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Characteristics, Stability and Reisolation of *nit* Mutant of *Fusarium oxysporum* from Strawberry

Dong Bum Shin*, Yeon Kyu Hong and Jae Min Cho
Plant Environment division, National Yeongnam Agricultural Experiment Station, RDA, Milyang 627-130, Korea

딸기로부터 분리된 *Fusarium oxysporum nit* 변이주의 특성과 안정성 및 재분리

신동범* · 홍연규 · 조재민
영남농업시험장 식물환경과

ABSTRACT : This study was conducted to investigate the potential of nitrate-nonutilizing mutants (nit mutants) in ecological studies of *Fusarium* disease of strawberry. *Nit* mutants of *Fusarium oxysporum* from strawberry were easily formed on chlorate-containing media. *Nit* mutants were assigned to three phenotypic classes, *nit*1, *nit*3, and NitM, on the basis of their growth on media containing one of the following five different nitrogen sources; nitrate, nitrite, hypoxanthine, ammonium and uric acid. Frequency of *nit* mutation and proportion of three phenotypes of *nit* mutants depended on the isolate. Mutation rate was 46.5% and ranged from 15.0% to 95.0%. The frequency of *nit*1 mutants was higher than that of *nit*3 or NitM. The complementary reaction between *nit*1 and NitM was higher than that of other combination. There has been no complementary response observed between *nit*3 and *nit*3. The *nit* mutants showed similar growth pattern as the that of wild type isolate on potato sucrose agar and potato sucrose liquid media. Most of the mutants retained pathogenicity, and maintained their phenotypes even after two year preservation through subculture on slanted PSA at room temperature. Nit mutants were selectively isolated from infested soil and infected plants on the selective medium (MMCPA) containing potassium chlorate with their original phenotypes, while naturally occurring isolates of *Fusarium oxysporum* were not grow on the medium. On the contrary, *nit* mutants showed very slight growth on the medium (MMPA) containing nitrate as a sole nitrogen source, and therefore could be distinguished from wild type isolates.

***Key words*: *Fusarium oxysporum*, *nit* mutant, strawberry.**

Fusarium oxysporum is the causal pathogen of wilt diseases in a broad range of host plants. *Fusarium* wilt of strawberry caused by *Fusarium oxysporum* f. sp. *fragariae* is manifested in young and mature plants throughout strawberry-growing regions. This disease is difficult to control, and especially cause severe damages on seedling. Accurate analysis of the population structure of this casual fungus would be helpful in developing effective control methods.

For ecological studies of *F. oxysporum* which causes a vascular disease to many agricultural crops, it is necessary to trace specific strains of pathogenic *F. oxysporum* by differentiating them from other formae speciales and races, and nonpathogenic strains of *F. oxysporum.*

*Corresponding author.

Several methods have been developed for the quantitative estimation of populations of *Fusarium* spp. in soil by means of selective media (4, 5, 11). These procedures are tedious, lengthy, and expensive. Puhalla (7) introduced the use of *nit* mutants (nitrate nonutilizing mutants) for studying heterokaryosis between strains of *F. oxysporum.* Hadar *et al.* (2) took the advantage of chlorate resistance of *nit* mutants and developed a chlorate-containing medium for the selective reisolation of *nit* mutants, and suggested the possibility of using *nit* mutants in ecological studies of *F. oxysporum.* It was also reported that *nit* mutants recovered from some formae speciales of *F. oxysporum* could be selectively reisolated from infected plants and infested soil on the chlorate-containing medium (8, 11). *Nit* mutants were used to monitor the behavior of a particular isolate in biological

control of cucumber fusarium wilt by nonpathogenic isolate of *F. oxysporum* (15). Another example of using *nit* mutants as markers for ecological study has been reported with *Colletotrichum acutatum* (3). And Takehara *et al.* (12) reported about stability of *nit* mutants of *Fusarium oxysporum*. In order to use *nit* mutants as markers for ecological studies of fungi, it is desirable for their mutants to have the same properties, much as pathogenicity, as wild-type strains, and to be stable during preservation. In this report, with *Fusarium oxysporum* isolated from strawberry plants, we examined the formation of *nit* mutant, the phenotype of obtained *nit* mutants and the complementary reaction between phenotypes of the *nit* mutants. We, also, examined the stability of *nit* mutants and reisolation of *nit* mutants from infected strawberry plants and infested soil.

MATERIALS AND METHODS

Tested isolates. The fourty isolates of *F. oxysporum* were isolated from strawberry plants collecting from several different fields at Milyang, which is main region of strawberry culture in Korea, and their *nit* mutants were used.

