Use of a Nitrate-Nonutilizing Mutant and Selective Media to Examine Population Dynamics of *Fusarium oxysporum* f. sp. *spinaciae* in Soil

Toshiaki Takehara, Katsuto Kuniyasu, Mitsutaka Mori, and Hiroshi Hagiwara

First, second, and fourth authors: Soil-borne Disease Laboratory, National Agricultural Research Center, Tsukuba, Ibaraki 305-8666, Japan; and third author: Kagawa Prefectural Agricultural Experiment Station, Takamatsu, Kagawa 761-8078, Japan.

Current address of K. Kuniyasu: 14-4 Higashi-tsuwa, Tsuchiura, Ibaraki 300-0067, Japan.

Current address of H. Hagiwara: National Agricultural Research Center for Tohoku Region, Morioka, Iwate 020-0198, Japan.

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ABSTRACT

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Determining the population density of the spinach wilt pathogen *Fusarium oxysporum* f. sp. *spinaciae* in soil with conventional *Fusarium*-selective media is quite difficult because nonpathogenic strains of *F. oxysporum* also grow on those media and are indistinguishable from the pathogen. Therefore, a nitrate-nonutilizing (*nit*) mutant of the pathogen and corresponding selective media were tested in an experimental approach to determine the population density of the pathogen. Colony forming units of the pathogen were countable after soil-dilution plating onto *nit* mutant-selective media MMCPA, CMP, and CGMBP. Colony forming units of wild-type *Fusarium* spp. were countable using a wild-

type *Fusarium*-selective medium, GMBP. By combining *nit* mutant- and wild-type-selective media, the population densities of pathogenic and nonpathogenic *F. oxysporum* in the same soil could be measured selectively. This method was useful in studying population dynamics of the pathogen after different soil treatments. Soil disinfested with hot water or chloropicrin was amended with the *nit* mutant pathogen, and subsequent changes in population densities of the pathogen were compared with those in nontreated field soil. The pathogen rapidly proliferated in disinfested soil and wilt developed faster than in nontreated soil. When a nonpathogenic isolate of *F. oxysporum* was added at high density to sterilized soil prior to the pathogen, growth of the pathogen was greatly suppressed. Nonpathogenic *F. oxysporum* could not, however, reduce the density of preexisting pathogen.

Additional keywords: hot-water disinfestation of soil.

Fusarium wilt of spinach, caused by *Fusarium oxysporum* f. sp. *spinaciae*, is a major production constraint in spinach-growing areas in the United States, Japan, Canada, and Sweden (2,13). In Japan, soil fumigation with chloropicrin or methyl bromide has been commonly used to control the disease. Soil disinfestation with hot water (8) has been proposed as a new alternative.

Even after soil disinfestation, severe infection of spinach may occur after several plantings (4). This infection is thought to be due to the proliferation of remaining or reintroduced pathogen in disinfested soil (16,19), but no study has actually reported changes in population density.

To determine pathogen density in soil, specific strains of pathogenic *F. oxysporum* must be identified by differentiating them from other formae speciales and races or nonpathogenic strains of *F. oxysporum*. On conventional *Fusarium*-selective media (7,14, 26) used in the soil-dilution plate technique, colonies of pathogens cannot be distinguished from other *Fusarium* colonies. The inoculation tests with host plants or race-differential cultivars for each colony that are needed to identify them are too labor- and time-intensive to be practical.

Nitrate-nonutilizing (*nit*) mutants of *F. oxysporum*, tolerant to chlorate, can be selectively reisolated from soil and plants using chlorate-containing media (3,20,22). We have generated *nit* mutants of the spinach wilt pathogen and reisolated them from soil on selective media (21-23). Based on these studies, we compared the proliferation of the pathogen added to soil disinfested with hot

water and chloropicrin or pretreated with nonpathogenic *F. oxysporum* with that in nontreated soil. The objectives of this study were to (i) examine the applicability of the *nit* mutant and its selective media for determining the population density of the spinach wilt pathogen in soil; (ii) examine the growth of the spinach wilt pathogen and disease occurrence in soil treated with hot water or chloropicrin, compared with nontreated field soil; and (iii) determine if pretreatment of soil with a nonpathogenic *F. oxysporum* delays reinfestation by the pathogen. Here, we report the successful use of *nit* mutants for determining pathogen density in soil and for studying the influence of soil treatments on the subsequent proliferation of the pathogen.

