Vegetative Compatibility Groups within
Fusarium oxysporum f. sp. *cepae* in Hokkaido-Japan

WIDODO[1*], NORIO KONDO[2], KIROKU KOBAYASHI[2], AND AKIRA OGOSHI[2]

[1]*Department of Plant Protection, Faculty of Agriculture, Institut Pertanian Bogor,
Kampus Darmaga, Bogor 16680, Indonesia*
[2]*Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan*

In Hokkaido, Fusarium basal rot, caused by *Fusarium oxysporum* f. sp. *cepae* is one of the important constrains since 1973 which contributes to a significant loss in onion production, either in the fields or during storage. Development of resistant cultivars is suggested as one of the effective control measures against the disease, however, this should be accompanied with the better understanding of the pathogen's population dynamics. This study was performed to investigate the population structure of *F. oxysporum* f. sp. *cepae* based on vegetative compatibility groupings (VCGs). Vegetative compatibility groups of *F. oxysporum* f. sp. *cepae* were characterized using nitrate non-utilizing (*nit*) mutants. Four VCGs and 2 single self-compatible (SSC) isolates were identified among 48 isolates, designated as VCG 0420 (33 isolates), 0421 (9 isolates), 0422 (2 isolates), 0423 (2 isolates), and 042-(2 isolates). VCG 0420, to which 4 ATCC isolates out of 6 belonged, was the predominant group within the growing region encompassing Hokkaido Japan. VCGs 0421 and artificial VCG 042- were found less frequently. Four isolates from Welsh onion were not compatible with any recovered VCGs and were assigned to 2 distinct VCGs (VCG 0422 and 0423).

Key words: *Allium cepa,* Fusarium basal rot of onions, *Fusarium oxysporum* f. sp. *cepae*, population

Hokkaido, the northernmost island of Japan, has the largest area under onion cultivation (*Allium cepa*) and accounts for 48% of national production. Fusarium basal rot, caused by *Fusarium oxysporum* f.sp. *cepae*, is an important soilborne disease of onion which occurs in Hokkaido. This fungus also causes seedlings damping-off and wilt. This disease was first reported in Japan in 1911, in the Ishikari district of western Hokkaido, but had no significant economic impact for many years (Kodama 1983). In 1970, the disease was reported in some areas of the central and eastern parts of Hokkaido i.e. Furano and Kitami (Kodama *et al*. 1974). By 1973, the disease was observed in most of the onion fields in Hokkaido (Kodama *et al.* 1974).

Although treating seedlings with fungicide (1-2% benomyl) prior to transplantation has significantly reduced losses (Kodama 1983), the planting of resistant onion cultivars has been suggested as a valuable control measure (Abawi and Lorbeer 1971; Simizu and Nakano 1995; Thornton and Mohan 1996). Often, however, the use of cultivars resistant to *F. oxysporum* is compromised by the appearance of new pathogenic strains. *F. oxysporum*'s ability to overcome host resistance may be related to its various strains existence and unstable genetic character (Abawi and Lorbeer 1965; Ploetz and Correll 1988; Jacobson and Gordon 1991). Since pathogens must constantly adapt to changes in their environment to survive (Abawi and Lorbeer 1972), an understanding of their population dynamics would be helpful in developing resistant cultivars.

Puhalla (1981) first made efforts to differentiate and classify *formae speciales* of *F. oxysporum* isolated from various plant species into vegetative compatibility groups (VCGs), using nitrate non-utilizing (*nit*) mutant (generated on media containing chlorate) that lacked the ability to use nitrate as a nitrogen source. This was refined by Correll *et al*. (1987). Since that time, numerous investigators have

classified *F. oxysporum* isolated from various host plants into distinct VCGs using *nit* mutants (Elmer and Stephens 1989; Larkin *et al*. 1990; Elias and Schneider 1991; Katan *et al.* 1991; Loffler and Rumine 1991; Venter *et al*. 1992; Fiely *et al.* 1995; Woudt *et al*. 1995; Woo *et al*. 1996; Harveson and Rush 1997; Kondo *et al.* 1997; Lori *et al.* 2004; Vakalounakis *et al.* 2005). Five VCGs of *F. oxysporum* f. sp. *cepae* from onion have been identified in Colorado, USA, and one of the VCGs was present in all three regions of Colorado (Swift *et al.* 2002). The use of VCGs has also been performed to determine the diversity of nonpathogenic populations of *F. oxysporum* isolated from carnation fields in Argentina (Lori *et al.* 2004). Beside for determining the VCGs of *Fusarium,* the use of *nit* mutant combining with selective media was also useful in studying population dynamics of pathogenic *F. oxysporum* f. sp. *spinaceae* in soil (Takehara *et al.* 2003). In the special case of *F. oxysporum,* the vegetative compatibility group (VCG) approach can be an effective diagnostic technique when the number of VCGs in *formae speciales* of interest is known to be relatively small since the tester strains needed are usually easy to obtain (Summerell *et al.* 2003).

