Characterization of *Fusarium oxysporum* f. sp. *fragariae* Based on Vegetative Compatibility Group, Random Amplified Polymorphic DNA and Pathogenicity

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Twenty-two isolates of *Fusarium oxysporum* f. sp. *fragariae* were obtained from diseased strawberry plants and their characteristics were investigated by vegetative compatibility group (VCG), random amplified polymorphic DNA (RAPD), and pathogenicity. Three major VCGs (A, B, and C) and one incompatible group were identified by nitrate reductase complementation test. The virulence pattern of the 22 isolates was studied in relation to four cultivars including Dochiodome, Red-pearl, Maehyang and Akihime. RAPD markers were used to determine genetic relationship, and created three major clusters among the 22 isolates of *F. oxysporum* f. sp. *fragariae*. Isolates belong to VCG-C were strongly pathogenic, and relatively high correlation was existed among VCG and RAPD, and virulence. In addition, VCG and RAPD pattern between pathogenic and non-pathogenic isolates were distinctly different.

Keywords : *Fusarium oxysporum* f. sp. *fragariae*, genetic variation, *nit* mutant, RAPD, VCG

Fusarium species probably cause more economic damage than any other plant pathogen (Correll et al., 1986). *Fusarium oxysporum* is a major vascular wilt pathogen and is responsible for wilt diseases on a variety of crop plants (Nelson, et al., 1981). Strawberry (*Fragariae* × *ananassa* Duch.) is an important crop in Korea, Japan and China. *Fusarium* wilt of strawberry caused by *F. oxysporum* f. sp. *fragariae* is responsible for dramatic yield losses in commercial strawberry production (Winks and Williams, 1965; Kim et al., 1982). *F. oxysporum* f. sp. *fragariae* is distinct from other *F. oxysporum* formae speciales in symptomtology, epidemiology and cultivar susceptibility

(Wilhelm, 1998).

Puhalla (1984) developed a method by which isolates within different formae speciales of *F. oxysporum* could be classified in vegetative compatibility groups (VCGs). VCGs also can be used to compare isolates among formae speciales of *F. oxysporum* (Ploetz, 1990). Vegetative compatibility provides a means of characterizing variation based on the genetics of the fungus rather than on the host pathogen interaction (Correll, 1991; Leslie, 1993). Isolates in the same VCGs often are linked with pathological and physiological attributes as well as geographic origins. However, VCGs have been used to study the origins and affinities among plant pathogenic fusaria (Ploetz and Shepard, 1989). Isolates of *F. oxysporum* in the same VCG are supposed to be a clone, even if they are geographically separated from each other. The loci and alleles that determine VCGs are presumed to be selectively neutral with respect to traits such as pathogenicity and vegetative compatibility (Leslie, 1993). Nitrate nonutilizing (*nit*) mutants can be used indirectly to assess vegetative compatibility among isolates of *F. oxysporum* (Puhalla, 1984). The mutant can be subdivided into three phenotypes (*nit* 1, *nit* 3 and *nit* M) that can be differentiated by their ability to utilize various nitrogen sources (Correll et al., 1987; Katan and Katan, 1988). If '*nit*' mutants of two isolates anastomose to form a wild-type heterokaryon when paired on nitrate minimal medium, they are assigned to the same VCG. The isolates of a given VCG typically posses very similar, or identical multilocus haplotypes; therefore, VCGs can be good predictors of genetic relatedness (Kistler et al., 1998). Isolates within a given VCG usually belong to the same forma specialis; however, a forma specialis may contain one or more VCGs.

In addition to VCG, molecular biological techniques such as random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) (Williams et al., 1990) overcome all these limitations and provide additional information for fungal characterization (Correll et al., 1993). RAPD has been widely used for assessing

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genetic diversity, genome mapping and molecular diagnostics of many species (Annamalai et al., 1995; Manulis et al., 1994).

The objectives of this study are to determine genetic variations of *F. oxysporum* f. sp. *fragarie* obtained from diseased strawberry plants from various locations in Korea based on VCG, RAPD, and pathogenicity. The results would be helpful to develop resistant cultivars against this destructive pathogen.

Materials and Methods

Fungal culture. Twenty-two isolates of *F. oxysporum* f. sp. *fragariae* used in this study were collected from different major strawberry cultivating areas in Korea (Table 1). Individual isolates were purified through the subculture of a single conidium repeatedly. All isolates used in this study were deposited in Korean Agricultural Culture Collection, National Institute of Agricultural Biotechnology, Suwon.