MATERIALS AND METHODS

Fungal strains. We used Spin-2 (wild type, MAFF GeneBank Accession No. 103060) and M2-1 (*nit* mutant derived from Spin-2: phenotype *nit1*) (23) as pathogenic strains of *F. oxysporum* f. sp. *spinaciae*. M2-1 is comparable to Spin-2 in growth and pathogenicity. We used S1HI1-W (wild type) and NA-12 (*nit* mutant derived from S1HI1-W: phenotype *nit1*) as nonpathogenic strains of *F. oxysporum*. Strain S1HI1-W was a contribution from Y. Akasaka, Iwate Horticultural Experiment Station, Japan (at that time). Isolated from a spinach root, it suppresses *Fusarium* wilt of spinach (1). Methods to generate *nit* mutants are described elsewhere (21). The pathogenic strains of *F. oxysporum* are abbreviated as PF and nonpathogenic strains of *F. oxysporum* as NPF.

Soil. All experiments used field soil from the National Agricultural Research Center (NARC) (i.e., low-humic andosols having no history of spinach planting). Before each experiment, the soil was processed as described below.

Corresponding author: T. Takehara; E-mail address: toshi@affrc.go.jp

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Chloropicrin-treated soil. A field plot was treated with chloropicrin on 16 September 1993 (treated area: 200 m²) and 5 September 2001 (treated area: 3 plots of 3 m²). After tilling and preparing the field plot, 15-cm-deep holes were made in every 900 cm² (30 by 30 cm), 3 ml of chloropicrin (80%) was injected into each hole, which was immediately filled with soil, and the entire plot was covered with transparent polyethylene film. Soil (down to 10 cm from soil surface) was collected 13 days after treatment in the 1993 experiment and 9 days after treatment in the 2001 experiment.

Hot-water-treated soil. As described for earlier experiments (8,9), hot water was sprinkled on the soil surface until the soil temperature reached >55°C at a depth of 20 to 30 cm. Soil (down to 10 cm from soil surface) was collected 13 days after treatment in the 1993 experiment and 9 days after treatment in the 2001 experiment.

Hot-water treatment in the 1993 experiment. A field plot (10 by 20 m) was disinfested with hot water on 16 September 1993. Hot water (90 to 95°C) was produced using a soil sterilizer (steam boiler) with a thermal output of 160,000 kcal/h (EC-350; Ishihara Kougyousho Co., Hamamatsu, Japan) and a steam-water thermal converter (Ebara Yutori Setto TB-880-10; Ebara Boiler Co., Fujisawa, Japan). The steam pressure of the boiler was set at 4.0 to 5.0 kg/cm² and steam temperature at 150 to 155°C. The steam-discharge valve was fully opened, and tap water was heated to 90 to 95°C with the thermal converter. A relatively heat-tolerant, horizontally sprinkling-type, plastic sprinkling tube (Sumisansui R Wing; Sumika Agrotech Co. Ltd., Osaka, Japan) with an inside diameter of 3.4 cm was used to sprinkle hot water on the soil. The tube had two pinholes (diameter, 0.25 mm) every 12 cm in a zigzag pattern on either side of the upper surface of the tube. Half of the pinholes were enlarged to 1 mm with heated needles to hasten the sprinkling. The tubes were laid in parallel on the soil surface (with pinholes upward) at intervals of 50 cm; transparent polyethylene film then was laid over them to cover the entire plot, and 75 liter/m² of the hot water was sprinkled on the soil surface. After sprinkling, the polyethylene film was kept on for 12 days to retain heat.

Hot water treatment in the 2001 experiment. The treatment (5 September 2001) was similar to that of 1993, except for the following points. Three replicate plots of 3 m² (1 by 3 m) each were treated separately. Hot water was produced directly by a hot-water soil-sterilizer with thermal output of 250,000 kcal/h (BW-30; Marubunseisakusho Inc., Hamamatsu, Japan) and hot water

sprinkled at 200 liter/m². The pinholes in the sprinkling tube (Sumisansui R Wing) were not enlarged.

Media and soil-dilution plating. The media listed in Table 1 were used in soil-dilution plating to selectively isolate *nit* mutants or wild-type *Fusarium* spp. Media MMCPA (22), CMP, and CGMBP (20) were used to detect *nit* mutants in soil. MMCPA is based on the FMMCPA of Hadar et al. (3), but has lower concentrations of KClO₃ and pentachloronitrobenzene (PCNB) and lacks chloramphenicol. CMP is MMCPA amended with chloramphenicol and miconazole nitrate. CGMBP was made by replacing sucrose in CMP with galactose and adding boric acid, based on the modified MMCPA of Ueda et al. (27). GMBP medium (20) was used to detect wild-type *Fusarium* spp.; *nit* mutants weakly grow on this medium, but their colonies are barely visible. To detect *Fusarium* spp. in general (*nit* + wild), we used Komada's *Fusarium*-selective medium (KM) (7) with reduced PCNB (75% wettable powder) concentration (0.25 g/liter), or PAC medium (i.e., a potato sucrose agar [PSA] medium amended with 0.25 g of chloramphenicol and 0.25 g of PCNB [75% wettable powder] per liter, acidified to pH 3.4). To detect fungi other than *Fusarium* spp., PAC or MMCPA was used. To detect bacteria and actinomycetes, we used 10% PTYG agar (PTYGA) medium (25). In soil-dilution plating, 10 g of soil was suspended in 90 ml of sterilized 0.05% water agar and serially diluted with 0.05% water agar, after which 0.7 to 1 ml (0.1 ml for PTYGA) of the serial dilutions were spread over solidified media in petri dishes. After dilution plating of the soil, media were incubated at 25°C in the dark or at room temperature for several days, and transferred to a lighted incubator at 25°C (except for PTYGA) to enable *Fusarium* colonies to develop pigment. Colonies were counted on MMCPA after 7 to 8 days, CMP after 7 to 9 days, CGMBP after 8 to 10 days, GMBP after 8 to 10 days, PAC after 5 to 7 days, KM after 6 to 8 days, and PTYGA after 3 to 5 days.

