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Vegetative Compatibility, Isozyme Polymorphisms and Pathogenicity of Isolates of *Fusarium oxysporum* f. sp. *fragariae*

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딸기 시들음병균(*Fusarium oxysporum* f. sp. *fragariae*)의 균주간 Vegetative Compatibility, 동위효소 Polymorphism과 병원성

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Abstract : Thirty-two isolates of *Fusarium oxysporum* from strawberry plants were examined for vegetative compatibility, isozyme polymorphism and pathogenicity. Four major vegetative compatibility groups (VCGs) were identified by means of a nitrate reductase complementation test, and four isozyme polymorphism groups were found with isozyme patterns of esterase, catalase, acid phosphatase and leucine aminopeptidase (LAP). There was significant correlation between the isozyme polymorphism and the vegetative compatibility; all isolates in VCGs A and D belonged to isozyme polymorphism group I and five of seven isolates in VCG B belonged to isozyme polymorphism group II. Two isolates in VCG C belonged to isozyme polymorphism groups II and III. Two isolates in VCG A were highly pathogenic to the Hokowase cultivar and moderately pathogenic to the Sistakara and Leiko cultivars. However, two isolates in VCG B and one isolate in VCG C were weakly pathogenic to all four cultivars except 1 isolate in VCG C which was highly pathogenic to the Leiko cultivar.

Key words **: cluster analysis, *Fusarium oxysporum* f. sp. *fragariae*, isozyme polymorphism, vegetative compatibility group.**

Fusarium oxysporum is a common soilborne plant pathogen with worldwide distribution and causes economic damage to agricultural crops. Within the species, however, there is a high level of host specificity (4). This specificity led Snyder and Hansen (28) to classify strains of the fungus into formae speciales on the basis of the host plants attacked.

Winks (32) reported that wilt of strawberry is caused by a new form of *Fusarium oxysporum*, and proposed the casual organism is *Fusarium oxysporum* Schlecht. ex Fr. f. sp. *fragariae*.

The leaves of strawberry infected with *F. oxysporum* f. sp. *fragariae* are generally rolled, chlorotic and suddenly wilted, and 1~2 of 3 leaflets are occasionally smaller than the others. The crown in all cases showed distinct reddish-brown vascular discoloration (3, 17).

To characterize the isolates of *F. oxysporum* from diseased strawberry plants in this study, the isozyme polymorphism and vegetative compatibility which is another potentially useful technique for studying genetic diversity in *F. oxysporum* were used. Nitrate nonutilizing (*nit*) mutants have provided a good marker for screening heterokaryotic colonies, and Puhalla (27) first adapted a technique developed by Cove (8) for *Aspergillus* to *Fusarium*, showing that the vegetative compatibility group (VCG) may be a handy tool for differentiating formae speciales of *F. oxysporum*.

Correll *et al.* (5) refined the heterokaryon technique by demonstrating that *nit* mutants of *F. oxysporum* could be subdivided into at least three phenotypic

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groups.

Venter *et al.* (31) assigned thirty-five South African isolates of *F. oxysporum* f. sp. *tuberosi* to six VCGs based on pathogenicity and vegetative compatibility. Kedera *et al.* (16) have shown that most maize plants are infected by two to three strains belonging to *Fusarium* section *Liseola* by using vegetative compatibility groups. Strausbaugh (29, 30) reported twenty-three strains of *Verticillium dahliae* could be subdivided into 4 VCGs based on pairings nitrate-nonutilizing mutants induced on medium containing chlorate.

In the past 20 years, electrophoretic separation of proteins has become an important tool in identification and differentiation of specific and subspecific taxa in various organisms, including fungi (23, 24). Electrophoretic separation of enzymes, which exploits the polymorphism of detected isozyme forms, is another molecular technique that has been widely used to generate a large number of markers for the assessment of genetic diversity in fungi (9).

Damaj *et al.* (9) reported that fifty isolates of binucleate *Rhizoctonia* representing 12 Japanese and 5 North American anastomosis groups (AGs) were divided into four district groups by cluster analysis of isozyme bands.

