The Use of Nitrate-Nonutilizing Mutants and a Selective Medium for Studies of Pathogenic Strains of *Fusarium oxysporum*

ESTHER HADAR and J. KATAN, Department of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Faculty of Agriculture, Rehovot 76100, Israel, and TALMA KATAN, Department of Plant Pathology, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

ABSTRACT

Hadar, E., Katan, J., and Katan, T. 1989. The use of nitrate-nonutilizing mutants and a selective medium for studies of pathogenic strains of *Fusarium oxysporum*. Plant Disease 73:800-803.

A chlorate-containing selective medium was developed for tracing chlorate-resistant, nitrate-nonutilizing (*nit*) mutants of pathogenic strains of *Fusarium oxysporum*. Neither wild-type strains of *Fusarium* spp. nor most of the fungi from six field soils could grow on this medium, whereas most *nit* mutants of six formae speciales grew on it. The *nit* mutants retained their pathogenicity. Survival in soil of *nit* mutants belonging to three formae speciales was comparable to that of their wild-type parents. The use of labeled *nit* mutants, combined with the chlorate-containing selective medium, can facilitate ecological studies of pathogenic strains of *F. oxysporum*.

Several methods have been developed for the quantitative estimation of populations of *Fusarium* spp. in soil by means of selective media (6,9,12,13). None of the conventional selective media enables the distinction between pathogenic and nonpathogenic strains, and once a specific strain of *F. oxysporum* is introduced into the soil, it often becomes morphologically indistinguishable from the natural *Fusarium* population when reisolation is attempted. Usually, the only way to estimate the frequency of a specific pathogenic strain in a mixed population of *Fusarium* spp. is to test the pathogenicity of random, representative individual cultures. This procedure is tedious, lengthy, and expensive. One approach to overcome this difficulty is to use strains labeled with specific markers. For example, mutants that are resistant to antimicrobial agents can be recovered and counted directly on a selective medium containing such chemicals (7). Another approach is the use of auxotrophic mutants frequently obtained through mutagenesis. However, such mutants might not be true representatives of the natural population of the pathogen.

Chlorate ion is toxic to many organisms, including *Fusarium* spp., but chlorate-resistant mutants frequently appear spontaneously as fast-growing sectors from restricted colonies on chlorate-amended media. Many chlorate-resistant mutants of *F. oxysporum* are auxotrophs, unable to utilize nitrate as a sole nitrogen source. Such nitrate-nonutilizing mutants, designated *nit*, form a thin, expansive mycelium on nitrate minimal medium but grow densely in the presence of ammonium or organic nitrogen sources. Puhalla (10) introduced the use of *nit* mutants for studying heterokaryosis between strains of *F. oxysporum*. We found that *nit* mutants of *F. o.* f. sp. *vasinfectum* retained their pathogenicity (4).

The purposes of this study were: 1) to develop a convenient method for estimating populations of pathogenic strains of *F. oxysporum* based on the use of chlorate-resistant *nit* mutants and a chlorate-containing selective medium and 2) to test the applicability of this method for studies of *F. oxysporum* in field soils.

Contribution 2526-E, 1988 series, from the Agricultural Research Organization, Volcani Center, Bet Dagan, Israel.

Accepted for publication 24 March 1989 (submitted for electronic processing).

MATERIALS AND METHODS

Media. Potato-dextrose agar (PDA) was used to maintain cultures and to grow inoculum for pathogenicity tests. Peptone-pentachloronitrobenzene (PCNB) medium (PPSM) (9), acidified with 1 ml/L of 90% lactic acid to suppress bacterial contamination, was used as a standard *Fusarium*-selective medium. The *Fusarium*-minimal medium (FMM) was Puhalla's MM (10), a sucrose-salt medium containing nitrate as the nitrogen source and consisting of (per liter): sucrose, 30 g; $NaNO_3$, 2 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; trace elements solution, 0.2 ml; and Difco agar, 20 g. Trace elements solution contains (per 95 ml of distilled water): citric acid, 5 g; $ZnSO_4 \cdot 7H_2O$, 5 g; $FeSO_4$, 4.75 g; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 1 g; $CuSO_4 \cdot 5H_2O$, 250 mg; $MnSO_4 \cdot H_2O$, 50 mg; H_3BO_3, 50 mg; and $Na_2MoO_4 \cdot 2H_2O$, 50 mg. FMM was used to recognize *nit* mutants. Chlorate-FMM (FMMC,

designated as MMC by Correll et al [2]), consisting of FMM amended with 15 g/L of $KClO_3$ and 1.6 g/L of L-asparagine, was used to generate *nit* mutants and served as the basis of the new selective medium, FMMCPA.