Comparison of media selectivity and rate of propagule recovery. To check media performance in the selectivity and recovery of *Fusarium* propagules, soil samples for *Fusarium* spp. detection were prepared and dilution plated on the media. The experiment was done twice. Preparation of each soil sample was as described below.

Nit mutant inoculum soil. The *nit* mutant strain (M2-1) of *F. oxysporum* f. sp. *spinaciae* was shake-cultured in potato sucrose (PS) liquid medium at 120 rpm, 28°C, for 7 days. Shake-culture suspension (2 ml) then was added to 400 ml (≈270 g) of auto-

TABLE 1. Composition of media used in this study to selectively isolate *nit* mutant and wild-type *Fusarium* spp.^a

Prescription	For <i>nit</i> mutant			For wild type
	MMCPA	CMP	CGMBP	GMBP
Sucrose	30 g	30 g	...	...
Galactose	...	...	30 g	30 g
NaNO ₃	2 g	2 g	2 g	2 g
KH ₂ PO ₄	1 g	1 g	1 g	1 g
MgSO ₄ ·7H ₂ O	0.5 g	0.5 g	0.5 g	0.5 g
KCl	0.5 g	0.5 g	0.5 g	0.5 g
L-Asparagine	1.6 g	1.6 g	1.6 g	...
KClO ₃	10 g	10 g	10 g	...
H ₃ BO ₃	...	...	0.5 g	0.5 g
Agar	20 g	20 g	20 g	20 g
Chloramphenicol	...	0.25 g	0.25 g	0.25 g
Sterile trace elements solution ^b	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Miconazole nitrate	...	50 mg ^c	50 mg	50 mg
Distilled water	1 liter	1 liter	1 liter	1 liter
After autoclaving				
PCNB (75% wettable powder)	0.25 g	0.25 g	0.25 g	0.25 g
pH ^d	3.2	3.4	3.8	3.8

^a *nit* = nitrate nonutilizing and ... = not added.

^b Distilled water, 95 ml; citric acid, 5 g; ZnSO₄·7H₂O, 5 g; FeSO₄·7H₂O, 4.75 g; Fe(NH₄)₂·(SO₄)₂·6H₂O, 1 g; CuSO₄·5H₂O, 250 mg; MnSO₄·H₂O, 50 mg; H₃BO₃, 50 mg; and Na₂MoO₄·2H₂O, 50 mg (17).

^c FLORID-D solution: 5 ml of 10 mg/ml (Mochida Pharmaceutical Co., Ltd., Tokyo).

^d pH was adjusted with 10% phosphoric acid solution.

claved soil in a flask, mixed well, and incubated for 1 month at 20°C in the dark.

Field soil. Soil was collected on 13 June 1995 in the first experiment and 25 August 2000 in the second experiment from the same field as that used in the soil disinfection experiment.

Field soil + *nit* mutant inoculum. The field soil described above was infested with the *nit* mutant (M2-1) inoculum soil at a low and high density. In the first experiment, the ratio of grams of field soil and inoculum soil was 19.8:0.2 and 18:2 for the low- and high-density infestations, respectively. In the second experiment, the ratio was 39.9:0.1 and 39:1 g for the low- and high-density infestations, respectively.

