NT

[a] Compiled from Garrett and Amy (20) and Marzluf (32) on the basis of analysis of mutants from *Aspergillus nidulans* and *Neurospora crassa*.
[b] Growth on basal medium with various nitrogen sources; + = typical wild-type growth, − = thin growth with no aerial mycelium.
[c] Nitrite excretion test as described by Cove (12). Yes = positive (bright purple) reaction, slight = some (light purple) reaction, no = negative (no purple) reaction, NT = not tested.
[d] Mutants were not recovered.

assimilation process in these fungi (at least 11 genes in *A. nidulans* and eight in *N. crassa*), and their regulation is complex (13,33). In general, the genes necessary for nitrate assimilation in *Aspergillus* and *Neurospora* appear to be similar in function. For example, both the nitrate and nitrite reductase structural polypeptides are encoded by single genes in these fungi. Both fungi also require several genes (at least five in *A. nidulans* and four in *N. crassa*) for the synthesis of a molybdenum-containing cofactor that is part of the nitrate reductase complex. This cofactor is also essential for purine dehydrogenase (37) activity; in the purine catabolism pathway, purine dehydrogenase hydroxylates hypoxanthine (Fig. 2). In addition to these structural loci, two regulatory loci are known in *A. nidulans* and *N. crassa*. The product of the nitrate-assimilation pathway-specific regulatory locus controls the induction of nitrate reductase and nitrite reductase. A second locus, the major nitrogen regulatory locus, represses the synthesis of both nitrate reductase and nitrite reductase whenever a preferred nitrogen source such as ammonium or glutamine is present (13,33).

The phenotypes of the *nit* mutants from *F. oxysporum* were determined by their colony morphology on media containing one of five different nitrogen sources (Fig. 1, Table 2). The *nit* mutants could be divided into three phenotypic classes. These classes presumably represent a mutation at a nitrate reductase structural locus (*nit1*), a nitrate-assimilation pathway-specific regulatory locus (*nit3*), and loci (at least five) that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity (NitM). To distinguish nitrite reductase mutants from pathway-specific regulatory mutants, *nit* mutants that grew as thin colonies on nitrite medium were checked for nitrate reductase activity by testing for nitrite excretion (Table 2).

The majority of *nit* mutants recovered on both PDC and MMC were *nit1* mutants (Table 3). However, the frequency of *nit1* mutants recovered was considerably higher on PDC (82–95%) than on MMC (59–66%). Consequently, the relative frequency of both the *nit3* and the NitM mutants recovered was considerably higher on MMC (10–28% were *nit3* and 10–25% were NitM) than on PDC (0–12% were *nit3* and 2–10% were NitM).

Two phenotypic classes that could affect nitrate assimilation were not recovered (Table 2). First, none of the *nit* mutants recovered were nitrite reductase mutants. Second, none of the chlorate-resistant sectors recovered were mutants at a major nitrogen regulatory locus, i.e., all of the chlorate-resistant sectors utilized uric acid.

Several sectors were recovered from each strain that were resistant to chlorate but had a wild-type colony morphology on MM. Several such sectors from each of the seven strains were examined in more detail to determine if these sectors resulted from a mutation in a single locus or from the heterokaryotic growth of complementary mutations in two or more different nuclei. Microconidia from these sectors were spread onto 3% water agar, and approximately 10 uninucleate microconidia were isolated as previously described. These conidia were transferred to MM, incubated for 5 days, and then scored for growth. Colonies resulting from each microconidium either remained thin on MM (typical *nit* mutant morphology) or had abundant aerial mycelium on MM (typical of wild-type morphology). The analysis of microconidia indicated that the chlorate-resistant nitrate utilizing sectors could be homokaryotic or heterokaryotic. Individual microconidia from homokaryotic sectors had a wild-type morphology on MM and presumably were mutants that were both chlorate-resistant and able to utilize nitrate (*crn* mutants). Microconidia recovered from heterokaryotic sectors were often a mixture of *nit* mutant conidia, wild-type conidia, and/or *crn* mutant conidia. Both homokaryotic (*crn* mutants) and heterokaryotic sectors were identified in each of the seven strains examined.