Pathogens. The pathogenic formae speciales of *Fusarium oxysporum* Schlecht. used were: *dianthi* (Prill. & Del.) Snyd. & Hans., *vasinfectum* (Atk.) Snyd. & Hans., *niveum* (E. F. Smith) Snyd. & Hans., *melonis* Snyd. & Hans., *melongenae* Matuo & Ishigami, and *radicis-lycopersici* Jarvis & Shoemaker.

Pathogenicity tests. Pathogenicity tests were carried out in a greenhouse by inoculating seedlings of the appropriate host plant (rooted cuttings in the case of carnation) with each isolate using the root-dip technique. Symptoms were visible on the inoculated plants within 10–20 days. Noninoculated seedlings, maintained as controls in the same greenhouses, remained healthy throughout the pathogenicity tests.

Isolation of *nit* mutants. All *nit* mutants were isolated on chlorate-amended media (2,4,10) and showed wild-type growth on PDA. Mutants FOD-E72, FOV-51, and FOV-210 were characterized as belonging to phenotypic class *nit* M (2); the other mutants have not been phenotyped.

Soils. Samples of field soils were collected from six sites in Israel (Table 1).

Production of inocula for survival studies. Mycelial disks from 5-day-old cultures of each isolate were placed in Erlenmeyer flasks (250 ml) containing 100 ml of liquid medium (5 g/L of yeast extract, 5 g/L of peptone, and 20 g/L of glucose). Flasks were shaken at 120 rpm at 30 C. After 4 days, the contents of the flasks were filtered through eight layers of sterile surgical gauze. The conidial suspensions were then washed with distilled water by centrifugation at 6,200× *g* for 10 min at 4 C and resuspended in sterile water.

Assay of populations of *Fusarium* spp. Conidial suspensions or soil samples were diluted with sterile 0.1% water agar. Aliquots (0.1 ml of a conidial suspension or 0.2 ml of a soil suspension) were spread over plates of PPSM or FMMCPA. After incubation at 27 C for 5–6 days, colonies were counted and results were expressed either as mean number of colonies per plate or as colony-forming units per gram of air-dried soil.

Survival of conidia of *F. oxysporum* in soil. Water suspensions of conidia were added to soil at 50,000–100,000 conidia per gram of soil, which resulted in soil moisture of 80% field capacity. Samples of 50 g of the infested soils were placed in 100-ml flasks, covered, and incubated at 27 C. Viability of the conidial inoculum was determined by dilution plating of soil samples taken at time zero and thereafter as indicated (Table 2). Percent survival was calculated by comparing population density from each sample date with that at time zero.

RESULTS

Developing the selective medium. Preliminary experiments were carried out to develop a selective chlorate-containing medium that would allow only the growth of chlorate-resistant *nit* mutants of *F. oxysporum*. Adding potassium chlorate (15 g/L) to PPSM, a commonly used *Fusarium*-selective medium, did not give satisfactory results, because both mutant and wild-type strains grew well on this medium. Apparently, the levels of organic nitrogenous compounds in PPSM masked the toxicity of chlorate to the wild-type strains. The medium used in the following experiments, designated FMMCPA, was based on FMMC, supplemented with PCNB (0.5 g a.i./L) to suppress fungi and with chloramphenicol (250 mg/L) and 90% lactic acid (0.5 ml/L) to suppress bacteria. The toxic effect of chlorate could be modulated by adjusting the amount of lactic acid in the medium. Adding 0.5 ml/L of 90% lactic acid (resulting in a pH level in the range of 2.9–3.1) suppressed bacteria and enabled reasonable growth of *nit* mutants. None of 50 randomly chosen wild-type isolates of *Fusarium* spp., originating from Rehovot and Eden soils, could grow on FMMCPA.