In this study, the genetic diversity of isolates of *F. oxysporum* f. sp. *fragariae* by VCGs and isozyme polymorphisms was investigated and the relationships among pathogenicity, isozyme patterns by electrophoresis and VCGs of isolates of *F. oxysporum* f. sp. *fragariae* were examined.

MATERIALS AND METHODS

Isolates of *F. oxysporum* f. sp. *fragariae*. All isolates of the pathogen were obtained from strawberry plants showing typical Fusarium disease symptoms (wilt and xylem discoloration). The diseased plants were collected from the fields of four districts in Korea (Table 1). The fungus was isolated by plating diseased plant tissues (surface-disinfested with 1% sodium hypochlorite for 5 min) on Komada's medium. Single spore cultures were established from microconidia of isolates that were identified as *F. oxysporum* on carnation leaf agar (CLA) medium by using the system of Nelson, Tousson, and Marasas (22) (Fig. 1). The isolates were maintained in soil culture at 4°C to minimize spontaneous mutation. Inoculum was obtained from these cultures as required by plating soil crumbs onto Czapeck-dox agar medium.

Pathogenicity test. Strawberry plants at 3~5 leaf-stage in pots (12.5×11 cm) were inoculated with the spore suspension (8×10^7 spores) of *F. oxysporum* f. sp. *fragariae* by soil drench using a 12 ml syringe. The spore suspension was prepared by shaking culture in Czapeck-dox medium. Control was Czapeck-dox medium without the fungus. Disease development was checked at 4 weeks after inoculation. To evaluate infection of inoculated plants, disease severity was rated based on, − : no wilting; + : edge of old leaves wilting; ++ : more than half of old leaves wilting and central leaves going to wilt or central leaves appearing deformed; and +++ : stems wilting.

Media. The minimal medium (MM) was composed of 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; Difco agar, 20 g in 1 liter distilled water. The trace elements solution was citric acid, 5 g; $ZnSO_4 \cdot 7H_2O$, 5 g; $FeSO_4 \cdot 7H_2O$, 4.75 g; $Fe(NH_4)_2SO_4 \cdot 6H_2O$, 1 g; $CuSO_4 \cdot 5H_2O$, 250 mg; $MnSO_4 \cdot H_2O$, 50 mg; H_3BO_3, 50 mg; $Na_2Mo_4 \cdot 2H_2O$, 50 mg in 95 ml distilled water. To generate nitrate non-utilizing mutants (*nit* mutants), the MM was amended with 1.6 g of L-asparagine and 15 g of $KClO_3$ per liter to produce chlorate medium (MMC). Phenotypic classes of the generated *nit* mutants were assigned on the basis of their growth on media containing different nitrogen sources. The media were nitrate medium (MM) and four other media; nitrite medium in which sodium nitrate was substituted with 0.5 g l^{-1} $NaNO_2$; hypoxanthine medium in which 0.2 g l^{-1} hypoxanthine; ammonium medium in which 1.0 g l^{-1} ammonium tartrate; uric acid medium in which 0.2 g l^{-1} uric acid.

Generation of nitrate non-utilizing (*nit*) mutants. Methods described by Puhalla (27), Correll *et al.* (6) and Katan *et al.* (15) were used. Soil crumbs of the culture were plated onto MM and grown for 3~4 days, then 2 mm^2 mycelial blocks were transferred on MMC and incubated for 15 days. Mycelial fragments from fast growing areas were transferred on MM and incubated for 3~4 days. Cultures producing a very thin but normally expansive growth with no aerial mycelium on MM were considered to be *nit* mutants. All *nit* mutants were resistant to chlorate (MMC) but showed wild-type growth on PDA.