Pathogenicity test. Isolates were screened for pathogenicity on four strawberry cultivars in Nonsan Strawberry Experiment Station, ARES, Korea. The cultivar Dochio-

Table 1. The isolates of *Fusarium oxysporum* f. sp. *fragariae* used in this study

Isolate	Strawberry Cultivar	Collection Site	VCG	RAPD pattern
Fo1	Nyoho	Nonsan, Chungnam	A	I
Fo3	Nyoho	Nonsan, Chungnam	A	I
Fo4	Nyoho	Buyeo, Chungnam	A	II
Fo6	Hokowase	Gongju, Chungnam	A	I
FF802	Hokowase	Damyang, Jeonnam	A	I
FF406	Hokowase	Damyang, Jeonnam	A	I
Fo23	Reiko	Nonsan, Chungnam	A	I
Fo30	Nyoho	Nonsan, Chungnam	A	I
Fo40	Redpearl	Iksan, Jeonbuk	A	I
Fo41	Redpearl	Iksan, Jeonbuk	A	I
FF801	Hokowase	Damyang, Jeonnam	B	II
FF408	Hokowase	Nonsan, Chungnam	B	II
FF409	Hokowase	Nonsan, Chungnam	B	II
Fo45	Akihime	Daedeok, Daejeon	B	I
Fo13	Hokowase	Hongseong, Chungnam	C	II
Fo47	Dochiodome	Nonsan, Chungnam	C	II
Fo48	Dochiodome	Nonsan, Chungnam	C	II
Fo50	Johong	Gangseo, Busan	C	II
Fo54	Dochiodome	Gongju, Chungnam	C	II
Fo56	Akihime	Yeongi, Chungnam	C	II
F011	Hokowase	Hongseong, Chungnam	Inc.[a]	III
Fo42	Akihime	Nonsan, Chungnam	Inc.[a]	III

[a]Incompatible with any other isolates.

dome, Redpearl, Maehyang and Akihime were used and five plants were inoculated per isolate. The pathogenicity of each isolate was tested on strawberry seedlings at the one leaf stage. The roots were dipped into a conidial suspension $(1\sim2 \times 10^{-6}$ microconidia per ml) for 10 min., then the seedlings were transplanted into sterilized soil and peat moss in plastic cups with 9 cm in diameter, and kept in growth chamber with a 12 hour photoperiod under mixed fluorescent and incandescent lamps. The photosynthetic photonflux density at plant canopy was 150 $Umol^{-1}$ m^2. Seedlings treated similarly and dipped in tap water served as control. Plants were watered daily, and no fertilizer were applied. Disease assessment was made 25 days after inoculation. The disease severity was evaluated according to the CIAT disease severity rating scale ranging from '−' (no symptoms) to '+' (death). Samples from diseased and control plants were surface sterilized, plated on PDA plates, and then incubated for 10-15 days to detect the pathogen in the symptomless plants. The experiments were repeated three times with consistent results were obtained.

Determination of compatibility group. VCGs were assigned by the complementation of nitrate nonutilizing (*nit*) mutants as a visual indicator of heterokaryon formation (Puhalla, 1984).

To determine the physiological phenotypes, a mycelial transfer (4 mm block) of the '*nit*' mutant was placed on each of the five media. The plates were incubated at 25°C and the colony morphology was scored related to the wild type parent. Isolates were evaluated after 3 to 4 days after incubation. Colonies with abundant aerial mycelia were considered as having '+' growth, colonies with sparse, transparent mycelium were considered as having '−' growth. The '*nit*' mutants were characterized as '*nit*1' (unable to utilize nitrate), '*nit*3' (unable to utilize nitrate or nitrite) and *nit*M (unable to utilize nitrate, hypoxanthin (or uric acid).

Complementation test. Vegetative compatible '*nit*' mutants may complement one another by forming a heterokaryon on minimal medium (Puhalla, 1986). Pairings were made by placing mycelia from each '*nit*' mutant 1-1.5 cm apart on MM. Pairing was incubated at 25°C in dark for 5 to 15 days and then scored for complementation. Dense aerial growth develops where mycelia of the two '*nit*' mutant colonies comes in contact, anastomose and form a heterokaryon. Heterokaryon was evident after 5-15 days by the formation of wild-type mycelia at the contact zone between two '*nit*' mutants. Absence of the wild-type growth between '*nit*' mutant derived from the same parent isolate indicated either allelic, or overlapping (otherwise, non complementary) mutation, or vegetative self incompatibility. In contrast, absence of the wild-type growth between

'*nit*' mutant from different parent isolates indicated either noncomplementary or inability to form heterokaryons due to lack of vegetative compatibility (Katan et al., 1991).