The objectives of this study were to investigate the population type and distribution of *F. oxysporum* f. sp. *cepae* from Hokkkaido Island, Japan, based on vegetative compatibility groups.

MATERIALS AND METHODS

Collection of Isolates and Pathogenicity Testing. *Fusarium oxysporum* were collected from infested-field-soil or diseased onion stem-plates from 6 locations (Mikasa, Furano, Kitami, Kunnenpu, Tsubetsu, and Tanno) in Hokkaido. Additional isolates were obtained from individuals (4 isolates of Welsh onion from Yakumo and 3 isolates of onion from Furano supplied by Dr. H. Abe and Dr. F. Kodama, respectively) and culture collections (American Type Culture

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*Corresponding author, Phone/Fax: +62-251-8423048,
E-mail: widodo@ipb.ac.id

Collection). Infected stem- plates with brown discoloration were surface sterilized using cotton wipes wetted with 70% ethanol and allowed to dry. Each stem-plate was cut with a sterile knife into small blocks (2 x 2 x 2 mm^3). Five pieces of each bulb were placed in Komada's medium (KM) (Komada 1975) and incubated at 26 °C for 5 to 7 days. The entire stem-plates were used for isolates recovered from damped-off seedlings. Soil dilution was used for isolation from soils. After 5-7 days incubation colonies, which were tentatively identified as *F. oxysporum* on the basis of colony morphology, were transferred to fresh plates of KM. Spores from 5-7-day-old cultures growing on KM were streaked onto water agar (20 g l^{-1}). After 18 h a single germinated spore was removed from the water agar and transferred to potato sucrose agar (PSA: potato 200 g, sucrose 15 g, agar 20 g, and distilled water 1000 ml). Identification was based on mycological characteristics as described by Nelson *et al.* (1983).

Pathogenicity Tests. Each isolate was tested for pathogenicity using a susceptible onion cultivar (Kitawase No. 3). A conidial suspension was prepared from 7-10-day-old cultures growing on PSA plates and used as inoculum. The suspension was filtered through 4 layers of cheesecloth to separate conidia from mycelium and adjusted to 1.0 x 10^6 spores ml^{-1}. Six- to 8-week-old seedlings were pulled up and shaken to remove excess soil, and then cut 5 mm below their stem plate. The roots of 15 seedlings were dipped for 24 h into 20 ml of a conidial suspension and then transplanted into a soil (Pot-ace, Katakura Chikkarin K. K., Tokyo, Japan)/ vermiculite mixture (3:1 v/v) in 12-cm-diameter plastic pots in triplicate. Each pot contained 5 seedlings. Other seedlings were dipped in sterile water and planted as above to act as controls. The number of damping-off plants was recorded over 5 weeks after transplantation. After 5 weeks, longitudinal sections of unwilted plants were observed for discoloration of stem-plates. These tests were conducted in a greenhouse with temperatures ranging from 20 to 28 °C. Of the isolates that induced plant damping-off more than 30% were considered as pathogenic. Re-isolation from the experimentally symptomatic plants was performed to verify that the recovered isolates had the same characteristics as the isolates in the initial inoculation.

Recovery of Nitrate Non-Utilizing (*nit*) Mutants. Mutants were selected using the method of Puhalla (1985). Each isolate of *F. oxysporum* was grown on PSA for 5-7 days at room temperature. Four small mycelial blocks (2 mm^3) of the fungus were placed onto a plate containing corn-meal-agar modified by adding 15 g l^{-1} KClO$_3$ (CMAC). Many isolates failed to form mutants on this medium, so chlorate (KClO$_3$) concentrations were often doubled to 30 g l^{-1}. The plates were incubated at 26 °C for 15 days and inspected

periodically for the appearance of fast growing hyphae developing from the initial colony. These fast growing hyphae were then transferred to a minimal medium (MM) that contained NaNO$_3$ (2 g l^{-1}) as the sole nitrogen source (Puhalla 1985). Mutants that grew on MM as thin expansive colonies with no aerial mycelium were considered as *nit* mutants.