Performance, range of applicability, and reliability of FMMCPA. Conidial suspensions of 12 *nit* mutants of six formae speciales of *F. oxysporum* were plated on FMMCPA and on PPSM, for comparison. Six experiments were conducted using various conidial concentrations of different *nit* mutants of each forma specialis, to evaluate the reliability of the technique. Representative results (Table 3) show that the recovery of 11 *nit* mutants on both media was comparable, although the average number of colonies was 8.5% lower on FMMCPA. Colonies of the mutants on FMMCPA were smaller than those on PPSM, their diameter usually being 20–30% of those on PPSM (Fig. 1A, C). One mutant (FOD-E10-6) did not grow on FMMCPA, and another (FOV-MR466) grew poorly and its colonies were difficult to detect.

Aqueous suspensions of soil from six fields, of different types and cropping history, were plated on PPSM and FMMCPA. The levels of natural

Table 1. Populations of *Fusarium* spp. from six field soils in Israel, estimated by soil dilution on two media[a]

Site	Soil properties: pH	Organic matter (%)	Clay (%)	Colony-forming units per gram of soil: PPSM	FMMCPA
Tirat Zevi	8.3	2.1	55.8	3,200	0
Eden	7.9	2.2	21.2	3,280	0
Kefar Warburg	8.1	0.1	5.0	3,800	0
Bet Dagan	7.5	1.4	52.5	5,350	0
Rehovot	7.6	0.4	3.8	5,940	0
Ein Dor	7.6	1.7	42.5	10,300	0

[a] PPSM = peptone-PCNB selective medium; FMMCPA = *Fusarium* minimal medium amended with chlorate, chloramphenicol, and PCNB and acidified.

Table 2. Survival in Rehovot soil of *nit* mutants of three formae speciales of *Fusarium oxysporum* and their wild-type parents[a]

Pathogen	Days after soil infestation	Survival (% ± SE of time zero): Wild type[b] PPSM	*nit* mutant[c] PPSM	*nit* mutant[c] FMMCPA
F. o. f. sp. *dianthi*	5	38.8 ± 2.1	39.4 ± 1.2	62.4 ± 4.6
	8	31.5 ± 2.9	20.7 ± 1.6	17.9 ± 0.8
	15	18.6 ± 1.3	10.2 ± 0.2	15.6 ± 1.1
	21	15.9 ± 0.5	13.4 ± 2.3	16.2 ± 2.1
F. o. f. sp. *lycopersici*	6	16.4 ± 1.1	21.2 ± 4.2	20.8 ± 3.9
	13	1.7 ± 0.1	6.8 ± 1.4	1.3 ± 0.8
	24	5.3 ± 0.8	3.4 ± 0.5	3.7 ± 0.6
F. o. f. sp. *niveum*	3	66.0 ± 4.6	65.0 ± 4.7	45.3 ± 12.1
	7	21.3 ± 2.2	21.9 ± 2.0	31.7 ± 5.0
	13	7.3 ± 2.2	7.8 ± 2.9	14.0 ± 1.6

[a] Soil samples were infested with conidial suspensions of the pathogens, sampled periodically, and plated on two media to recover populations of *F. oxysporum*. Survival was calculated by comparison with the population level at zero time. PPSM = peptone-PCNB selective medium; FMMCPA = *Fusarium* minimal medium amended with chlorate, chloramphenicol, and PCNB and acidified.

[b] Isolates FOD-1547, FOL-1, and FON-AA-O were used as wild types of f. sp. *dianthi*, *lycopersici*, and *niveum*, respectively. No colonies of *Fusarium* spp. were evident on FMMCPA.

[c] Strains FOD-E72, FOL-M, and FON-C were used as mutants of f. sp. *dianthi*, *lycopersici*, and *niveum*, respectively.

populations of *Fusarium* spp. recovered on PPSM ranged from 3,200 to 10,300 cfu/g (Table 1). No growth of *Fusarium* spp. was observed on FMMCPA (Table 1, Fig. 1), and the growth of bacteria was suppressed totally. The growth of most fungi other than *Fusarium* spp. on FMMCPA was greatly restricted, but a few fungi gave rise to colonies 4–30 mm in diameter. These included species of *Aspergillus* (e.g., *A. niger*), *Penicillium, Trichoderma*, and nonsporulating fungi; *Trichoderma* spp. produced thin, expansive, nonsporulating distinct colonies on FMMCPA.