Genomic DNA extraction. Three to four mycelial plugs (each 4 mm in diameter) from PDA cultures were transferred to flasks containing 150 ml of potato dextrose broth, which were incubated at 25±1°C on orbital shaker (125 rpm) for 4 days. Mycelia from cultures were collected onto Mira cloth by vacuum filtration, washed with sterile distilled water, lyophilized, and ground in liquid nitrogen. Total genomic DNA was extracted from powdered sample of mycelium using the method described previously by Lee and Taylor (1990). The DNA was resuspended in 50 μl of TE buffer and stored at −20°C.

RAPD analysis. Genomic DNA from *F. oxysporum* f. sp. *fragariae* was amplified by the RAPD using the SRILS uniprimer kit of random primers purchased from Seoulin Bio-Science Ltd., Seoul, Korea. The genomic DNA from 22 isolates were used to screen 12 universal rice primers (URPs) to determine if banding patterns produced by the arbitrary amplification could differentiate between the isolates. The primers URP-5 and URP-12 were selected among 12 primers tested on the basis of reproducible bands obtained. Preliminary amplification was conducted to determine the optimal concentration of the component in the PCR reaction mixture. The most intense bands were considered for the analysis. PCR amplifications were performed in a total volume of 20 μl containing 20 ng genomic DNA, 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris/HCl (pH 8.3), 200 μM dATP, dCTP, dGTP and dTTP, primer 100 ng, and 1U of *Taq* DNA polymerase (Promega Corp. Madison, WI). Each reaction was overlaid with 1 drop of mineral oil. PCR was carried out in PTC 100 programmable thermo cycler (MJ Research, Water Town, MA, USA). The program included an initial denaturation at 94°C for 4min, 40 cycles with denaturation at 94°C for 1 min, annealing 56°C for 1 min, an extension at 72°C for 2 min, and a final extension at 72°C for 7 min. Negative controls (no template DNA) were used for each set of experiment to test for the presence of nonspecific reaction. All experiments were repeated at least three times. The PCR products were electrophoresed on 1.2% agarose gel using 0.5X TBE buffer, stained with ethidium bromide and visualized under UV and photographed.

Data analysis. Each profile was compared on the basis of the presence (1) versus absence (0) of RAPD product of the same electrophoretic mobility. Relative relatedness among isolates was determined. Pairwise comparison was made between all isolates and the values used to generated a

similarity matrix (Sneath and Sokal, 1973). A dendrogram was constructed a representing phenetic relationship between the isolates from the matrix of dissimilarities by the unweighted pair-group method algorithm (UPGMA). All calculations were conducted using the computer program NTSYS-pc.

Results

Vegetative compatibility analysis. Twenty out of the 22 isolates were compatible with each other to some extent (Table 1). Fo1, Fo3, Fo4, Fo6, Fo23, Fo30, Fo40, Fo41, FF406 and FF802 were strongly compatible with these ten isolates, but not with the others tested. All of these isolates were grouped in to VCG-A. Isolates Fo45, FF408, FF409 and FF801 were compatible only with one another. Therefore, these isolates were assigned as VCG-B. Isolates Fo13, Fo47, Fo48, Fo50, Fo54 and Fo56 were compatible with each other, not with other isolates. Therefore, these isolates were designated VCG-C, while Fo11 and Fo42 were incompatible with any other isolates.

RAPD analysis. Genomic DNA obtained from the 22 isolates of *F. oxysporum* f. sp. *fragariae* was subjected to RAPD-PCR analysis. Twelve random URPs were tested, using genomic DNAs from the *F. oxysporum* isolates f. sp. *fragariae* as templates. Five primers yielded reproducible RAPD pattern, two of these were used for a comparative analysis. The primers including URP-5 and URP-12 generated a distinct banding pattern for all of the isolates. RAPD banding patterns generated for the isolates with two primers were shown in Fig. 1. Amplified fragments ranged from 150 bp to 2.8 kb. RAPD assays of the isolates with two primers yielded 151 bands were scored, of which 120 (79.4%) polymorphic. The number of DNA fragments amplified and scored per isolates for individual primer ranged from 5 to 12. The RAPD analysis revealed genetic differences between the isolates (Fig. 2). Dendrogram which was obtained from RAPD analysis divided the fungal isolates into three major groups, and its pattern was very similar to vegetative compatibility of the same isolates.