Proliferation of *nit* mutant pathogen in hot water-, chloropicrin-, and non-treated soils. *First experiment (1993).* Thirteen days after treatment, soil was collected from the treated and nontreated plots. The next day, each soil was placed in plastic pots (30 by 23 cm, 12 cm deep, 3 kg of soil per pot) and infested with the *nit* mutant pathogen (M2-1) as follows. The *nit* mutant M2-1 was shake-cultured in PS liquid medium at 120 rpm, 28°C, for 7 days, and the bud-cell suspension was diluted to $\approx 10^6$ and 10^4 bud cells/ml. In all, 10 ml (1-ml samples $\times$ 10 spots) of the 10^6 (high-density) or 10^4 (low-density) inoculum (bud-cell suspension) was added to a pot of soil and mixed well to produce initial pathogen densities of $\approx 5,000$ and 50 bud cells/g of soil, respectively. The next day, 40 spinach seed (cv. Majikku-hourensou; Sakata Seed Co., Yokohama, Japan) were sown per pot and placed in a greenhouse (15 to 30°C) to observe disease development. Two pots were used for each treatment. Soil was sampled as described below for soil-dilution plating at 1, 13, 25, 48, and 76 days after *nit* mutant inoculation. Soil was sampled from five locations between spinach plants in each pot, combined (≈ 20 g total), and mixed well to serve as one sample for each pot. Population densities of the *nit* mutant, wild-type *Fusarium* spp., other fungi, and bacteria were measured by the soil-dilution plate method. MMCPA medium was used to detect *nit* mutant and fungi other than *Fusarium* spp. KM was used to detect *Fusarium* spp. in general. The population density of wild-type *Fusarium* spp. was calculated by subtracting the *nit* mutant population from the general population of *Fusarium* spp. obtained on KM.

Second experiment (2001). The experimental designs and methods in the second experiment were almost the same as in the first. Soil was collected 9 days after hot water and chloropicrin treatments. One pot (3 kg) of soil was collected from each field plot (three plots of 3 m² for each treatment), resulting in three replicates of pots for each treatment. Inoculum was applied only at the low density to produce an initial pathogen density of ≈ 50 bud cells/g of soil. Spinach seed was sown at 30 seed/pot. CMP

medium was used to detect the *nit* mutant and PAC medium to detect *Fusarium* spp. in general and other fungi.

Antagonistic effect of nonpathogenic *F. oxysporum* on pathogen proliferation in sterilized soil. *First experiment.* Field soil (100 g) was placed in 200-ml Erlenmeyer flasks, which were then capped with double-layered aluminum foil and autoclaved at 120°C for 60 min. A nonpathogenic strain of *F. oxysporum* (NPF: strain S1HI1-W) was shake-cultured in PS liquid medium at 28°C, 120 rpm for 7 days, then 3 ml of the 100-fold diluted culture fluid (1.5×10^5 bud cells/ml) was added to the sterilized soil in flasks and mixed well to produce an initial NPF density of $\approx 7 \times 10^3$ bud cells/g dry soil. The *nit* mutant pathogen (PF: strain M2-1) was cultured in PS liquid medium at 28°C, 120 rpm for 8 days (to 3.5×10^7 bud cells/ml), and 1 ml of the 30-fold or 3,000-fold diluted culture fluid was inoculated into the soil 8 days after NPF pretreatment to produce an initial PF density of $\approx 1.8 \times 10^2$ and 1.8×10^4 bud cells/g dry soil, respectively. Soil with no NPF pretreatment also was amended with PF in the same way. No spinach seed were sown and two flasks per treatment were incubated at 25°C in the dark. At 6 and 13 days after PF inoculation, soil in flasks was aseptically stirred, and soil samples (10 g) were taken to measure the population densities of PF and NPF by soil-dilution plating on CGMBP and GMBP media. The caps were kept on the flasks throughout the experiment, except during the addition of NPF and PF and sampling of soil. Water content of soil throughout was $\approx 35\%$ (wt/wt).

Second experiment. The experimental design was the same as in the first experiment, except for the following details. Soil (200 g) was placed in 300-ml Erlenmeyer flasks. The initial density for NPF was adjusted to $\approx 10^4$ bud cells/g dry soil, and for PF to $\approx 10^2$ and 10^4 bud cells/g dry soil. Soil (15 g) was sampled immediately after PF inoculation, and 6, 13, and 28 days after PF inoculation. At the end of the experiment, soil in all flasks was checked for contamination by fungi other than *Fusarium* spp. or by bacteria using soil-dilution plating on PAC and PTYGA media.

Third experiment. The third experiment was identical to the second, except for the use of a *nit* mutant of NPF and a wild type of PF. A *nit* mutant of NPF (strain NA-12) was added 8 days before the wild-type PF (strain Spin-2). Population densities of PF and NPF were measured by soil-dilution plating on GMBP and CGMBP, respectively.

After the completion of all experiments, *nit* mutant-infested soil was sterilized before disposal to prevent release of the mutant strains into the natural environment.