***Nit* Mutant Phenotypes.** The phenotypic classes of all *nit* mutants were determined by their growth on media containing nitrate (2 g l^{-1}), nitrite (0.5 g l^{-1}), hypoxanthine (0.2 g l^{-1}), or ammonium tartrate (1 g l^{-1}) as the sole nitrogen source (Correll 1991). A small block of each mutant colony was transferred onto each medium. The plates were incubated as described above and colony growth was scored relative to the parental wild-type after 4 days growth. The *nit* mutants were placed into phenotypic classes (Klittich and Leslie 1988; Correll 1991) based on their ability to utilize the various nitrogen sources (Fig 1).

Complementation Test. Prior to complementation tests among isolates, the vegetative self-compatibility of each isolate was examined by the method of Jacobson and Gordon (1988). Ten isolates were initially selected for vegetative compatibility tests. They were paired in all possible combinations and grown on minimal medium to determine the number of vegetative compatibility groups (VCGs). Vegetatively compatible *nit* mutants that carry the mutated gene on different loci may complement one another by the formation of a dense aerial wild-type mycelium at the position where mycelia of *nit1* and NitM colonies touch. A NitM from one isolate in each of the VCGs thus identified was selected to serve as a tester. After VCGs were established for the initial 10 isolates, the remaining isolates were treated in the same manner. For isolates that did not match with any of the established VCGs, a tester for a second group was obtained and the procedure was repeated.

RESULTS

When the isolates of *F. oxysporum* inoculated into cultivars Kitawase No. 3 using the root dip method were examined, some of them induced a progressive yellowing and die back from the tips of their leaves. In these cases the aerial part died completely within two or three weeks after inoculation. All the isolates inducing such symptom were taken to be as *F. oxysporum* f. sp. *cepae* based on the description of Kodama (1983). The degree of disease incidence induced within isolates having this symptom varied from 20-100%. A total of 38 isolates of *F. oxysporum* pathogenic to onion and 173 nonpathogenic isolates were recovered from soil samples and diseased onion bulbs (Table 1).

Table 1 Geographical location and pathogenicity tests of isolates of *Fusarium oxysporum* collected from onion fields

Location	Source (year)	*F. oxysporum* population (cfu g^{-1} dry soil)	No. of isolates tested	No. of pathogenic isolates	No. of nonpathogenic isolates
Mikasa	Soil (1996)	5.3 x 10^3	77	11	66
Furano	Bulb (1997)		53	6	47
Tanno	Soil (1997)	1.2 x 10^3	26	2	24
Kitami	Soil (1997)	1.9 x 10^3	24	3	21
Tsubetsu	Soil (1997)	0.4 x 10^3	6	1	5
Kunnenpu	Bulb (1997)		25	15	10

Nit mutants emerged from restricted growth on corn meal agar medium amended with chlorate after 4-15 days. Most of the isolates produced mutants when generated on medium containing chlorate 15 g l^{-1}. Some other isolates had to be generated on media containing 30 g l^{-1} to obtain the mutants. All the isolates used in this study are able to produce all the 3 mutant phenotypes as indicated on their ability on using different nitrogen source (Fig 1).

The 38 isolates of *F. oxysporum* f. sp. *cepae* recovered from soils in Hokkaido Island-Japan along with the ten known isolates of *F. oxysporum* f. sp. *cepae* were grouped into 4 distinct VCGs and 2 SSC isolates based on complementation of *nit1* and NitM mutants. They consisted of one large VCG with 33 members, including 4 isolates from ATCC. This one large group was first reported by Yoo *et al*. (1993), and was numbered using Puhalla's numbering system (Puhalla 1985) as VCG 0420 by Katan and Di Primo (1999). Therefore, the three other VCGs and two isolates of single self-compatibility were an additional group for this *formae speciales*. These three other VCGs were then designated as VCG 0421 (9 isolates), VCG 0422 (2 isolates), and VCG 0423 (2 isolates), respectively. The two isolates of SSC with members of ATCC 11850 and ATCC 46076 were assigned to artificial VCG 042- (Table 2). The population of *F. oxysporum* f. sp. *cepae* assigned as VCG 0420 was mostly distributed throughout the sampling area in Hokkaido (Fig 2).