We determined the physiological phenotypes of complementary *nit* mutants (designated as *nitA* and *nitB*) from 31 strains of *F. oxysporum* recovered by other investigators in three previous studies (8,9,41). These mutants had been designated as either *nitA* or *nitB* to indicate they were different and could complement one another. Therefore, these designations were operational and implied no physiological phenotype. Not surprisingly, neither the *nitA* nor the *nitB* mutants corresponded to a particular *nit* mutant phenotype; all three phenotypes were found among both the *nitA* and *nitB* mutants from the various strains. We subsequently isolated a NitM mutant from any strain in which neither the *nitA* nor the *nitB* mutation fell into the NitM class. All wild-type strains and a NitM mutant derived from each wild-type strain have been deposited at the Fusarium Research Center (The Pennsylvania State University, University Park).

Complementation tests. Physiological complementation between *nit* mutants with different mutations was indicated by the development of dense aerial growth where the mycelia of the *nit* mutant colonies came in contact and anastomosed to form a heterokaryon. Complementation occurred between *nit* mutants with different phenotypes in six of the seven strains used in the current study. Complementation occurred more rapidly and growth of the resulting heterokaryon was more robust in pairings of NitM with *nit1* or *nit3* mutants than in pairings of *nit1* with *nit3* mutants (Table 4). When *nit1* and *nit3* mutants were paired, complementation often was not evident for 2–3 wk. Frequently, even after 3 wk, the complementation reaction was weak (very little aerial mycelium). In some pairings of *nit1* with *nit3* mutants

TABLE 3. Frequency and phenotype of nitrate nonutilizing (*nit*) mutants recovered from two media

Strain[a]	Medium[b]	Sectors per colony[c]	*nit*[d] (%)	Number of *nit* mutants examined	*Nit* mutant classes[e] *nit1* (%)	*nit3* (%)	NitM (%)
O-1078	PDC	1.25	94	118	90	0	10
	MMC	0.78	87	102	65	10	25
O-1139	PDC	1.44	97	139	94	4	2
	MMC	0.33	96	67	59	28	13
O-1222	PDC	1.65	90	149	85	11	4
	MMC	0.96	80	77	62	27	10
NP-G16	PDC	1.20	83	100	95	1	4
	MMC	0.91	95	86	66	16	17
C42	PDC	0.92	89	82	92	2	6
	MMC	0.38	78	59	64	19	17
E660A	PDC	1.31	98	121	96	1	3
	MMC	0.58	97	65	62	23	15
T62	PDC	1.15	96	86	82	12	6
	MMC	0.91	92	84	63	24	13

[a] See Table 1.
[b] PDC = potato-dextrose agar medium plus chlorate, MMC = minimal agar medium plus chlorate.
[c] Mean frequency of chlorate-resistant sectors per colony.
[d] Percent of chlorate-resistant sectors that grew as thin expansive colonies on minimal medium.
[e] *Nit* mutant phenotypes determined according to growth on basal medium amended with different nitrogen sources (see Table 2). *nit1* = a mutation in a nitrate reductase structural locus, *nit3* = a mutation in a nitrate-assimilation pathway-specific regulatory locus, NitM = a mutation in one of five loci that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity.

TABLE 4. Complementation reactions between nitrate nonutilizing (*nit*) mutants of *Fusarium oxysporum*[a]

	nit1	*nit3*	NitM
nit1	− or ±	± or −	+
nit3	± or −	−	+
NitM	+	+	+ or −

[a] The three phenotypic classes recovered from *F. oxysporum* presumably reflect mutations in a nitrate reductase structural locus (*nit1*), a nitrate-assimilation pathway-specific regulatory locus (*nit3*), and one of five loci that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity (NitM). + = Complementation occurs readily, − = no complementation occurs, ± = weak and/or slow complementation occurs.

recovered from the same parental strain, no complementation was observed. Some *nit1* mutants were able to complement one another, as could some NitM mutants. No complementation was observed between any of the *nit3* mutants.