Statistical analyses. StatView for Windows (version 5.0; SAS Institute Inc., Cary, NC) was used for statistical analysis. Population density data were transformed before analysis using the

TABLE 2. Number of colonies of *nit* mutant *Fusarium oxysporum*, wild-type *Fusarium* spp., and other fungi on five selective media (first experiment)^a

Soil sample	MMCPA	CMP	CGMBP	GMBP	KM ^b
<i>Fusarium</i> spp. ^c					
Field soil ^d	0	0	0	45.0 $\pm$ 6.2	40.2 $\pm$ 6.9
<i>nit</i> mutant inoculum ^e	143.6 $\pm$ 9.4	142.6 $\pm$ 7.3	119.6 $\pm$ 9.3	0	153.4 $\pm$ 21.1
Field soil + <i>nit</i> (low density) ^f	14.8 $\pm$ 3.3	14.6 $\pm$ 3.0	13.4 $\pm$ 3.9	45.0 $\pm$ 5.6	56.6 $\pm$ 8.5
Field soil + <i>nit</i> (high density) ^g	109.8 $\pm$ 7.6	119.8 $\pm$ 12.5	95.8 $\pm$ 15.0	38.2 $\pm$ 7.3	156.6 $\pm$ 14.3
Other fungi ^h					
Field soil ^d	97.2 $\pm$ 8.2	37.2 $\pm$ 7.0	2.8 $\pm$ 3.1	0	0.6 $\pm$ 0.5
<i>nit</i> mutant inoculum ^e	0	0	0	0	0
Field soil + <i>nit</i> (low density) ^f	77.4 $\pm$ 1.7	23.4 $\pm$ 4.5	0.2 $\pm$ 0.4	0	0.2 $\pm$ 0.4
Field soil + <i>nit</i> (high density) ^g	38.6 $\pm$ 10.0	4.8 $\pm$ 2.4	0	0.2 $\pm$ 0.4	0.2 $\pm$ 0.4

^a Aliquots (1 ml per petri dish) of soil dilution in sterilized 0.05% water agar were spread over the solidified media; *nit* = nitrate nonutilizing; numbers are means of five replicates.

^b KM = Komada's *Fusarium*-selective medium (7) with reduced pentachloronitrobenzene (75% wettable powder) concentration (0.25 g/liter of the medium).

^c Colonies of *Fusarium* spp. per petri dish $\pm$ standard deviation (SD).

^d Field soil; 200-fold dilution.

^e *Nit* mutant inoculum (strain M2-1 incubated in sterilized soil); 2,000-fold dilution.

^f Field soil (19.8 g) amended with 0.2 g of the above *nit* inoculum; 200-fold dilution.

^g Field soil (18 g) amended with 2 g of the above *nit* inoculum; 200-fold dilution.

^h Colonies of fungi other than *Fusarium* spp. per petri dish $\pm$ SD.

formula $\log([CFU/g \text{ of dry soil}] + 1)$. Wilt data expressed as percentages were divided by 100 and square roots obtained for arcsine transformation before analysis. Data were analyzed by analysis of variance (ANOVA) and, when a significant ($P < 0.05$) F value was observed, Student's t tests were used to compare differences between two treatment means. Tukey-Kramer's method was used to compare multiple treatment means.

RESULTS

Media selectivity and rate of propagule recovery. Similar results were obtained from the two experiments to check media performance in selectivity and recovery of *Fusarium* propagules (Tables 2 and 3; Fig. 1). Naturally occurring *Fusarium* spp. in field soil did not grow at all on MMCPA, CMP, or CGMBP.

TABLE 3. Number of colonies of *nit* mutant *Fusarium oxysporum*, wild-type *Fusarium* spp., and other fungi on five selective media (second experiment)^a

Soil sample	MMCPA	CMP	CGMBP	GMBP	KM ^b
<i>Fusarium</i> spp. ^c					
Field soil ^d	0	0	0	52.8 ± 4.2	54.2 ± 6.3
<i>nit</i> mutant inoculum ^e	62.6 ± 11.6	58.8 ± 3.9	52.6 ± 12.8	0	65.4 ± 8.5
Field soil + <i>nit</i> (low density) ^f	9.8 ± 1.9	7.8 ± 1.6	9.8 ± 3.9	56.4 ± 6.5	72.0 ± 8.2
Field soil + <i>nit</i> (high density) ^g	71.8 ± 10.2	83.4 ± 8.2	86.0 ± 8.9	54.2 ± 5.8	153.8 ± 20.8
Other fungi ^h					
Field soil ^d	40.2 ± 3.9	1.4 ± 0.5	0	11.4 ± 4.0	5.6 ± 0.5
<i>nit</i> mutant inoculum ^e	0	0	0	0	0
Field soil + <i>nit</i> (low density) ^f	27.6 ± 3.6	0	0	12.2 ± 2.8	5.2 ± 1.3
Field soil + <i>nit</i> (high density) ^g	20.4 ± 6.2	0	0.2 ± 0.4	12.8 ± 1.9	1.8 ± 0.4

^a Aliquots (0.7 ml per petri dish) of soil dilution in sterilized 0.05% water agar were spread over the solidified media; *nit* = nitrate nonutilizing; numbers are means of five replicates.

^b KM = Komada's *Fusarium*-selective medium (7) with reduced pentachloronitrobenzene (75% wettable powder) concentration (0.25 g/liter of the medium).

^c Colonies of *Fusarium* spp. per petri dish ± standard deviation (SD).

^d Field soil; 100-fold dilution.