Media. Minimal medium (MM) was formulated as sucrose 30 g, $NaNO_3$ 2 g, $KH_2PO_47H_2O$ 0.5 g, KCl 0.5 g, sterile trace elements solution 0.2 ml in a liter of distilled water. The chlorate-containing medium (MMC) was MM amended with L-asparagine 1.6 g and $KClO_3$ 15 g in a liter of distilled water. For classification of the phenotype of *nit* mutant, we used the MM amended with one of the following five nitrogen sources: $NaNO_3$, $NaNO_2$, hypoxanthine, ammonium tartrate, and uric acid. To detect *nit* mutants in soil and plants, we used MMCPA medium which is a MM amended with $KClO_3$ 10 g, L-asparagine 1.6 g and PCNB 0.25 g in a liter of distilled water. For the detection of wild-type *F. oxysporum*, MMPA medium, which is MM amended with $KClO_3$ 10 g and PCNB 0.25 g in a liter of distilled water, was used. Komada's medium was used to detect *Fusarium* spp. in general.

***Nit* formation.** Isolates of *F. oxysporum* were grown on potato sucrose agar (PSA) for 7 days at 25°C. Twenty small mycelial blocks (3 mm in diameter) were then cut from these colonies, and placed well spaced four mycelial blocks on each plate of MMC medium. After incubating at 25°C for 7 days, mycelial blocks were cut from normally grown colonies, transferred to MM and incubated at 25°C. Mycelial block shown very thin but normally expansive growth was designated as nit mutant.

Classification of phenotype of *nit* mutant. The phenotypes of nit mutants obtained were classified on the basis of growth on amended MM media with one of the five nitrogen sources by incubating at 25°C for 7 days.

Survey of the complementary reaction. Mycelial blocks of *nit* mutant shown different phenotypes were equidistantly placed on MM plate. This plate was incubated at 25°C for 7 days, and then observed whether a line of dense growth was formed where they contacted.

Comparison of growth and pathogenicity between nit mutants and wild-type strains.

Growth on PSA and PS liquid media. Mycelial disks (5 mm in diameter) form three phenotypes of nit mutants (*nit*1, *nit*3, and NitM) and wild-type isolates were placed on the center of PSA medium in a plate (90 mm in diameter) with three replications, and incubated at 20°C. The radius of the colony was measured after 4 days of incubation. Mycelial disks were also placed in 100 ml of potato sucrose (PS) liquid medium and incubated at 28°C with shaking condition (120 rpm). After 7 days, bud cell was counted using a hematocytometer under light microscope.

Pathogenicity. Tested plants were cultivated in autoclaved soil in a vinyl pot (9 cm in diameter). The pathogen was cultured in 100 ml of PS liquid medium for 7 days with shaking condition (120 rpm). Fourteen days after transplanting the runner of strawberry (susceptible cultivar Hokowase), each pot was inoculated with 30 ml of the pathogen culture fluid (10^8 cfu/ml). Three pots for each treatment were used. Thirty days after inoculation, disease severity was evaluated. All of these plants were grown in greenhouse at 20~30°C in temperature range. The experiment was conducted twice.

Stability and reisolation of *nit* mutants.

Stability during preservation. Thirty isolates of each phenotype were preserved by subculture on slanted PSA for two years at room temperature. Their phenotypes, based on the utilization of five nitrogen sources after preservation, were compared to their original phenotypes.

Reisolation in soil and plants. Tested plants were cultivated in nursery soil (natural and autoclaved) in a vinyl pot (9 cm in diameter). The wild type and nit mutants (*nit*1, 3, M) of pathogen isolates were cultured in 100 ml of PS liquid medium for seven days at 25°C with shaking condition (120 rpm). Fourteen days after transplanting, each pot was inoculated with 30 ml of the pathogen culture fluid (10^8 cfu/ml). After strawberry seedlings died, the pots were maintained in greenhouse

and watered occasionally. Six months after inoculation, soil was sampled and the pathogen population was measured by soil dilution plating onto MMCPA and MMPA medium. Fifteen days after inoculation, segments of the crowns from the inoculated strawberry were surface disinfected with 70% ethanol for one minute and placed on MMCPA and MMPA medium. The reisolatied nit nutants were tested for their phenotypes by the utilization of five nitrogen sources.