Complementation between different *nit1* mutants usually was slow and quite weak. The *nit1* complementation pattern was typical of complementation between mutants within the same gene (intragenic) rather than of complementation between different genes (intergenic) (18). For example, if three *nit1* mutants, x, y, and z, were paired to test for complementation, x and y could both fail to complement z, indicating that x and y were in the same locus as z. It is still possible, however, for x and y to form a complementing heterokaryon with each other if they alter the *nit1* gene product in different ways. Such intragenic complementation usually produces a heterokaryon intermediate between one in which no complementation occurs and one in which complete complementation occurs (18). Since similar complementation patterns have been observed between *nit1* mutants of *F. moniliforme* that map to the same locus (26), we expect all *nit1* mutants of *F. oxysporum* to be allelic as well.

The complementation reactions between different NitM mutants, on the other hand, were rapid and robust and resulted in a heterokaryon with dense aerial mycelium. For six of the seven strains, two to six complementation groups were identified among the NitM mutants. With NitM mutants, intergenic complementation patterns prevailed.

No complementation occurred between any *nit* mutant testers (a *nit1* or *nit3* mutant and a NitM mutant) recovered from different strains when these testers were paired in all combinations. Thus, each VCG listed in Table 1, with the possible exception of strain T62, represents a distinct VCG.

Heterokaryon self-incompatibility. No complementation occurred between any *nit* mutants of *F. o.* f. sp. *melonis* (strain T62), even after repeated attempts. A total of 166 *nit* mutants (124 *nit1*, 30 *nit3*, and 12 NitM mutants) failed to complement either a *nit1* mutant, a *nit3* mutant, or four NitM mutants, all of which were recovered from strain T62. Furthermore, no complementation was observed when the *nit1* or NitM mutants were paired among themselves. The lack of complementation between phenotypically distinct *nit* mutants recovered from strain T62 lead us to designate this strain "heterokaryon self-incompatible."

DISCUSSION

***Nit* mutants in fungi.** Most fungi can utilize nitrate as a nitrogen source by reducing it to ammonium (Fig. 2) via nitrate reductase and nitrite reductase (19). Some fungi unable to utilize nitrate, such as the higher Basidiomycetes, the Saprolegniaceae, and the Blastocladiales, apparently cannot synthesize nitrate reductase (47). Chlorate, a nitrate analogue, has been very useful for studying nitrate assimilation in fungi as well as bacteria, algae, and plants (15). The reduction of chlorate to chlorite by nitrate reductase can presumably result in chlorate toxicity in these organisms. Although other modes of action may be possible (11), in general, chlorate-sensitive strains can reduce nitrate to nitrite but chlorate-resistant strains cannot.

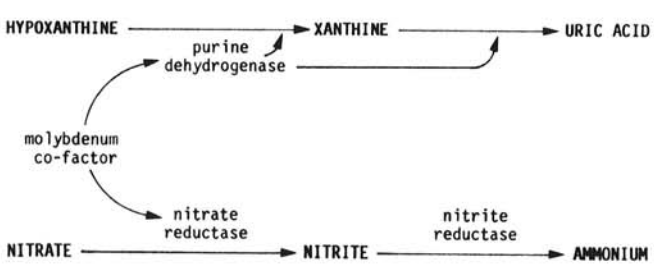

Fig. 2. Nitrate utilization pathway in *Aspergillus nidulans* and *Neurospora crassa*.

In the current study, nitrate nonutilizing (*nit*) mutants were recovered from *F. oxysporum*. *Nit* mutants have also been recovered from a number of other fungi, including *N. crassa* (32), *Ustilago maydis* (31), *A. nidulans* (12), *A. flavus* (36), *F. moniliforme* (26,44), *F. solani* (6), and *Verticillium albo-atrum* (21). All the *nit* mutants recovered from *F. oxysporum* could be divided into three distinct phenotypic classes. These classes presumably reflect mutations at a nitrate reductase structural locus (*nit1*), a nitrate-assimilation pathway-specific regulatory locus (*nit3*), and loci (at least five) that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity (NitM). These phenotypic classes correspond with those recovered from *Gibberella fujikuroi* (*F. moniliforme*) (26).

In a genetic analysis of *nit* mutants recovered from *F. moniliforme*, Klittich et al (26) have mapped specific *nit* loci; *nit1* and *nit3* mutants each map to a single locus, whereas five *nit* loci are known to affect the synthesis of the molybdenum-containing cofactor. Since *F. oxysporum* has no known teleomorph available for genetic analysis, we assume that the mutations we observed correspond to those found in *F. moniliforme* and are responsible for the three phenotypes observed in *F. oxysporum*. Therefore, we have chosen to use the same *nit* mutant gene name and number designation used with *F. moniliforme* (26). Since NitM mutants in *F. oxysporum* cannot be distinguished from one another genetically, they have not been given genotype designations. It is likely, on the basis of complementation reactions (18), that they represent mutations at more than one locus.