Among the ATCC isolates tested, ATCC 46076 and ATCC 46077 were originally isolated from Hokkaido, Japan, in the 1970's. Complementation assays of ATCC 46077 placed it into the largest vegetative compatibility group, while ATCC 46076 was unable to form heterokaryons with any other isolates collected from Hokkaido, Japan (Table 2). The ATCC 46076 isolate was also not vegetatively compatible with the most predominant isolates collected from Hokkaido in this study as showed in the complementation tests with the isolate SM 025 (Fig 3).

Table 2 Vegetative compatibility groups among isolates of *Fusarium oxysporum* f. sp. *cepae*

VCGa	Source/Origin	No. of isolates
0420	Mikasa	5 (SM 024, SM 025, 1-1026, 1-2015, 1-2017)
	Furano	6* (NK 041, TR 001, TR 005, 93-37-3, 93-37-4, nKF 900)
	Kitami	2 (KTM 517, KTM 519)
	Kunnenpu	15 (KARS 9801, Kn-O-01, Kn-O-02, Kn-O-04, Kn-O-06, Kn-O-10, Kn-O-11, Kn-O-12, Kn-O-14, Kn-O-17, Kn-O-18, Kn-O-19, Kn-H-03,989-01, 989-02)
	Tsubetsu	1(9812-04)
	ATCC	4 (ATCC 11711, ATCC 46077, ATCC 62592, ATCC 60768)
0421	Mikasa	6(SM 037, SM 044, SM 045, SM 046, MH 019, 3-1024)
	Kitami	1 (KTM 503)
	Tanno	2(TN 98-01, K-3-19)
0422	Yakumo	2** (WO-F-41 and WO-F-46)
0423	Yakumo	2** (WO-F-10 and WO-F-34)
042-^{b}	ATCC	2 (ATCC 11850 and ATCC 46076)

* Including three isolates from Dr. F. Kodama (nKF-900, 93-37-3, and 93-37-4); ** Isolates of Welsh onion from Dr. H. Abe; ^{a}VCG's are numbered according to Puhalla's numbering system as proposed by Katan and Di Primo (1999); ^{b}042- is an artificial group containing isolates that are single member of a VCG.

Fig 2 Distribution of *Fusarium oxysporum* f. sp. *cepae* population in Hokkaido based on vegetative compatibility groups. A. Mikasa, B. Furano, C. Kunnenpu, D. Kitami, E. Tanno, F. Tsubetsu, G. Yakumo. ● VCG 0420, ◐ VCG 0421, ◑ VCG 0420 and 0421, ◯ VCG 0422, ◇ VCG 0423.
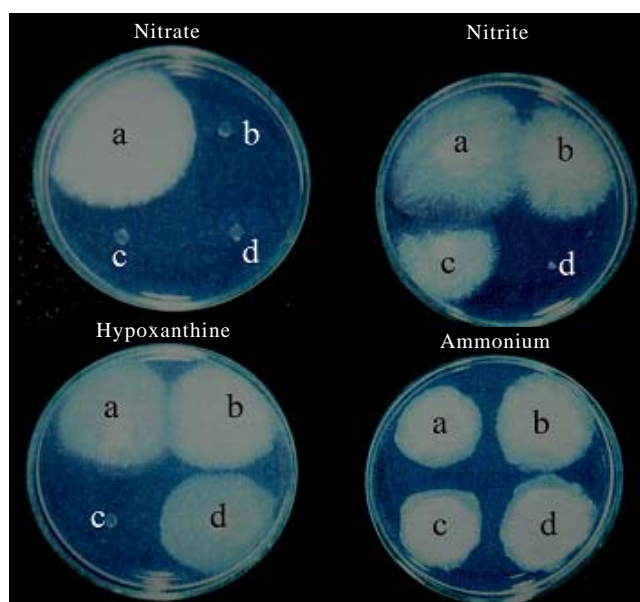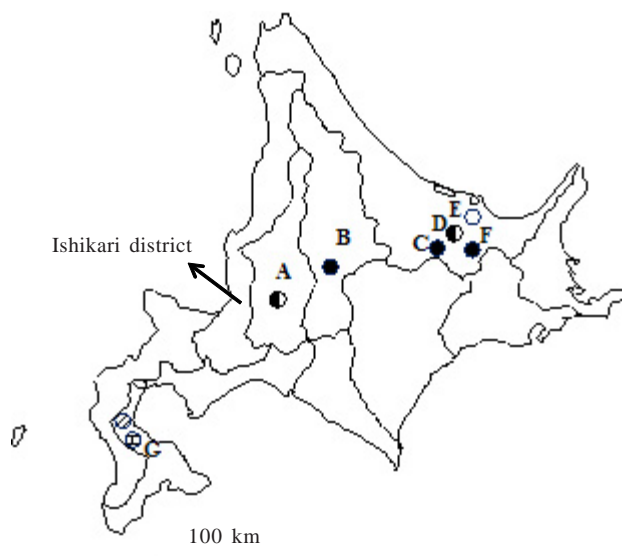