RESULTS

Characterization of *nit* mutants. The nit mutants of *F. oxysporum* isolates from strawberry were formed on chlorate-containing media. Mutation rate was 46.5% and ranged from 15.0 to 95.0%. Nit mutants were assigned to three phenotypic classes, such as *nit*1, *nit*3, and NitM on the basis of their growth on media containing one of the following five different nitrogen sources; nitrate, nitrite, hypoxanthine, ammonium and uric acid. The frequency of *nit*1 was 51.3% and higher than that of *nit*3 or NitM (Table 1). The complementary reactions between *nit*1 and NitM mutant were higher than those of any other combinations. No complementary response was observed in *nit*3 and *nit*3 combination (Fig. 1).

Comparison of growth and pathogenicity between *nit* mutants and wild-type strains.

Growth on PSA and PS liquid medium. When mycelial disks from three phenotypes of *nit* mutants and wild-type isolate of *F. oxysporum* were incubated on PSA medium, *nit* mutants developed the same colonies as wild-type isolates, and *nit* mutants could not be distinguished from wild type isolates in morphologically. The growth rates were also the similar. For liquid culture on PS liquid medium, no difference in the number of bud cell was observed among tested isolates (Table 2).

Pathogenicity. *Nit* mutants retained pathogenicity to strawberry, and the difference in virulence between *nit* mutants and wild-types was not clear. The relationship between their phenotypes and pathogenicities was also not clear (Table 3).

Table 1. Frequency of *nit* mutation and with different phenotype of *Fusarium oxysporum* isolates from strawberry when induced on the chlorate containing medium

No. of tested isolates	% of nit mutation[a]	% of different phenotype[b]		
		*nit*1	*nit*3	NitM
40	46.5 (15~95)	51.3	23.7	25.0

[a]The twenty mycelial blocks per each isolate were used when induced on the medium containing chlorate.
[b]The phenotypes of *nit* mutants obtained were classified based on the growth on amended MM media with one of the five nitrogen sources.

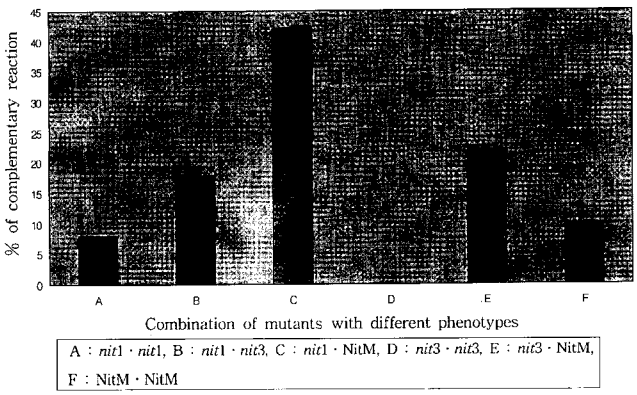

Fig. 1. Frequency of complementary reaction between phenotypes of obtained nit mutant from *Fusarium oxysporum* when cultured on the MM medium at the same plate.

Table 2. The growth of nit mutants and wild type isolate of *F. oxysporum* on PSA and PS liquid medium

Strain	Phenotype	Radius of colony (mm)[a]	No. of bud cell (10^8 cell/ml)[b]
YNS 055n-1	*nit*1	46.7 a[c]	1.13 a
YNS 179n-5	*nit*3	45.3 a	1.20 a
YNS 371n-6	NitM	46.7 a	1.27 a
YNS 215	Wild type	47.3 a	1.23 a

[a]After 4 days of incubation at 25°C on PSA medium. Average of 3 replications.
[b]After 7 days of incubation at 28°C on PS liquid medium with shaking culture (120 rpm).
[c]Means followed by a common letter are not significantly different at the 5% level by DMRT.

Stability and reisolation of *nit* mutants.

Stability during preservation. All tested *nit* mutants maintained their original phenotypes even after two years of preservation by subculture on slanted PSA at room temperature (Table 4).

Reisolation in soil and plants. When the tested isolates, which were inoculated on test plants cultivated in natural and autoclaved soil, were reisolated at 15 days and 6 months from plants and soil after inoculation, respectively, on the potassium chlorate-containing selective medium (MMCPA), *nit* mutants were selectively isolated from infected plants and infested soil, respectively (Table 5). Naturally occurring isolates of *F. oxysporum* did not grow on this medium. On the medium containing nitrate as a sole nitrogen source (MMPA), *nit* mutants showed very slight growth and could be dis-

Table 3. Pathogenicity of *nit* mutants and wild type isolate of *F. oxysporum* from strawberry

Strain	Phenotype	% of disease occurrence[a]	
		Experiment 1	Experiment 2
YNS 206n-1	*nit*1	100.0	100.0
YNS 206n-5	*nit*3	100.0	100.0
YNS 206n-10	NitM	66.7	100.0
YNS 215n-1	*nit*1	100.0	100.0
YNS 215n-2	*nit*3	100.0	66.7
YNS 215n-4	NitM	100.0	100.0
YNS 363n-2	*nit*1	66.7	100.0
YNS 363n-10	*nit*3	100.0	100.0
YNS 363n-6	NitM	100.0	66.7
YNS 206	wild-type	100.0	100.0
YNS 215	wild-type	100.0	100.0
YNS 363	wild-type	100.0	100.0
Control(uninoculated)	–	0.0	0.0

[a]The browning of tissue in crown was observed 30 days after inoculation.