Conspicuous by their absence are mutants that affect the major nitrogen regulatory locus such as *areA* in *A. nidulans* and *nit-2* in *N. crassa*. In these two fungi, these mutants are often chlorate-resistant and occur relatively frequently (12,13,32,33). A possible explanation for this absence in *F. oxysporum* is that the mutants at an equivalent locus are either lethal or chlorate-sensitive. This explanation would be consistent with observations of Leslie (30) on the *nnu* mutant of *G. zeae* (*F. graminearum*). This putative regulatory mutant is unable to utilize most of the nitrogen sources catabolized by its wild-type parent, including nitrate (29), but is chlorate-sensitive and produces chlorate-resistant sectors when cultured on MMC (J. F. Leslie, *unpublished*). Since the *nnu* mutant differs significantly from its counterparts in *N. crassa* and *A. nidulans*, it is possible that at least some aspects of nitrogen regulation in the Fusaria are different from those in these better-studied fungi.

Application of *nit* mutants for vegetative compatibility tests. Virulence has been, and undoubtedly will continue to be, a very useful trait for the characterization of diversity among strains of *F. oxysporum*. Vegetative compatibility, however, is another useful tool for identifying diversity among strains of *F. oxysporum*. Vegetative, or heterokaryon, incompatibility is widespread in many fungi (1,2,5,6,14,21,38,44) including *F. oxysporum* (4,8–10,16,17,23,24,41). As a result, vegetative incompatibility phenotypes are naturally occurring genetic markers that could be used to differentiate strains of *F. oxysporum*.

To determine if two isolates are vegetatively compatible, the isolates must be marked with complementary mutants so that heterokaryotic growth can be detected on a selective medium. In the past, the generation of such auxotrophic mutants employed traditional mutagenesis techniques that were so laborious as to render the procedure almost useless for screening a population of field isolates for vegetative compatibility. *Nit* mutants, on the other hand, can readily be recovered from *F. oxysporum*. Since these mutations probably affect at least seven loci with three distinct phenotypes, the *nit* mutants can be used as forcing markers in the formation of heterokaryons. The nature and relative frequency of these mutants play an important role in determining the most efficient and reliable procedure for screening natural populations for vegetative compatibility.

In three previous studies (8,9,41), complementary *nit* mutants were arbitrarily designated as *nitA* and *nitB*. These *nit* mutants were then used as tester strains in vegetative compatibility tests with other isolates of *F. oxysporum*. Some problems, however, have been encountered with use of certain *nit* mutant tester strains

in vegetative compatibility tests. For example, Correll et al (8) showed that a *nit* mutant from 64% of the isolates of *F. o.* f. sp. *apii* race 2 examined did not complement either the *nitA* (*nit1*) or the *nitB* (*nit3*) testers from a standard *F. o.* f. sp. *apii* race 2 isolate. However, the *nit* mutant from each of these isolates did complement a third *nit* mutant, designated *nitC*, strain C42 (Table 1). Also, both the *nitA* (*nit1*) and the *nitB* (*nit3*) testers generated from strain C42 by Correll et al (8) readily complemented this *nitC* mutant. This *nitC* mutant, which was considered to be a good tester, showed thin growth on hypoxanthine medium and has been renamed NitM. Thus, it is possible to obtain misleading results if neither tester strain is a NitM mutant.

With the exception of heterokaryon self-incompatible strain T62, all NitM mutants from a given strain of *F. oxysporum* readily complemented all of the *nit1* and *nit3* mutants from the same strain (Table 4). Furthermore, some NitM mutants also complemented each other. Our data suggest that the NitM mutants are the most reliable *nit* mutant tester strains in vegetative compatibility tests. Thus, investigators using *nit* mutants to determine the vegetative compatibility of field isolates of *F. oxysporum* should identify a NitM tester strain for each different VCG. This procedure should help to reduce the occurrence of false-negative complementation reactions between vegetatively compatible isolates.