^e *Nit* mutant inoculum (strain M2-1 incubated in sterilized soil); 10,000-fold dilution.

^f Field soil (39.9 g) amended with 0.1 g of the above *nit* inoculum; 100-fold dilution.

^g Field soil (39 g) amended with 1 g of the above *nit* inoculum; 100-fold dilution.

^h Colonies of fungi other than *Fusarium* spp. per petri dish ± SD.

Fig. 1. Colony formation of nitrate-nonutilizing (*nit*) mutant *Fusarium oxysporum* and wild-type *Fusarium* spp. in soil on three selective media (first experiment). Samples (1 ml per petri dish) of each soil dilution in sterile 0.05% water agar were spread over solidified media and incubated for 10 days at 25°C. Soil samples from top to bottom are: field soil (200-fold-dilution solution); *nit* mutant inoculum (strain M2-1 incubated in sterilized soil, 2,000-fold-dilution solution); field soil (19.8 g) amended with 0.2 g of the described *nit* inoculum (200-fold-dilution solution); field soil (18 g) amended with 2 g of the *nit* inoculum (200-fold-dilution solution). Media are from left to right: CGMBP (*nit* mutant-selective medium); GMBP (wild-type *Fusarium*-selective medium); KM (Komada's *Fusarium*-selective medium [7] with reduced pentachloronitrobenzene (75% wettable powder) concentration [0.25 g/liter of the medium]). Note that *nit* mutants produced colonies on CGMBP, wild-type *Fusarium* spp. produced colonies on GMBP, and both did on KM.

Selectivity (suppression of colony formation of other fungi) increased, in order, on MMCPA, CMP, and CGMBP. Propagule recovery of *nit* mutant inoculum on these media was nearly comparable to KM, although the average number of colonies was 19.6 to 22.0% lower on CGMBP compared with KM. On GMBP, colonies of *nit* mutants grew very weakly and were barely visible for lack of available nitrogen source, facilitating the counting of wild-type *Fusarium* colonies. To confirm phenotypes of *Fusarium* colonies on CGMBP and GMBP, 50 randomly chosen colonies arising from field soil infested with a low density of *nit* mutants

were transferred to *Fusarium* minimal medium (MM) (17) containing nitrate as the sole nitrogen source. All 50 of the tested *Fusarium* colonies on CGMBP proved to be *nit* mutants, as indicated by thin expansive growth on MM, and all 50 tested *Fusarium* colonies on GMBP were wild type. Thus, the population densities of the added *nit* mutant and wild-type *Fusarium* spp. could be determined separately using these media.

Proliferation of *nit* mutant pathogen in hot water-, chloropicrin-, and non-treated soils. To examine the influence of hot water and chloropicrin treatments on the proliferation of the patho-

Fig. 2. Wilt occurrence in spinach plants and population densities of microorganisms after nitrate-nonutilizing (*nit*) mutant of *Fusarium oxysporum* f. sp. *spinaciae* was added to hot-water-, chloropicrin-, or non-treated soils (first experiment). **A**, Progress of wilt; **B**, changes in population densities of inoculated *nit* mutant; **C**, population densities of microorganisms one day after *nit* inoculation. Left graphs in each graph pair are results from low-density inoculation of the *nit* pathogen (≈ 50 bud cells/g of soil), and right graphs are from high-density inoculation ($\approx 5,000$ bud cells/g of soil). The population density of "other fungi" represents colonies other than *Fusarium* spp. on MMCPA medium. Mean of two replicates. Vertical lines represent the standard error. There are no significant differences between treatments with the same letter according to Tukey-Kramer's method (5% level). Abbreviations: Inoc. *nit* = inoculated *nit* mutant; Bact. = bacteria; Act. = actinomycetes; and n.d. = not detected.

gen introduced after the treatments, the *nit* mutant pathogen was added to the treated soil and nontreated field soil (raw soil). Spinach wilt developed faster and more severely in hot-water- and chloropicrin-treated soil than in raw soil (Figs. 2A and 3A).

Fig. 3. Wilt occurrence in spinach plants and population densities of microorganisms after nitrate-nonutilizing (*nit*) mutant *Fusarium oxysporum* f. sp. *spinaciae* was inoculated into hot-water-, chloropicrin-, or non-treated soils (second experiment). **A**, Progress of wilt; **B**, changes in population densities of inoculated *nit* mutant; **C**, population densities of microorganisms 1 day after *nit* inoculation. The inoculation density of the *nit* pathogen was ≈ 50 bud cells/g of soil. The population density of "other fungi" represents colonies other than *Fusarium* spp. on PAC medium. Mean of three replicates. Vertical lines represent the standard error. There are no significant differences between treatments with the same letter according to Tukey-Kramer's method (5% level). Abbreviations: Inoc. *nit* = inoculated *nit* mutant; Bact. = bacteria; Act. = actinomycetes; and n.d. = not detected.