Conidial suspensions of *nit* mutants of *F. oxysporum*, belonging to four formae speciales, were plated directly on PPSM and FMMCPA. In parallel, the conidial suspensions were mixed with soils from three sites, and samples of the infested and noninfested soils were then plated on both media. The numbers of colonies of the mutants, upon direct plating on PPSM and FMMCPA (Table 4), were similar on both media (averages 31.0 and 30.8, respectively), in agreement with earlier results (Table 3). As expected, wild-type *Fusarium* spp. of the natural soil populations did not grow on FMMCPA. When conidia of *nit* mutants were mixed with soil and plated on FMMCPA, the numbers of resulting colonies ranged between 79 and 119% compared with direct plating of the same conidial suspensions on this medium, except for one mutant (FOV-210). This mutant of *F. o.* f. sp. *vasinfectum* (4) could not be recovered after its conidia had been mixed with Rehovot soil and grew poorly when mixed with Ein Dor soil.

Colonies of *Fusarium* spp. that grew on FMMCPA previously inoculated with mixtures of soil and conidia of *nit* mutants of the various pathogens (Fig. 1E) were transferred to FMM to verify their mutant phenotype. Of 355 colonies tested, 336 (95%) were confirmed as *nit* mutants. Sixty randomly chosen colonies that grew on PPSM inoculated with soil and conidia of two *nit* mutants of *F. o.* f. sp. *vasinfectum* (on which both the mutants and the natural population of *Fusarium* can grow) were also tested for phenotype. Thirty-eight (63%) of these colonies were confirmed as being the original mutants. Thirty colonies of *Fusarium* spp. that grew on PPSM previously inoculated with noninfested natural (control) soils were also tested, and none appeared to be a mutant.

Table 3. Number of colonies of *nit* mutants of *Fusarium oxysporum* on two media[a]

Pathogen	Mutant strain	Colonies per petri dish (± SE) PPSM	FMMCPA
F. o. f. sp. *dianthi*	FOD-E72	23.5 ± 2.3	29.6 ± 4.1
	FOD-E72	25.6 ± 0.7	24.6 ± 1.9
	FOD-E72	54.6 ± 2.7	52.4 ± 2.9
	FOD-E72	60.1 ± 5.3	53.8 ± 3.5
	FOD-E10-6	24.2 ± 1.9	0
	FOD-E10-6	45.6 ± 2.8	0
F. o. f. sp. *lycopersici*	FOL-M	32.6 ± 2.3	26.5 ± 2.2
	FOL-M	48.7 ± 3.1	50.0 ± 2.4
	FOL-P	33.8 ± 3.3	33.0 ± 1.7
F. o. f. sp. *melongenae*	FOMG-690G-3	38.6 ± 4.2	31.0 ± 3.9
	FOMG-690G-3	53.0 ± 2.4	45.6 ± 3.1
F. o. f. sp. *melonis*	FOM-V	36.2 ± 2.0	32.8 ± 2.6
	FOM-S	48.4 ± 2.1	36.6 ± 3.1
F. o. f. sp. *niveum*	FON-U	34.6 ± 2.7	35.2 ± 2.2
	FON-U	76.0 ± 3.6	70.2 ± 2.1
	FON-C	31.2 ± 2.6	26.0 ± 2.1
F. o. f. sp. *vasinfectum*	FOV-CT201	19.1 ± 1.6	19.6 ± 3.2
	FOV-MR466	38.6 ± 2.0	32.2[b] ± 1.0
	FOV-51	42.0 ± 2.2	38.0 ± 4.2
Average[c]		41.0	37.5

[a] Aliquots (0.1 ml per petri dish) of conidial suspension were spread over the solidified medium. Concentration of conidia was adjusted by counting with a hemacytometer to give 20–80 conidia per petri dish. Numbers are means of five replicates. PPSM = peptone-PCNB selective medium; FMMCPA = *Fusarium* minimal medium amended with chlorate, chloramphenicol, and PCNB and acidified.

[b] The colonies were small and hyaline and difficult to detect and count.

[c] *F. o.* f. sp. *dianthi* (FOD-E10-6) excluded.