Table 4. Mutations of phenotypes during the preservation of *nit* mutants from *F. oxysporum*[a]

Phenotype	No. of mutants which changed in phenotype		
	6 month	12 month	24 month
*nit*1	0/30	0/30	0/30
*nit*3	0/30	0/30	0/30
NitM	0/30	0/30	0/30

[a]Preserved on slanted PSA at room temperature(subcultured two or three times).

tinguished from wild type strains of *F. oxysporum*. The population densities of *nit* mutants in the soil were approximately $30\sim40\times10^3$ cfu/g dry soil, which were the same level as the wild type isolates (Table 5). *Nit* mutants which were reisolated on MMCPA from infected plants and infested soil, retained their original phenotypes (Table 6.)

Table 6. The stability of *nit* mutants in the infected plants and infested soil

Strain	Phenotype	Treatment	% of altered phenotypes[a]	
			Plants	Soil
YNS 501n-1	*nit*1	natural soil	0.0	0.0
YNS 504n-4	*nit*3	natural soil	0.0	0.0
YNS 506n-3	NitM	natural soil	0.0	0.0
YNS 501n-1	*nit*1	autoclaved soil	0.0	0.0
YNS 504n-4	*nit*3	autoclaved soil	0.0	0.0
YNS 506n-3	NitM	autoclaved soil	0.0	0.0

[a]The *nit* mutants were reisolated from infected plants and infested soil 15 days and 6 months after inoculation, respectively. The phenotypes of *nit* mutants were tested on the basis of growth on amended MM media with one of the five nitrogen sources.

DISCUSSION

Several methods have been developed for the quantitative estimation of populations of *Fusarium* spp. in soil by means of selective media (4, 5, 13). This procedure is tedious, lengthy and expensive. One approach to overcome this difficulty is to use strains labeled with specific markers. Another approach is the use of auxotrophic mutants frequently obtained through mutagenesis. However, such mutants might not be true representation of the natural population of the pathogen.

Nitrate nonutilizing mutants, designated as *nit*, form a thin and expansive mycelium on nitrate minimal medium

Table 5. The selective reisolation of nit mutants of *F. oxysporum* from infected strawberry plants and infested soil

Strain	Phenotype	Treatment	Frequency of reisolation[a]			
			Plants (reisolation)		Soil (10^3 cell/dry soil/g)	
			MMCPA	MMPA	MMCPA	MMPA
YNS 501n-1	*nit*1	natural soil	+	+	37.3	2.7
YNS 504n-4	*nit*3	natural soil	+	+	39.7	3.0
YNS 506n-3	NitM	natural soil	+	+	41.7	2.7
YNS 501n-1	*nit*1	autoclaved soil	+	–	37.0	0.0
YNS 504n-4	*nit*3	autoclaved soil	+	–	34.7	0.0
YNS 506n-3	NitM	autoclaved soil	+	–	33.7	0.0
YNS 501	wild-type	natural soil	–	+	0.0	32.7
YNS 504	wild-type	natural soil	–	+	0.0	35.3
YNS 506	wild-type	natural soil	–	+	0.0	39.0
YNS 501	wild-type	autoclaved soil	–	+	0.0	36.3
YNS 504	wild-type	autoclaved soil	–	+	0.0	30.0
YNS 506	wild-type	autoclaved soil	–	+	0.0	28.3

[a]Reisolated from infected plants and infested soil 15 days and 6 months after inoculation, respectively.

but grow densely in the presence of ammonium or organic nitrogen sources. Puhalla (7) introduced this mutants for studying heterokaryosis between strains of *F. oxysporum*. Hadar *et al.* (2) suggested the possibility of using *nit* mutants in ecological studies of *F. oxysporum* by using a chlorate containning selective medium.