Several procedures also should reduce the time needed to screen a large population of field isolates of *F. oxysporum* for vegetative compatibility. Culturing the parental strain on 10 MMC plates should be sufficient to generate a NitM mutant tester. Other isolates could be tested for vegetative compatibility with this particular strain by pairing a single *nit* mutant from each uncharacterized isolate with the NitM tester. The *nit* mutants from uncharacterized isolates should be generated on PDC, not MMC, as sectors occur more frequently on this medium and a higher proportion of these sectors are *nit1*. A *nit* mutant from up to five different isolates can be paired with a NitM tester on a single 6-cm petri plate containing MM. If a *nit* mutant from an uncharacterized isolate complements a NitM mutant tester strain from a known VCG, then that isolate can be assigned to the known VCG. Isolates with *nit* mutants that fail to complement with any of the NitM mutant testers should be handled as follows: First, the phenotype of the *nit* mutant from the uncharacterized isolates should be determined. If this *nit* mutant is NitM, then a *nit1* or *nit3* mutant should be generated and the pairings with the NitM testers repeated. Once nonidentity with the known VCGs is established, at least one *nit* mutant from each phenotypic class (*nit1, nit3*, and NitM) should be generated in each uncharacterized isolate. If complementation occurs between phenotypically distinct mutants recovered from an uncharacterized isolate, a new VCG has been identified and a NitM mutant should be retained and used as a tester for other uncharacterized isolates. If complementation does not occur between phenotypically distinct *nit* mutants, the parental strain can be designated "heterokaryon self-incompatible."

Neither the frequency nor the importance of heterokaryon self-incompatibility in *F. oxysporum* is known. Although heterokaryon self-incompatibility may be due to some anomaly of the *nit* mutants themselves, it seems unlikely. Puhalla (40) apparently observed a similar phenomenon with a strain of *F. oxysporum* when color mutants were used for complementation tests. Papa (36) also observed a lack of complementation between two different *nit* mutant phenotypes in each of two isolates of *A. flavus*. He suggested, however, that the lack of complementation may have been the result of a double mutation in some of the *nit* mutants.

The lack of complementation observed may be due to a strain's inherent inability to anastomose. For example, preliminary observations on heterokaryon self-incompatibility in several strains of *F. oxysporum* (24), *F. moniliforme* (7), and *V. albo-atrum* (J. C. Correll, *unpublished*) indicate that hyphae of these wild-type strains rarely anastomose. In *F. moniliforme*, heterokaryon self-incompatibility is a heritable trait and may be controlled by a single nuclear gene (7). If heterokaryon self-incompatibility is widespread in *F. oxysporum*, then other measures of genetic diversity, such as isozyme patterns (4) and DNA restriction fragment length polymorphisms (25,46), could be employed to determine strain relationships.

The use of *nit* mutants should enable researchers to compare strains of *F. oxysporum* (formae speciales, races, and nonpathogens) for vegetative compatibility worldwide. These techniques, coupled with virulence tests, should provide valuable information on the genetic diversity of natural populations of this ubiquitous soil inhabitant.