Analysis of pathogenicity. Twenty-two isolates of *F. oxysporum* f. sp. *fragariae* from diseased strawberry plants were tested. Twenty isolates produced symptoms (*i.e.* leaf rolled, yellow leaf, chlorosis, wilting and death) on inoculated plants. Therefore, these isolates were considered to pathogenic to strawberry plants. The 20 isolates were collected from diseased strawberry in different strawberry cultivating area in Korea. There was a significant association between the pathogenicity of the isolate collected (Table 2). Four strawberry cultivars, Dochiodome, Redpearl, Maehyang
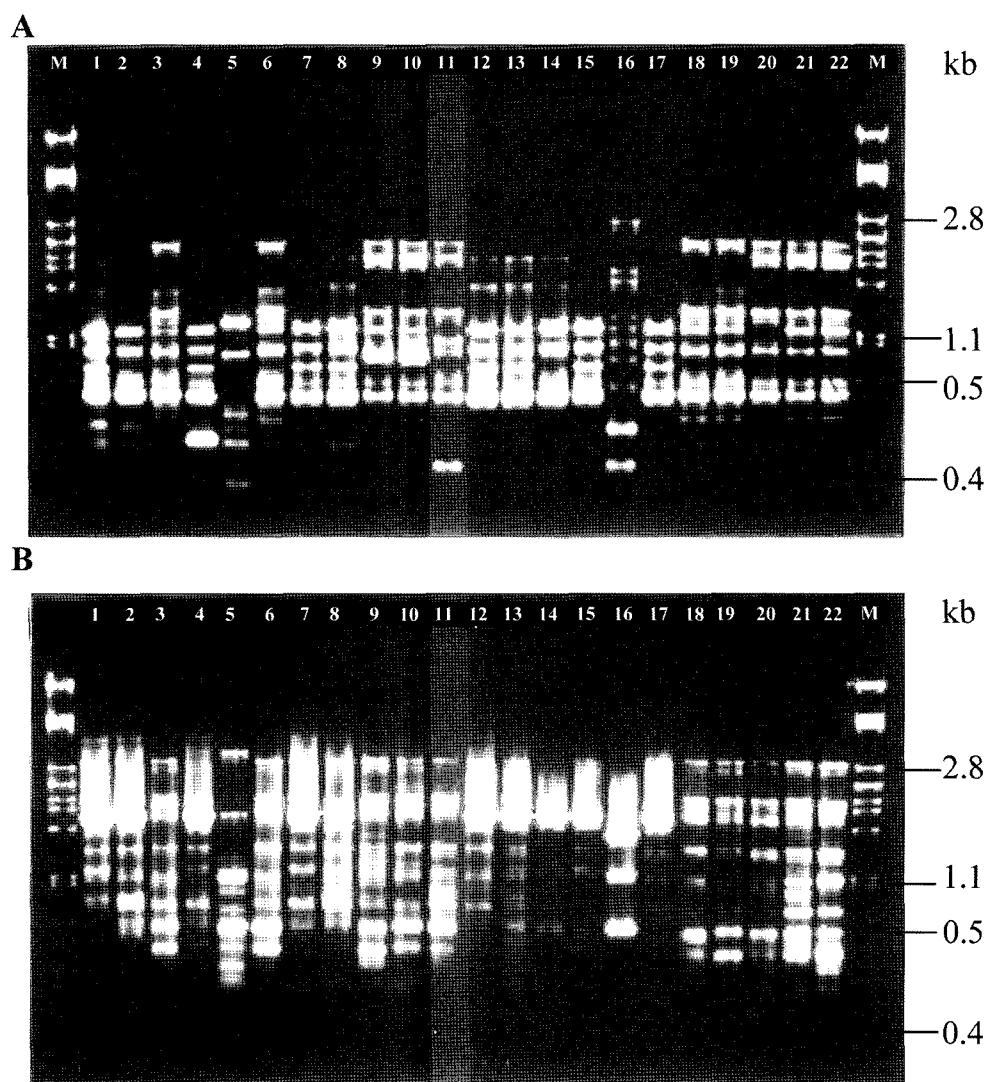
Fig. 1. Electrophoretic patterns on an 1.2% agarose gel of amplified fragment generated from the 22 isolates of *F. oxysporum* f. sp. *fragariae* with random primer (A) URP-5 and (B) URP-12. Lane M, DNA marker (λ DNA digested with *PstI*); lane 1, Fo1; 2, Fo3; 3, Fo4; 4, Fo6; 5, Fo11; 6, FF801; 7, FF802; 8, FF406; 9, FF408; 10, FF409; 11, Fo13; 12, Fo23; 13, F030; 14, Fo40; 15, Fo41; 16, Fo42; 17, Fo45; 18, Fo47; 19, Fo48; 20, Fo50; 21, Fo54; and 22, Fo56.