Nit mutants of some formae speciales of *F. oxysporum* except *fragariae* were easily formed on chlorate containing medium, assigned to three phenotypes (*nit*1, *nit*3, NitM). Frequency of *nit* mutation and proportion of three phenotypes in the *nit* mutants depend on the strain of each formae speciales (10). Shin *et al.* (8) also reported about *nit* mutation of *F. oxysporum* from spinach that the frequency of *nit*1 mutant was higher than that of *nit*3 and NitM, and the frequency of complementary reaction was higher at *nit*1 · NitM and NitM · NitM combination than other combination. There were no complementary responses observed between *nit*3 · *nit*3 combination (1, 8, 10). In this study, we have obtained same tendency result. Corell *et al.* (1) reported that all *nit*1 and *nit*3 mutants readily complemented the NitM mutants, and NitM mutant should be used as one of the *nit* mutant testers to identify the vegetative compatibility group to which isolates of *F. oxysporum* belong.

In order to use nit mutants as markers for ecological studies of *F. oxysporum*, it is desirable for *nit* mutants to have the same properties, such as pathogenicity, as those of wild type strains. In this study, in terms of growth on solid or liquid media and pathogenicity, most mutants showed the same growth pattern or pathogenicity as the wild type strain. Takehara *et al.* (12) reported that although some mutants of *F. oxyspoum* f. sp. showed slow growth on solid media or weakened pathogenecity, most mutants showed the same growth pattern and pathogenicity. Watanabe (14) reported that *nit* mutants of non-pathogenic *F. oxysporum* available for cross protection against *Fusarium* wilt in sweet potatoes retained their ability for cross protection.

Although we did not observe the recovery of nitrate utilization by *nit* mutants during their preservation on slanted PSA, Takehara *et al.* (12) reported the recovery rate was ranged from 0 to 23.1%. The cause of this reversion is not known, but this indicates some instability of *nit* mutants, and it may have to be careful in this respect when using them as markers. It is to be noted that none of *nit*3 mutants recovered the ability to use nitrate. It is thought to represent the mutation at a nitrate assimilation pathway specific regulatory locus. *Nit*3 might be a more stable mutant than *nit*1 or NitM (1). In this study, although all tested *nit* mutants did not show the reversion of their phenotype, further study is needed to consider the stability and preservation of *nit* mutants.

Stability tests in the soil showed that *nit* mutants can survive for a long period in soil without changing their phenotypes (12). In our study, no changes in phenotypes of *nit* mutants were observed 15 days after infection on plants and 6 months after inoculation in soil.

Nit mutants were selectively isolated from infested soil and infected plants on a potassium chlorate containing selective medium (MMCPA), while naturally occurring isolates of *F. oxysporum* could not grow on the same medium. On the contrary, *nit* mutants showed very slight growth on a medium nitrate as a sole nitrogen source (MMPA), and therefore could be distinguished from wild type isolates.

From all of these experiments, we concluded that *nit* mutants can be used as markers in ecological studies of *F. oxysporum* related strawberry. In the future, it will be necessary to further investigate whether nit mutants can proliferate and survive in natural soil as wild-type strains survive.

요 약

딸기 시들음병 발생생태 연구에 있어서 *nit* 변이균주의 이용가능성을 구명하고자 연구를 수행하였는데, 그결과는 다음과 같다. 딸기 식물체로부터 분리된 *Fusarium oxysporum* 균주의 *nit* 변이주는 쉽게 작출되었으며 그 작출율은 15.0~95.0%이었다. 작출된 *nit* 변이주는 *nit*1, *nit*3, NitM의 3가지 표현형으로 유별되었으며, 3가지 표현형 중에서 *nit*1의 분포비율이 *nit*3, NitM 보다 높았다. 그리고 *nit* 변이주 상호간에 균사보완반응이 관찰되었는데, *nit*1과 NitM의 상호간의 균사보완반응 비율이 다른 조합에서 보다 높았으며, *nit*3과 *nit*3의 상호간에는 균사보완반응이 관찰되지 않았다. *nit* 변이주들은 야생주와 거의 같은 생육을 보였으며, 병원성에 있어서도 야생주와 같은 경향을 보였다. 그리고 *nit* 변이주를 실온에서 2년간 보존하여도 그들의 표현형을 유지하였다. 또한 염소산 염($KClO_3$)을 함유한 선택분리배지(MMCPA)에서 야생주는 생육되지 않았으나, 감염토양 및 식물체로부터 *nit* 변이주가 선택적으로 분리되었으며, *nit* 변이주 본래의 표현형은 유지되었다. 한편 질소원으로써 질산염을 함유한 분리배지(MMPA)에서 *nit* 변이주는 거의 자라지 않아 야생균주와 식별이 가능하였다.

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