LITERATURE CITED

1. Anagnostakis, S. L. 1982. Genetic analyses of *Endothia parasitica*: Linkage data for four single genes and three vegetative compatibility types. Genetics 102:25-28.
2. Anagnostakis, S. L. 1987. Chestnut blight: The classical problem of an introduced pathogen. Mycologia 79:23-27.
3. Armstrong, G. M., and Armstrong, J. K. 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. Pages 391-399 in: Fusarium: Diseases, Biology, and Taxonomy. P. E. Nelson, T. A. Toussoun, and R. J. Cook, eds. Pennsylvania State University Press, University Park. 457 pp.
4. Bosland, P. W., and Williams, P. H. 1987. An evaluation of *Fusarium oxysporum* from crucifers based on pathogenicity, isozyme polymorphism, vegetative compatibility and geographical origin. Can. J. Bot. 65:2067-2073.
5. Brasier, C. M. 1983. Inter-mycelial recognition systems in *Ceratocystis ulmi*: Their physiological properties and ecological importance. Pages 451-497 in: The Ecology and Physiology of the Fungal Mycelium. D. H. Jennings and A. D. M. Rayner, eds. Cambridge University Press, London.
6. Correll, J. C. 1986. Differential growth response of *Fusarium oxysporum* and *Fusarium solani* on a medium containing potassium chlorate. (Abstr.) Phytopathology 76:1145.
7. Correll, J. C., Leslie, J. F., and Klittich, C. J. R. 1987. Observations on the heritability of heterokaryon "self-incompatibility" in *Gibberella fujikuroi* (*Fusarium moniliforme*). (Abstr.) Phytopathology 77:1710-1711.
8. Correll, J. C., Puhalla, J. E., and Schneider, R. W. 1986. Identification of *Fusarium oxysporum* f. sp. *apii* on the basis of colony size, virulence, and vegetative compatibility. Phytopathology 76:396-400.
9. Correll, J. C., Puhalla, J. E., and Schneider, R. W. 1986. Vegetative compatibility groups among nonpathogenic root colonizing strains of *Fusarium oxysporum*. Can. J. Bot. 64:2358-2361.
10. Correll, J. C., Puhalla, J. E., Schneider, R. W., and Kraft, J. M. 1985. Differentiating races of *Fusarium oxysporum* f. sp. *pisi* based on vegetative compatibility. (Abstr.) Phytopathology 75:1347.
11. Cove, D. J. 1976. Chlorate toxicity in *Aspergillus nidulans*: Studies of mutants altered in nitrate assimilation. Mol. Gen. Genet. 146:147-159.
12. Cove, D. J. 1976. Chlorate toxicity in *Aspergillus nidulans*: The selection and characterization of chlorate resistant mutants. Heredity 36:191-203.
13. Cove, D. J. 1979. Genetic studies of nitrate assimilation in *Aspergillus nidulans*. Biol. Rev. 54:291-327.
14. Croft, J. H., and Jinks, J. L. 1977. Aspects of the population genetics of *Aspergillus nidulans*. Pages 339-360 in: Genetics and Physiology of *Aspergillus*. J. E. Smith and J. A. Pateman, eds. Academic Press, New York.
15. Dunn-Coleman, N. S., Smarrelli, J., Jr., and Garrett, R. H. 1984. Nitrate assimilation in eukaryotic cells. Int. Rev. Cytol. 92:1-50.
16. Elias, K. S., and Schneider, R. W. 1986. Genetic diversity within *Fusarium oxysporum* f. sp. *lycopersici* as determined by vegetative compatibility (heterokaryosis) tests. (Abstr.) Phytopathology 76:1129.
17. Elmer, W. H., and Stephens, C. T. 1986. Vegetative compatibility groupings in *Fusarium oxysporum* f. sp. *asparagi*. (Abstr.) Phytopathology 76:1114.
18. Fincham, J. R. S. 1966. Genetic Complementation. Benjamin, New York. 143 pp.
19. Garraway, M. O., and Evans, R. C. 1984. Fungal Nutrition and Physiology. John Wiley & Sons, New York. 401 pp.
20. Garrett, R. H., and Amy, N. K. 1978. Nitrate assimilation in fungi. Adv. Microb. Physiol. 18:1-65.
21. Gordon, T. R., Correll, J. C., and McCain, A. H. 1986. Host specificity and vegetative compatibility in *Verticillium albo-atrum*. (Abstr.) Phytopathology 76:1111.
22. Hart, L. P., and Endo, R. M. 1981. The effect of time of exposure to inoculum, plant age, root development, and root wounding on Fusarium yellows of celery. Phytopathology 71:77-79.
23. Ireland, K. F., and Lacy, M. L. 1986. Identification of pathogenic isolates of *Fusarium oxysporum* f. sp. *apii* race 2. (Abstr.)