and Akihime were inoculated and disease severity was assigned after 45 days of inculation and the virulence pattern of the 22 isolates in relation to four cultivars have been depicted in Table 2. Isolates Fo4, FF801, Fo47, Fo50, Fo54 and Fo56 are highly pathogenic in all cultivars (Group 1). All other isolates were moderately, or weakly pathogenic to cultivars Dochiodome, Redpearl and Maehyang. However, the isolates of Fo6, Fo30, and Fo13 were pathogenic in the cultivar Dochiodome and moderately pathogenic to other cultivars (Group 2). The isolates Fo1, FF 406, Fo23, Fo40, FF408, FF409 and Fo48 were caused no symptoms on cultivar Akihine tested (Group 3). Isolates Fo3, and Fo45 were pathogenic on cultivars Akihime but not pathogenic to other cultivars. The

isolate FF802 and Fo41 were pathogenic on cultivars Redpearl and Dochiodome, respectively but not other cultivars. Therefore, the isolates of Fo3, Fo45, FF802, and Fo41 are considered as Group IV. Isolates Fo42 and Fo11 were produced no symptoms in all four cultivars tested. These isolates were considered as non pathogenic.

Pathogenicity test were repeated three times with consistent results were obtained. On the basis of reaction on cultivars of all isolates could be grouped into 4 pathotypes groups. The findings in this study clearly demonstrated that variation was existed in pathogenicity of *F. oxysporum* f. sp. *fragariae* isolates in Korea.

Correlation of VCG and RAPD, pathogenicity. The
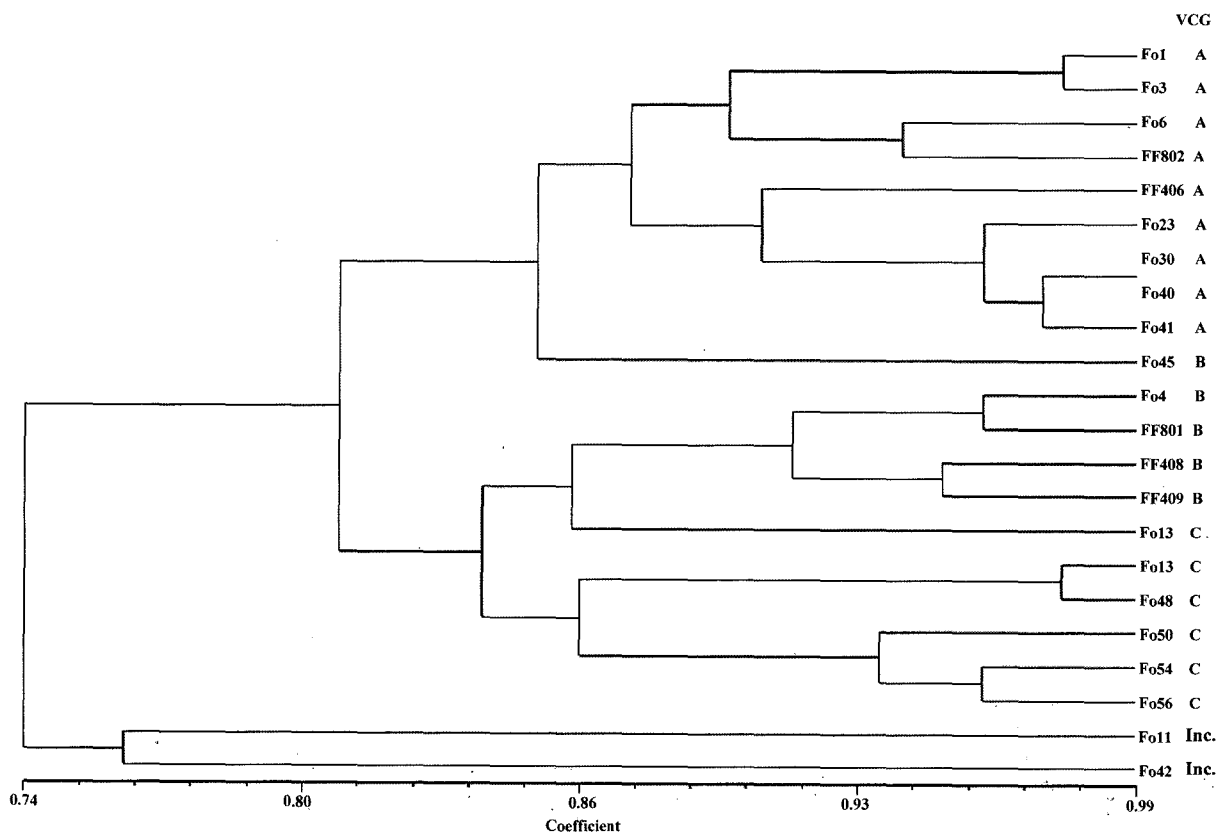
Fig. 2. Dendrogram showing relationship among the 22 isolates of *F. oxysporum* f. sp. *fragariae*. Genetic similarity was obtained by RAPDs in relation to VCGs.