When the pathogen was inoculated at a low density (≈ 50 bud cells/g of soil), pathogen growth and wilt occurrence was very limited until ≈ 25 days after inoculation in all treatments (Fig. 2A and B, left graph; Fig. 3A and B). Thereafter, the pathogen proliferated rapidly in hot-water- and chloropicrin-treated soils, but grew very slowly or not at all in raw soil, with wilt following a similar pattern.

When the pathogen was inoculated at a high density ($\approx 5,000$ bud cells/g of soil), most spinach plants had wilted by 46 days after inoculation in hot-water- and chloropicrin-treated soil, while wilt occurrence was delayed in raw soil. The pathogen population did not increase detectably in raw soil and increased gradually up to $\approx 10^4$ CFU/g of dry soil in hot-water- and chloropicrin-treated soils (Fig. 2A and B, right graph).

In all cases, the fungal and bacterial flora of disinfested soil obviously was simplified, judging from the morphology of colonies on media. The proportion of *Trichoderma* spp. was higher after chloropicrin treatments. In the first experiment, the populations of wild-type *Fusarium* spp. and other fungi were significantly lower in disinfested soils compared with those in raw soil. The population of actinomycetes was >10 times greater in raw soil than in the other two soil plots (Fig. 2C). A similar tendency was observed in the second experiment (Fig. 3C); however, the difference was not always significant because of the rather large variance that may have resulted from imperfect disinfecting of one field plot in each of the three replicate plots of the hot water and chloropicrin treatments.

Antagonistic effect of nonpathogenic *F. oxysporum* on pathogen proliferation in sterilized soil. To examine the possibility of competition between populations of PF and NPF in soil, we studied their population dynamics in more controlled conditions (i.e., in sterilized soil in flasks). An example of soil-dilution plating from the second experiment is shown in Figure 4, and changes in population densities of PF and NPF in the three similar experiments are shown in Figure 5. When incubated alone, PF (*nit* mutant) proliferated rapidly, reaching a density of 1.01 to 3.82×10^5 CFU/g of dry soil in 13 days (Fig. 5A and C). When NPF (wild type) was added to soil 8 days before PF, PF proliferation was significantly suppressed, and PF did not change significantly, remaining at approximately the initial density (Fig. 5B and D). Furthermore, in the third experiment, in which a wild type was used as PF and *nit* mutant as NPF (Fig. 5E and F), PF proliferation also was suppressed as in the previous two experiments. Thus, the *nit* mutant is not competitively inferior to wild type because of its lack of nitrate utilization. The density of PF added later did not affect NPF proliferation (Fig. 5B, D, and F). No fungal or bacterial contamination of the soil in flasks was detected throughout the experiments.

DISCUSSION

Nit mutants of *F. oxysporum* most commonly have been used to define vegetative compatibility groupings of this important plant pathogen (5,6) since the first such report by Puhalla in 1985 (17). Although Hadar et al. (3) developed a chlorate-containing selective medium for chlorate-resistant *nit* mutants in 1989 and suggested the possibility of using *nit* mutants as marker strains for ecological studies of *F. oxysporum*, only a few studies (18,24) actually have used *nit* mutants for this purpose. As shown in our experiments, the population density of the *nit* mutant pathogen could be measured using *nit* mutant-selective media MMCPA, CMP, and CGMBP. CGMBP had the highest selectivity of the three media. The miconazole nitrate in CMP and CGMBP greatly contributes to their high selectivity. The population density of wild-type NPF was countable on GMBP. Combining CGMBP and GMBP would be especially useful for analyzing population dynamics of pathogenic and nonpathogenic *F. oxysporum* by using a *nit* mutant for one strain and a wild type for another. However,

for unknown reasons, sometimes fewer colonies than expected grew on MMCPA, CMP, and CGMBP (T. Takehara, *unpublished data*). A decrease in water content and light exposure during the preservation of those media may adversely affect *Fusarium* propagule recovery. Therefore, after pouring and solidifying media in plates, plates should be stored in a sealed box in a dark, cool place and used within a month. Recovery of *nit* mutant propagules on the media also should be compared each time of use to that on a more reliable medium, such as PAC, for *Fusarium* detection. Some *nit* isolates also may be more susceptible to antifungal agents such as miconazole nitrate and PCNB in these media; decreasing the agents may be useful for obtaining higher recovery. The choice of medium to detect a specific *nit* mutant should be determined according to the recovery rate of the *nit* mutant on those media and the population density of other fungi in a particular soil.