Characterization of *nit* mutant phenotypes. The *nit* mutants were assigned to different phenotypic classes on the basis of their growing on media con-

Table 1. Host cultivar, geographic origin and VCGs of isolates of *Fusarium oxysporum* f. sp. *fragariae*

Isolate	Host cultivar	Geographic origin	VCG[a]	I.P.T.[b]
Y1	Hokowase	Chungchongnam-do Yongi	A	I
K1	Hokowase	Kyonggi-do Kimpo	A	I
K2	Hokowase	Kyonggi-do Kimpo	A	I
K3	Hokowase	Kyonggi-do Kimpo	A	I
K4	Hokowase	Kyonggi-do Kimpo	A	I
N2	Hokowase	Chungchongnam-do Nonsan	A	I
N3	Hokowase	Chungchongnam-do Nonsan	A	I
N4-1	Hokowase	Chungchongnam-do Nonsan	A	I
N6-1	Hokowase	Chungchongnam-do Nonsan	A	I
N6-2	Hokowase	Chungchongnam-do Nonsan	A	I
N7	Hokowase	Chungchongnam-do Nonsan	A	I
N8	Hokowase	Chungchongnam-do Nonsan	A	I
N9	Hokowase	Chungchongnam-do Nonsan	A	I
N10	Hokowase	Chungchongnam-do Nonsan	A	I
M1-2-1	Sistakara	Kyongsangnam-do Miryang	A	I
M1-1	Sistakara	Kyongsangnam-do Miryang	B	IV
M2-2-1	Sistakara	Kyongsangnam-do Miryang	B	IV
M2-4-1	Sistakara	Kyongsangnam-do Miryang	B	IV
M2-4-2	Sistakara	Kyongsangnam-do Miryang	B	IV
M3-2	Sistakara	Kyongsangnam-do Miryang	B	IV
M3-3-2	Sistakara	Kyongsangnam-do Miryang	B	III
M4-1	Leiko	Kyongsangnam-do Miryang	B	I
M5	Leiko	Kyongsangnam-do Miryang	C	III
N11	Hokowase	Chungchongnam-do Nonsan	C	IV
N1	Hokowase	Chungchongnam-do Nonsan	D	I
Y2	Hokowase	Kyonggi-do Kimpo	D	I
N5	Hokowase	Chungchongnam-do Nonsan	E	III
M1-2-2	Sistakara	Kyongsangnam-do Miryang	F	II
M1-3	Sistakara	Kyongsangnam-do Miryang	G	III
M2-1-1	Sistakara	Kyongsangnam-do Miryang	H	II
M2-1-2	Sistakara	Kyongsangnam-do Miryang	I	III
M3-1	Sistakara	Kyongsangnam-do Miryang	J	III

[a] Vegetative compatibility group.
[b] Isozyme polymorphism type.

taining one of five different nitrogen sources (Table 2) (21). To determine the physiological phenotype, mycelium of the *nit* mutant was grown on each of the five media, and the colony morphology was scored relative to the wild-type parent after 4-day culture.

Vegetative compatibility tests. Complementation was evident by the formation of the dense aerial wild-type mycelium at the contact zone between two *nit* mutants (Fig. 2).

All mutants were paired on MM. Heterokaryon formation between mutants of different parental isolates was checked after incubation at 28°C for 4~10 days to determine their vegetative compatibility groups (VCGs).

Extraction of protein. Mycelial mats of 10-day-old culture in PDB were harvested by suction-filtering through filter paper. The mycelial mats were macerated in 0.1 M Tris-HCl buffer (0.1 M Tris, 2 mM EDTA), pH 7.2, by mortar and pestle with sea sand. The homogenates were centrifuged at 15,000 g for 30 min at 4°C. The supernatants were used for electrophoresis.

Electrophoresis and isozyme staining. Electrophoresis was carried out in 6~26% polyacrylamide gradient slab gel (200×150×2 mm) with the discontinuous buffer system.

Esterase, catalase, acid phosphatase and leucine aminopeptidase (LAP) were detected on gel by various staining methods (23).