dendrogram generated using UPGMA which was based on the RAPD analysis provided distinct difference with the VCG of the *F. oxysporum* f. sp. *fragariae* isolates that recovered from different regions of strawberry cultivating area in Korea. On the other hand, the isolates belong to VCG-C as well as RAPD pattern II group were very strong pathogenic to be 2.65 of disease index, while the others that had weak virulence were belong to different RAPD groups, and relatively high correlation was found between VCG, RAPD, and virulence of the isolates (Table 3). Three clusters of pathogenic and nonpathogenic isolates contained five clonal individuals based on RAPD profile (Fig. 2). From the results of pathogenicity test using 4 strawberry cultivars, VCGs and RAPD patterns between pathogenic and nonpathogenic isolates were much different.

Discussion

Genetic characterization of *F. oxysporum* f. sp. *fragariae* is important for the efficient management of Fusarium wilt through use of resistant cultivars in strawberry growing area. This study demonstrated that pathogenicity, VCG and RAPD provided valuable information regarding to the

degree of variation and level of genetic relatedness among the 22 isolates of *F. oxysporum* f. sp. *fragariae*. Nagarajan et al., (2004) reported that RAPD and restricted fragment length polymorphism (RFLP) of intergenic spacer (IGS) region has been used successfully and delineated into 8 groups within *F. oxysporum* f. sp. *fragariae*. Hyun and Park (1996b) have shown that RAPD profile separates 24 isolates of *F. oxysporum* f. sp. *fragariae* into two distinct groups. In order to determine the genetic variation of *F. oxysporum* f. sp. *fragariae*, it was studied using molecular markers such as RAPD and RFLP of IGS region. In the previous study RAPD analysis has been used successfully and delineated groups within formae speciales of *F. oxysporum*. One group was delineated with *F. oxysporum* f. sp. *albedinis* (Tantaoui et al., 1996); two groups within f. sp. *dianthi* (Manulis et al., 1994; Manicom et al., 1990) and two to three groups within f. sp. *pisi* (Grajal-Martin et al., 1993). Nelson et al. (1997) considered that the isolates of *F. oxysporum* f. sp. *erythroxyli* appeared to be distinguished from other formae speciales by RAPD analysis, because distinct banding patterns were observed between *F. oxysporum* f. sp. *erythroxyli* and the other formae speciales tested.