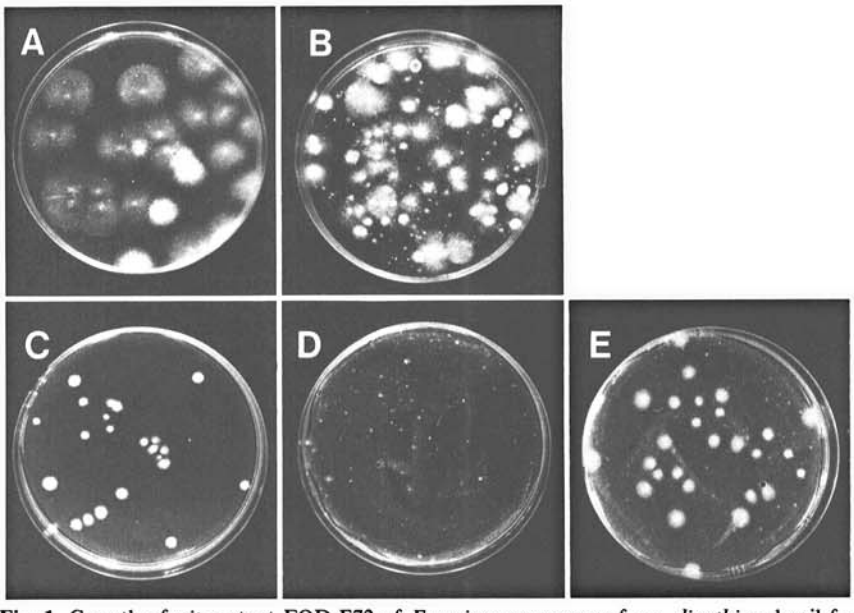

Fig. 1. Growth of *nit* mutant FOD-E72 of *Fusarium oxysporum* f. sp. *dianthi* and soil fungi on selective media, after 5 days at 27 C: Conidial suspension of FOD-E72 plated on **(A)** peptone-PCNB selective medium (PPSM) and **(C)** *Fusarium* minimal medium amended with potassium chlorate, PCNB, chloramphenicol, and lactic acid (FMMCPA); aqueous suspension of Eden soil plated on **(B)** PPSM and **(D)** FMMCPA; and mixture of FOD-E72 conidia and Eden soil suspension plated on FMMCPA **(E)**.

Recovery and identification of *nit* mutants from inoculated plants. The suitability of the FMMCPA for the selective isolation of *nit* mutants from diseased plants was examined. Root or stem segments of diseased tomato or carnation plants, previously inoculated with wild-type strains and *nit* mutants of four isolates of *F. o.* f. sp. *radicis-lycopersici* or two isolates of *F. o.* f. sp. *dianthi*, were surface-disinfested with 1% sodium hypochlorite for 1 min and plated on PDA and FMMCPA. On PDA, most of the segments gave rise to typical colonies of *F. oxysporum*. On FMMCPA, growth of *Fusarium* was evident only from segments of plants inoculated with the *nit* mutants. The growing colonies of *F. oxysporum* from FMMCPA were then verified as *nit* mutants by their phenotype on FMM.

Survival of wild-type pathogenic strains of *F. oxysporum* and their *nit* mutants in soil. The purpose of this experiment was to compare the behavior of *nit* mutants and their wild-type parents, by following their populations in a natural soil, in order to see if such mutants can serve as true representatives of the pathogens. Changes in time in soil of populations of wild-type strains of three formae speciales of *F. oxysporum* and of their respective *nit* mutants were followed. The typical pattern of a rapid initial decline of the introduced *F. oxysporum* (61–84% reduction in the

first 5–7 days) was observed (Table 2). The rate and extent of decline were similar within each pair of a wild-type strain and its *nit* mutant. The enumeration of populations of the *nit* mutants was easier and more accurate with FMMCPA than with PPSM, because only the mutant's colonies could grow on FMMCPA. No colonies of *Fusarium* spp. were observed when soils infested with the wild-type pathogens were plated on FMMCPA. Of 121 colonies that developed on the FMMCPA plates and were transferred to FMM, 116 (95%) were confirmed by their phenotype as being the original mutants.

Pathogenicity of the *nit* mutants. Seventy-three *nit* mutants, belonging to seven formae speciales of *F. oxysporum* (*dianthi, lycopersici, melongenae, melonis, niveum, radicis-lycopersici*, and *vasinfectum*), were tested for pathogenicity on their respective hosts. Seventy-one (97%) were pathogenic, confirming earlier results with *F. o.* f. sp. *vasinfectum* (4).