Cluster analysis. Comparison between isolates for

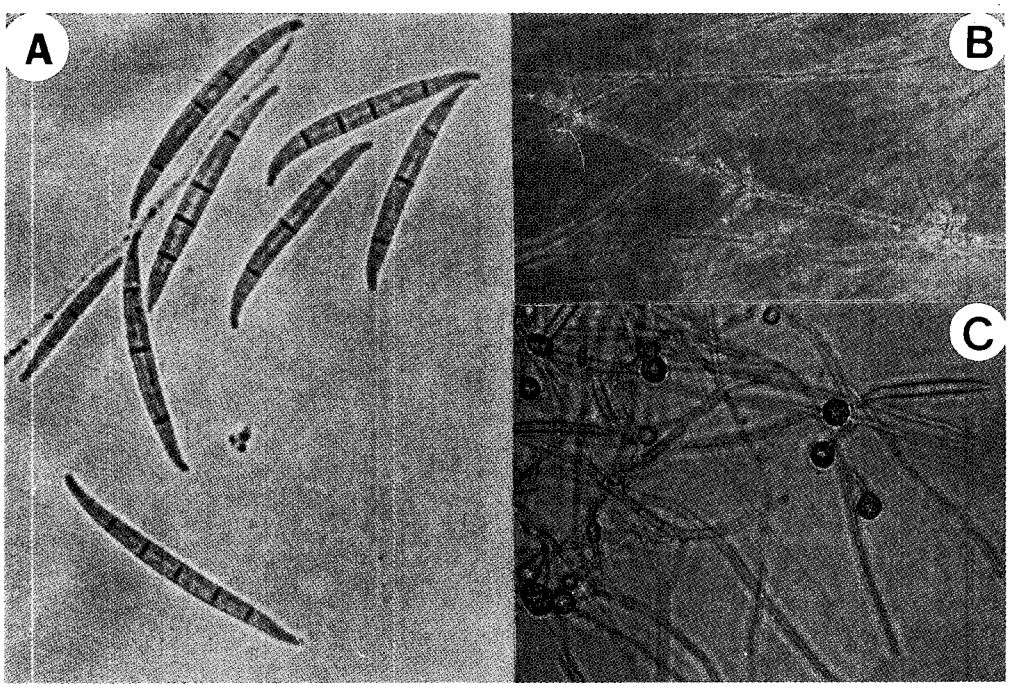

Fig. 1. *Fusarium oxysporum* isolated from diseased strawberry. A : Macroconidia (×400) on CLA medium. B : Microconidia and microconidiophore (×100) on CLA medium. C : Chlamydospores (×200) on CLA medium.

Table 2. Identificatin of nitrate nonutilizing (*nit*) mutant phenotypes from *Fusarium oxysporum* f. sp. *fragariae* by growth on different nitrogen sources

Mutant designation	Growth on nitrogen sources[a]					Mutation[b]
	Nitrate	Nitrite	Ammonium	Hypoxanthine	Uric acid	
Wild-type	+	+	+	+	+	None
Nit 1	−	+	+	+	+	Nitrate reductase structural locus
Nit 3	−	−	+	+	+	Pathway-specific regulatory locus
Nit M	−	+	+	−	+	Molybdenum cofactor locus

[a] Growth on basal medium with various nitrogen sources; + : typical wild-type growth, − : thin growth with no aerial mycelium.

[b] Complied from Marzluf (1981) on the basis of analysis of mutants from *Neurospora crassa*.

each isozyme profile was done on the basis of the presence versus absence of isozyme of the same *Rf* value. Bands of the same *Rf* value were scored as identical. A phenogram was derived from the similarity matrix by the unweighted pair-group method algorithm contained in the SAS (20).

RESULTS

***Nit* mutant isolation.** Spontaneous chlorate-resistant sectors were readily recovered from all isolates of *F. oxysporum* f. sp. *fragariae* when cultured on MMC. A majority (60~100%) of the chlorate-resistant sectors were unable to utilize nitrate as a sole nitrogen source and consequently grew as thin expansive colonies with no aerial mycelium on MM; these were regarded as *nit* mutants. Finally, 367 *nit* mutants were isolated from the isolates.

***Nit* mutant phenotypes.** The *nit* mutants could be divided into three phenotypic classes by the sites of mutation; at the nitrate reductase structural locus (*nit 1*), a nitrate assimilation pathway specific regulatory locus (*nit 3*) and loci (at least 5) that affect the assembly of a molybdenum containing co-factor necessary for nitrate