Table 2. Pathogenicity of *Fusarium oxysporum* f. sp. *fragariae* isolates from wilted strawberry plants to four strawberry cultivars

Isolate	Disease index[a]			
	Dochiodome	Redpearl	Maehyang	Akihime
Fo1	+	+	−	−
Fo3	−	−	−	+
Fo4	++++	++	++++	+
Fo6	+++	+++	+++	+
FF802	−	+	−	−
FF406	+	+	−	−
Fo23	++	−	−	−
Fo30	+	+	+	−
Fo40	+	+	−	−
Fo41	+	−	−	−
FF801	++++	+	+	+
FF408	+	+	−	−
FF409	+	+	+	−
Fo45	−	−	−	+
Fo13	+	+	+	−
Fo47	+++++	+++	+++++	++++
Fo48	+	+	+	+
Fo50	+++++	++	+++	+
Fo54	++++	+++	++	+
Fo56	++++	+++	+++	+++
Fo11	−	−	−	−
Fo42	−	−	−	−

[a]Disease index: − = no symptom, + = 1-2 leaf rolled and yellowed leaf, ++ = all leaf rolled and deformed leaf, +++ = chlorosis and wilting, ++++ = wilting, +++++= death. Rating of 45 days after inoculation.

Table 3. Comparison of virulence and RAPD pattern of *F. oxysporum* f. sp. *fragariae* between different VCGs

VCG	Virulence[a]	RAPD pattern[b]
A	0.92 ab	I, II
B	0.76 ab	I, II
C	2.65 a	II
Inc.[c]	0.00 b	III

[a]Disease index was the mean disease severity value on artificially inoculated plants, which was calculated as described in Materials and Methods (Nam et al. (2005)). Means followed by the same letter are not significantly different, according to Duncan's multiple range tests (P = 0.01). The numbers indicate the degree of virulence of isolates in each VCG.
[b]As determined by RAPD-PCR using URP primers.
[c]Inc. was incompatibility.

The previous studies demonstrated that the RAPD analysis could separated strains of *F. oxysporum* f. sp. *vasinfectum* into three genetic groups corresponding to races A3 and A4 (Fernandez et al., 1994; Assigbetse et al., 1994). These races of *F. oxysporum* f. sp. *vasinfectum* were classified on the basis of pathogenicity toward three differential cotton species *Gossibium hirsutum, G. barbodense* and *G. arboreum* (Assigbetse et al., 1994). Most of the studies failed to show a positive correlation between pathogenic variation and DNA polymorphisms (Fernandez et al., 1994). The comparative virulence of *F. oxysporum* f. sp. *fragariae* from different VCGs should be useful for selection of resistant cultivars in strawberry like as the report of Hyun et al. (1996a). Several studies showed that VCGs is a good indicator of the evolutionary lineage of *F. oxysporum* f. sp. *lycopersici* (Elias and Schneider, 1991; Elias et al., 1993) and other formae speciales of *F. oxysporum* (Apple and Gorden, 1994, 1996; Koenig et al., 1997). In this study, VCGs were assigned among 22 iso-

lates of *F. oxysporum* f. sp. *fragariae* based on complementation of *nit* mutants. On the other hand, Woo et al., (1996) have reported that there was a good correspondence of the RFLP and RAPD banding patterns among 20 isolates of pathogenic strains of *F. oxysporum* f. sp. *phaseoli* and these results were correlated to the vegetative compatibility and showed the isolates to be genetically similar. Mes et al., (1998) also demonstrated that comparison of RAPD profiles of *F. oxysporum* f. sp. *lycopersici* revealed two groups that coincide with compatibility groups. Similar investigation was reported in *F. oxysporum* f. sp. *cubense* (Ploetz, 1990).

In this study, the correlation among VCG was not precise. Several researchers have been observed that a strong correlation was existed between the restriction patterns of mtDNA and VCGs in *F. oxysporum* f. sp. *melonis* and indicate that the isolates must have a high degree of genetic homology (Gordon and Okamoto, 1992; Jacobson and Gordon, 1990). Manicom and Baayen (1993) reported that two major coincident groups of VCG and RFLP existed between the populations of *F. oxysporum* f. sp. *dianthi* in Israel. Similar results have been known in *F. oxysporum* ff. sp. *gladioli, lycopersici* and *dianthi* (Elias et al., 1993; Mes et al., 1994). Other studies using *F. oxysporum* from carnation and muskmelon suggested that there were no similarities between the isolates when analyzed by RAPD, or RFLP markers (Gordon and Okamoto, 1992; Manulis et al., 1994). In our study, RAPD generated distinct banding patterns according to the VCGs. In addition, VCG and RAPD pattern between pathogenic and non-pathogenic isolates of this fungus were distinctly different. Therefore, we concluded that the differentiations among the isolates of *F. oxysporum* f. sp. *fragariae*, which were determined by VCG, RAPD, and pathogenicity, respectively, were correlated each other in some extent.

Acknowledgment

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