Phytopathology 76:956.
24. Jacobson, D. J., and Gordon, T. R. 1987. Vegetative compatibility and self-incompatibility within *Fusarium oxysporum* f. sp. *melonis*. (Abstr.) Phytopathology 77:1696.
25. Kistler, H. C., Bosland, P. W., Benny, U., Leong, S., and Williams, P. H. 1987. Relatedness of strains of *Fusarium oxysporum* from crucifers measured by examination of mitochondrial and ribosomal DNA. Phytopathology 77:1289-1293.
26. Klittich, C. J. R., Leslie, J. F., and Wilson, J. D. 1986. Spontaneous chlorate-resistant mutants of *Gibberella fujikuroi (Fusarium moniliforme)*. (Abstr.) Phytopathology 76:1142.
27. Kraft, J. M., and Haglund, W. A. 1978. A reappraisal of the race classification of *Fusarium oxysporum* f. sp. *pisi*. Phytopathology 68:273-275.
28. Leach, J., and Yoder, O. C. 1983. Heterokaryon incompatibility in the plant-pathogenic fungus, *Cochliobolus heterostrophus*. J. Hered. 74:149-152.
29. Leslie, J. F. 1986. Utilization of nitrogen sources by *Gibberella zeae*. Mycologia 78:568-576.
30. Leslie, J. F. 1987. A nitrate non-utilizing mutant of *Gibberella zeae*. J. Gen. Microbiol. 133:1279-1287.
31. Lewis, C. M., and Fincham, J. R. S. 1970. Regulation of nitrate reductase in the Basidiomycete *Ustilago maydis*. J. Bacteriol. 103:55-61.
32. Marzluf, G. A. 1981. Regulation of nitrogen metabolism and gene expression in fungi. Microbiol. Rev. 45:437-461.
33. Marzluf, G. A., Perrine, K. G., and Nahm, B. H. 1985. Genetic regulation of nitrogen metabolism in *Neurospora crassa*. Pages 83-94 in: Molecular Genetics of Filamentous Fungi. W. E. Timberlake, ed. Alan R. Liss, New York.
34. Nelson, P. E. 1981. Life cycle and epidemiology of *Fusarium oxysporum*. Pages 51-80 in: Fungal Wilt Diseases of Plants. M. E. Mace, A. A. Bell, and C. H. Beckman, eds. Academic Press, New York. 640 pp.
35. Nelson, P. E., Toussoun, T. A., and Cook, R. J., eds. 1981. Fusarium: Diseases, Biology, and Taxonomy. Pennsylvania State University Press, University Park. 457 pp.
36. Papa, K. E. 1986. Heterokaryon incompatibility in *Aspergillus flavus*. Mycologia 78:98-101.
37. Pateman, J. A., Cove, D. J., Rever, B. M., and Roberts, D. B. 1964. A common cofactor for nitrate reductase and xanthine dehydrogenase which also regulates the synthesis of nitrate reductase. Nature 201:58-60.
38. Perkins, D. D., Radford, A., Newmeyer, D., and Björkman, M. 1982. Chromosomal loci of *Neurospora crassa*. Microbiol. Rev. 46:426-570.
39. Pound, G. S., and Fowler, D. L. 1953. Fusarium wilt of radish in Wisconsin. Phytopathology 43:277-280.
40. Puhalla, J. E. 1984. A visual indicator of heterokaryosis in *Fusarium oxysporum* from celery. Can. J. Bot. 62:540-545.
41. Puhalla, J. E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Can. J. Bot. 63:179-183.
42. Puhalla, J. E., and Hummel, M. 1983. Vegetative compatibility groups within *Verticillium dahliae*. Phytopathology 73:1305-1308.
43. Puhalla, J. E., and Spieth, P. T. 1983. Heterokaryosis in *Fusarium moniliforme*. Exp. Mycol. 7:328-335.
44. Puhalla, J. E., and Spieth, P. T. 1985. A comparison of heterokaryosis and vegetative incompatibility among varieties of *Gibberella fujikuroi (Fusarium moniliforme)*. Exp. Mycol. 9:39-47.
45. Snyder, W. C., and H. N. Hansen. 1940. The species concept in *Fusarium*. Am. J. Bot. 27:64-67.
46. Taylor, J. W. 1986. Fungal evolutionary biology and mitochondrial DNA. Exp. Mycol. 10:259-269.
47. Whitaker, A. 1976. Amino acid transport in fungi: An essay. Trans. Br. Mycol. Soc. 67:365-376.
48. Williams, P. H., ed. 1981. Fusarium yellows. Pages 124-129 in: Screening Crucifers for Multiple Disease Resistance. University of Wisconsin, Madison.
49. Yoder, O. C., Valent, B., and Chumley, F. 1986. Genetic nomenclature and practice for plant pathogenic fungi. Phytopathology 76:383-385.