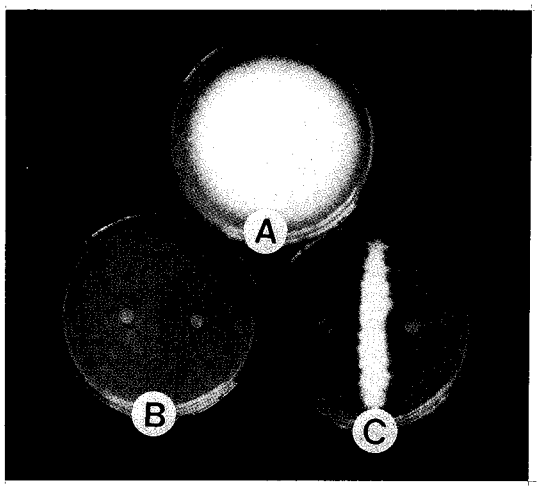

Fig. 2. Heterokaryon formation between nitrate nonutilizing mutants of *Fusarium oxysporum* on a minimal medium (MM). A : Typical wild-type culture, B : No complementation, C : Strong complementation.

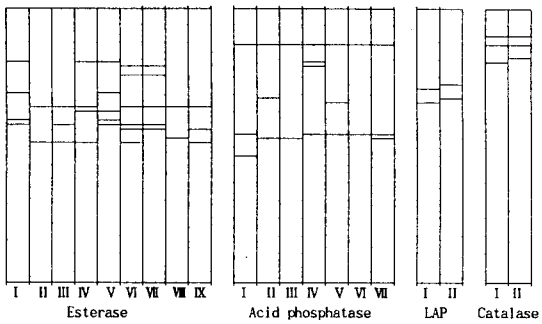

Fig. 3. Zymorgrams of esterase, acid phosphatase, LAP and catalase from mycelium of *Fusarium oxysporum* f. sp. *fragariae*. Each lane represents a separate isozyme pattern type.

reductase activity (nit M).

Consequently, 174 *nit 1* mutants, 114 *nit 3* mutants and 79 nit M mutants were recovered, and the recovering frequency was 47.3%, 31.1% and 21.6% for *nit 1*, *nit 3* and nit M mutants, respectively.

Vegetative compatibility of isolates. Twenty-six isolates out of 32 isolates were compatible with each other to some extent. Fourteen isolates, Y1, K1, K2, K 3, N2, N3, N4-1, N6-1, N6-2, N7, N8, N9, N10 and M1-2-1, were strongly compatible with isolate K4. All these isolates are therefore classified as VCG A.

Isolates M1-1, M2-2-1, M2-4-1, M2-4-2, M3-2, M3-3-2 and M4-1 were compatible with one another, but not with other isolates. We therefore assigned them as

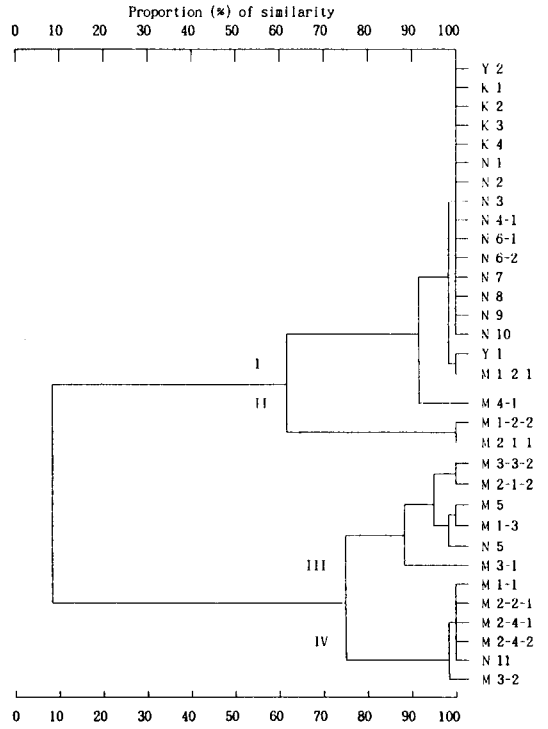

Fig. 4. Phenogram demonstrating relationships among 32 isolates of *Fusarium oxysporum* f. sp. *fragariae* by numerical cluster analysis.

VCG B.

Isolates N1 and Y2 and isolates M5 and N11 were compatible with each other but not with any other isolates; therefore, they were assigned as VCG C and VCG D.