Table 4. Recovery of propagules of formae speciales of *Fusarium oxysporum* from soil or conidial suspension on two selective media[a]

Expt. no.	Source of inoculum		Colonies per petri dish	
	Soil[b]	Pathogen and *nit* mutant strain	PPSM	FMMCPA
1	Rehovot	None	40.8	0
	None	*F. o.* f. sp. *dianthi*, FOD-E72	32.0	28.2
	Rehovot	*F. o.* f. sp. *dianthi*, FOD-E72	58.0	28.6
2	Rehovot	None	39.6	0
	None	*F. o.* f. sp. *lycopersici*, FOL-M	34.4	31.6
	Rehovot	*F. o.* f. sp. *lycopersici*, FOL-M	36.4	29.4
	None	*F. o.* f. sp. *vasinfectum*, FOV-210	19.6	22.6
	Rehovot	*F. o.* f. sp. *vasinfectum*, FOV-210	44.4	0
3	Ein Dor	None	22.0	0
	None	*F. o.* f. sp. *vasinfectum*, FOV-MBR2181	55.0	54.8
	Ein Dor	*F. o.* f. sp. *vasinfectum*, FOV-MBR2181	61.4	43.4
	None	*F. o.* f. sp. *vasinfectum*, FOV-W	26.6	27.6
	Ein Dor	*F. o.* f. sp. *vasinfectum*, FOV-W	36.0	21.2
4	Eden	None	65.6	0
	None	*F. o.* f. sp. *dianthi*, FOD-E72	25.6	24.6
	Eden	*F. o.* f. sp. *dianthi*, FOD-E72	73.4	29.2
	None	*F. o.* f. sp. *niveum*, FON-U	31.6	33.8
	Eden	*F. o.* f. sp. *niveum*, FON-U	88.0	24.6
5	Eden	None	58.6	0
	None	*F. o.* f. sp. *lycopersici*, FOL-M	34.4	31.6
	Eden	*F. o.* f. sp. *lycopersici*, FOL-M	56.0	31.4
	None	*F. o.* f. sp. *vasinfectum*, FOV-210	19.6	22.6
	Eden	*F. o.* f. sp. *vasinfectum*, FOV-210	60.8	5.0[c]

[a]Petri dishes were inoculated with 0.2 ml of a soil suspension (1:10–1:100 dilution) or 0.1 ml of a diluted conidial suspension of the indicated strain, or with a mixture thereof. PPSM = peptone-PCNB selective medium; FMMCPA = *Fusarium* minimal medium amended with chlorate, chloramphenicol, and PCNB and acidified.
[b]Soil properties are given in Table 1.
[c]The colonies were small and difficult to detect and count.

DISCUSSION

The selective medium FMMCPA enabled the recovery from soils and the growth of *nit* mutant strains belonging to various formae speciales of *F. oxysporum*. It suppressed bacteria and fungi, including wild-type *Fusarium* spp. of six different soils. Apparently, the combination of PCNB, which is a selective agent commonly used in fungal selective media (1,9,12,13), and chlorate, which is highly toxic to many organisms but not to the *nit* mutants, contributes to the selectivity of this medium. The *nit* mutations condition resistance to chlorate (a nitrate analogue) pleiotropically. *Nit* mutants appear spontaneously, without mutagenic treatments, and are indistinguishable from the wild-type strains when grown on PDA or on media with organic nitrogen or ammonium. They retained their pathogenicity, confirming previous results with *F. o.* f. sp. *vasinfectum* (4), and the pattern and rate of their survival in soil were similar to those of their wild-type parents. *Nit* mutants seem similar to their wild-type parents in pathogenicity and survival in Rehovot field soil under laboratory conditions, and their easily detectable genetic markers may make them potentially useful for ecological studies. Preliminary screening for suitable *nit* mutants is essential, however, since some mutants may not be adequate for this purpose (e.g., *F. o.* f. sp. *vasinfectum* strains FOV-210 and FOV-MR466 and *F. o.* f. sp. *dianthi* strain FOD-E10-6).

FMMCPA is a simple and easy to handle medium. The use of this selective medium is much less cumbersome than pathogenicity tests and is reliable, since no naturally occurring *nit* mutants have been detected on this medium and at least 95% of the colonies directly observed on the plates were later confirmed as mutants by their phenotype. Besides pathogenicity tests, only a few means have been suggested for the identification of pathogenic strains of *Fusarium* spp., e.g., vegetative-compatibility grouping (2,4,10) and molecular approaches (5,8). Chlorate as a selective agent might possibly be used with media other than FMM, provided that no interference from nitrogenous compounds occurs. FMMCPA might also be used for tracing and identifying suitable *nit* mutants of nonpathogenic forms of *Fusarium* with important traits, such as those related to soil suppressiveness (3,11).

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