It may be argued that *nit* mutants are not truly representative of wild-type *F. oxysporum*, because they cannot use nitrate. However, we have reported previously that *nit* mutants share many of the properties, such as growth, pathogenicity, benomyl sensitivity, and survival in soil, of wild-type strains (23). Many types of nitrogen sources in soil are available to *nit* mutants, and no difference in saprophytic ability has been reported so far between *nit*

mutants and wild-type strains of *F. oxysporum*. Moreover, in field experiments in plastic houses, population densities of *nit* mutants of *F. oxysporum* f. sp. *spinaciae* and f. sp. *lycopersici* were monitored for 2 years and correlated with disease severity (T. Takehara, *unpublished data*). However, a *nit* mutant of *Colletotrichum gloeosporioides* was reported to be less competitive than the wild type on northern joint vetch (a leguminous weed) (28). Thus, more study is needed on the saprophytic ability of *nit* mutants in raw soil.

Presently, the number of years *nit* mutants can survive in field soil is not known. *Nit* mutant-infested soil, media, and infected plants must be sterilized before disposal to avoid mutant dispersion, because once *nit* mutants become present in natural soil, they no longer can be used as marker strains. For the same reason, extreme care should be taken when using *nit* mutants in field experiments, and *nit* mutants should not be used in open fields.

Hot-water disinfestation and chloropicrin fumigation of soil are highly effective against *Fusarium* wilt of spinach (8). In field soil infested with the pathogen, the pathogen was killed by using hot water to elevate soil temperature to $>55^{\circ}\text{C}$ at a depth of 20 to 30 cm and holding that temperature for 3 to 4 h. However, the possibility of proliferation of the remaining or reintroduced pathogen after disinfestation has not been examined. We used a *nit*

Fig. 4. Influence of the pretreatment of nonpathogenic *Fusarium oxysporum* (NPF, strain S1HI1-W) on the proliferation of *F. oxysporum* f. sp. *spinaciae* (PF, nitrate-nonutilizing [*nit*] mutant M2-1) in sterilized soil. Soil dilution plates (10 days after plating) from the second experiment 13 days after inoculation of PF. From left column to right, soil samples were: PF alone (initial density: 10^2 bud cells/g of dry soil); PF alone (initial density: 10^4 bud cells/g of dry soil); NPF was treated at 10^4 bud cells/g of dry soil 8 days before PF inoculation at 10^2 CFU/g of dry soil; NPF was treated at 10^4 bud cells/g of dry soil 8 days before PF inoculation at 10^4 bud cells/g of dry soil. Soil samples at 10^{-1} , 10^{-2} , and 10^{-3} dilutions were plated on GMBP (wild-type *Fusarium*-selective medium, upper three lines) and on CGMBP (*nit* mutant-selective medium, lower three lines). Colonies of pretreated NPF (wild type) are growing on GMBP, and colonies of PF (*nit*) inoculated later are growing on CGMBP. Note that PF densities in NPF-treated soils are significantly lower than those in PF-alone soils.

Fig. 5. Influence of the pretreatment of nonpathogenic *Fusarium oxysporum* (NPF) on the proliferation of *F. oxysporum* f. sp. *spinaciae* (PF) in sterilized soil. **A and B**, first experiment; **C and D**, second experiment; **E and F**, third experiment. **A to D**, Nitrate-nonutilizing (*nit*) mutant of PF (M2-1) and wild type of NPF (S1HI1-W) were used. **E and F**, Wild type of PF (Spin-2) and *nit* mutant of NPF (NA-12) were used. **A, C, and E**, PF was incubated alone (initial density: 10^2 or 10^4 bud cells/g of dry soil); **B, D, and F**, NPF was treated at 10^4 bud cells/g of dry soil 8 days before PF inoculation at 10^2 or 10^4 bud cells/g of dry soil. Mean of two replicates. Vertical lines represent the standard error. **A and B**, Initial densities for PF and **B, D, and F**, initial densities for NPF are estimated values calculated from the number of incorporated bud cells.

mutant to study population dynamics of the pathogen in disinfested soils that contained relatively limited diversity of microorganisms, and demonstrated rapid proliferation of the pathogen compared with raw soil.

The pathogen in large root residues of spinach survives chloropicrin fumigation (4). Thus, survivors in roots after chloropicrin treatment may be an important source of reinfestation. Although rapid reinfestation with the pathogen in hot-water-treated fields has not been observed yet, care should be taken to avoid pathogen reintroduction.

To examine the possibility of delaying pathogen proliferation with preinoculation of different microorganisms, we pretreated soil with a nonpathogenic strain of *F. oxysporum* (NPF strain S1HI1-W) 7 days before the pathogen (PF) using a high NPF/PF density ratio. Spinach wilt then was suppressed (T. Takehara, unpublished data). Because the NPF strain is not antagonistic against PF in dual culture on PSA plates, the disease suppression is thought to be caused by competition for nutrition or infection sites or by induced systemic resistance (10,11,15). In flasks, NPF clearly suppresses PF proliferation but cannot reduce the density of already existing PF. Because NPF at least delays pathogen proliferation, introducing a highly proliferative NPF (12) after soil disinfestation may be useful.

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