Isolates N5, M1-2-2, M1-3, M2-1-1, M2-1-2- and M 3-1, however, were not compatible with any other isolates; we assigned each of the isolates to a single VCG, VCGs E, F, G, H, I and J, respectively.

Isozyme analysis. When the 32 isolates of *F. oxysporum* f. sp. *fragariae* were surveyed for isozyme polymorphism by electrophoresis, 27 putative loci were detected in the 4 enzyme systems assayed.

There were 9 esterase, 7 acid phosphatase, 2 LAP and 2 catalase zymogram types (Fig. 3).

Based on the isozyme analysis, a phenogram was constructed that depicts mean similarities between groups of isolates with identical isozyme patterns (Fig. 4). Four groups are evident. Group I consisted of isolates K1, K2, K3, K4, Y1, Y2, N1, N2, N3, N4-1, N4-2, N6-1, N6-2, N7, N8, N9 and N10 from cultivar Hokowase, and isolate M1-2-1 and isolate M4-1 from

Table 3. Pathogenicity of isolates of *Fusarium oxysporum* f. sp. *fragariae* representing each VCG to four cultivars at 3~4 leaf stage grown in each pot

Cultivar	Isolate				
	VCG A		VCG B		VCG C
	K4	M1-2-1	M1-1	M2-4-1	N11
Hokowase	+[a]	+++	+	+	+
Soohong	−	+	−	−	+
Sistakara	+	++	+	+	n
Leiko	++	++	+	−	+++

[a] − : No symptoms; + : Edge of old leaves showed wilting; ++ : More than half of old leaves showed wilt and central leaflets were going to wilt, or central leaflets appeared to be deformed; +++ : Stem was wilted; n : No test.

cultivar Sistakara and cultivar Leiko.

Group II consisted of isolates M1-2-2 and M2-1-1 from cultivar Sistakara. Group III consisted of isolate N5 from cultivar Hokowase, isolates M1-3, M2-1-1, M 3-1 and M3-3-2 from cultivar Sistakara and isolate M5 from cultivare Leiko. Group IV consisted of isolates M 1-1, M2-2-1, M2-4-1, M2-4-2 and M3-2 from cultivar Sistakara.

Relationship between VCG and isozyme polymorphism groups. All isolates in VCG A and D were isozyme polymorphism group I, 5 isolates out of 7 isolates in VCG B were isozyme polymorphism group IV. And 2 isolates in VCG C were isozyme polymorphism group III and IV. Each isolae in VCG E, F, G, H, I and J was isozyme polymorphism group II and III (Table 1).

Relationship between VCG and pathogenicity of isolates. Four strawberry cultivars, Hokowase, Soohong, Sistakara and Leiko were inoculated with 2 isolates of VCG A, isolates K4 and M1-2-1, 2 isolates of VCG B, isolates M1-1 and M2-4-1, isolate N11 of VCG C and isolate M1-3 of VCG G. After four weeks, disease severity was rated.

Isolates K4 and M1-2-1 were highly pathogenic to cultivar Hokowase and moderately pathogenic to cultivars Sistakara and Leiko, relatively (Table 3). However, isolates M1-1 and M2-4-1 of VCG B and isolate N11 of VCG C were little or weakly pathogenic to the 4 cultivars except isolate N11 which was highly pathogenic to cultivar Leiko, relatively.

DISCUSSION

Venter *et al.* (31) characterized thirty-five South African isolates of *F. oxysporum* f. sp. *tuberosi* by pathogenicity and vegetative compatibility, and found a complex pattern of association among disease symptoms caused in potatoes, VCG, and geographic distribution.

Strausbaugh *et al.* (30) reported when 26 strains of *Verticillium dahliae* representing 16 VCGs assayed using microsclerotial color mutants were reassessed using *nit* mutants, only four VCGs were identified.

In this study at least four VCGs were distinguished among the isolates. A similar situation has been reported for other formae speciales (6, 7, 15), but some have numerous VCGs (10, 25).

Some isolates did not pair with some other isolates in the same VCG or did so in a weak manner. Similar infidelity of vegetative compatibility within a VCG has been reported elsewhere (1, 25).

This could be due to a lack of complementation between certain combinations of mutants (12), or it may reflect as yet undescribed complexities in the genetic basis for vegetative compatibility.

Correll *et al.* (6), Flood *et al.* (11) and Katan *et al.* (15) reported races were related to VCGs. On the other hand, it is reported that no correlations were found between race and VCG (7, 10, 13, 14, 18, 25, 26).

We found that the correlation among VCGs, host cultivars and geographic origins was not precise. This shows that isolates of *F. oxysporum* f. sp. *fragariae* in Korea may have no specificity to the host cultivars and geographic origins.

But in a result of pathogenicity test to the 4 strawberrry cultivars, we assumed that though more number of isolates should be tested, virulence of isolates between VCGs might be different.

Therefore, even though races of *F. oxysporum* f. sp. *fragariae* were not clearly defined, variation may reflect different levels of aggressiveness in this study.

Accordingly, awareness of the comparative virulence of populations of *F. oxysporum* f. sp. *fragariae* from

different VCGs is suggested when selecting isolates to use for resistance screening and when considering the release and distribution of resistant material bred.

Isozyme variation has been used to determine the taxonomic status of organisms when the morphological characteristics were not distinctive. This has been possible because isozymes are consistent within an individual, unaffected directly by environmental factors, and often show considerable differentiation between different populations of individuals. Since each locus of an enzyme can be used as an independent source of taxonomic information, many traits can be examined in an organism (2).

All isolates tested were differentiated into 4 zymogram types by electrophoretic patterns of the four enzymes. There was a correlation between isozyme patterns and VCGs except some isolates. These suggest that there is sufficient differentiation among the pathotypes to support the division of *F. oxysporu* f. sp. *fragariae* into four or more.

In this study, sufficient differentiation among isolates of *F. oxysporum* f. sp. *fragariae* by VCG, isozyme patterns and pathogenicity, and some correlation between VCGs and isozyme patterns were found, but race was not clearly defined.

요 약

우리나라 여러 딸기 재배지에서 전형적인 시들음 증상을 나타내는 이병식물에서 분리한 32개의 *Fusarium oxysporum* 균주들을 vegetative compatibility와 전기영동에 의한 동위효소상의 차이에 의해 분류하고 이들과 병원성과의 관계를 알아보았다. Nitrate-nonutilizing(*nit*) mutant를 이용하여 vegetative compatibility group으로 분류해 본 결과 크게 A, B, C, D의 4개 그룹으로 나눌 수 있었으며 A그룹에는 15개 균주, B그룹에는 7개 균주, C와 D그룹에는 각각 2개 균주, 그리고 single VCG인 6개 균주가 존재하였다. 전기영동에 의한 esterase, catalase, acid phosphatase, leucin-aminopeptidase(LAP)의 동위효소상을 비교하여 본 결과 I, II, III, IV의 4개 그룹으로 나눌 수 있었으 I 그룹에는 18개 균주, II 그룹에는 2개 균주, III 그룹에는 6개 균주, IV 그룹에는 6개 균주로 분류할 수 있었다. 또한, VCG의 A와 D그룹에 속하는 모든 균주들은 동위효소상의 I 그룹에 속하였으며 VCG B그룹의 7개 균주들 중에서 5개 균주가 동위효소상의 IV 그룹, 나머니 2개 균주는 I 그룹과 III 그룹에 속하였으며 C 그룹의 균주는 III과 IV 그룹에 속하였고, single VCGs들은 III 그룹에 4개 균주, II 그룹에 2개 균주가 속하므로 VCG와 동위효소상 간에는 밀접한 관계가 있음을 알 수 있었다. 4개 딸기 품종에 대해 병원성을 비교하여 본 결과 VCG A에서 선발된 2개 균주들은 보교조생에 대해 가장 병원성이 강하였으며 정보나 여홍에 대해서도 강한 병원성을 가지고 있었다. 반면에 B 그룹에서 선발된 2개 균주들은 4개 품종에 대해 병원성이 약하거나 거의 없었으며 C 그룹에서 선발된 1개 균주는 여홍에 대해 상대적으로 가장 강한 병원성을 가지고 있었다.

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