Fig 1 Growth of wild-type parental strain (SM 025) of *Fusarium oxysporum* f. sp. *cepae* and its three nitrate non-utilizing (*nit*) mutant phenotypes on four different nitrogen resources. a. Wild–type, b. *Nit* 1, c. NitM, d. *nit* 3. Dense aerial mycelium growth indicating the ability on using nitrate resources.
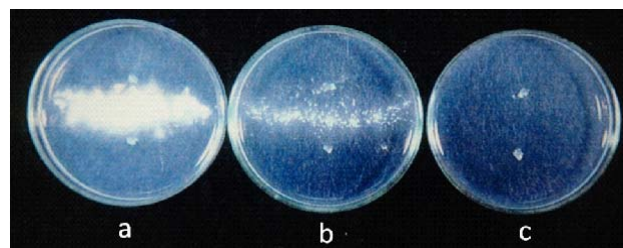

Fig 3 Heterokaryon formation between complementary nitrate non-utilizing mutants of *Fusarium oxysporum* f. sp. *cepae* on minimal medium containing nitrate as the sole source of nitrogen. a. Complementation reaction between isolate SM 025 and 1-1026, b. Complementation between isolate SM 025 and nKF 900, c. No complementation between isolate SM025 and ATCC 46076.

DISCUSSION

Thirty eight isolates of *F. oxysporum* f. sp. *cepae* collected from seven locations in Hokkaido and 10 isolates from the ATCC which included 4 isolates from Hokkaido, were grouped into 4 VCGs and 2 SSC isolates. Within 42 isolates of *F. oxysporum* f. sp. *cepae* from Hokkaido, one predominant VCG containing 33 isolates was identified. The isolates obtained from onion were widely distributed across most of the sampling sites (Fig 1). The presence of this predominant group in different onion fields indicates that this population has been selectively maintained. A similar phenomenon has been observed for other *formae speciales* of *F. oxysporum* (Elias and Schneider 1991; Katan *et al.* 1996; Ahn *et al.* 1998). The other VCG of onions from Hokkaido (VCG 0421) and one of the members of artificial VCG 042- (ATCC 46076) may lack the competitive traits necessary to insure their dispersal and survival. These results also showed that population structure of *F. oxysporum* f. sp. *cepae* based on VCGs was dynamic over time as indicated by replacing the predominant population of Hokkaido's isolate in 1970's (ATCC 46076) grouped into VCG042- with the new group VCG 0420 identified in this study. The change population predominance of *F. oxysporum* f. sp. *cepae* in Hokkaido might be affected by the shift of onion cultivars planted and other abiotic factors.

Isolates within *formae speciales* are genetically similar, probably originating from a single pathogenic genotype or clonal origin. Kistler (1997) proposed that a VCG within a population of *F. oxysporum* indicates a distinct clonal lineage as reported by Harveson and Rush (1997) on *F. oxysporum* f. sp. *betae*. They showed that VCGs of *F. oxysporum* f. sp. *betae* represented distinct isolated populations indigenous to their respective areas. Other researchers (Bosland and Williams 1987; Woo *et al.* 1996), however, reported that the *formae speciales* they were investigating did not exhibit a correlation between VCG and geographical origin. Although welsh onion isolates tested in this study was not comprehensive, these isolates tended to be distinct VCGs from onion isolates and were found to be limited in their geographical origin. Swift *et al.* (2002) reported that 9 out of 19 *F. oxysporum* f. sp. *cepae* isolates collected from diseased onion in Colorado, USA were grouped into 4 different VCGs and were also found in the limited area as indicated in our study. The extent of the distribution in Hokkaido of VCGs 0422 and 0423, is still unclear, as we did not have an adequate sampling of Welsh onion isolates from other locations. It is possible that these VCGs are dispersed via seeds, seedlings, plant debris, and/or human activities. Therefore, it would be useful to monitor those groups that have the potential to cause a serious problem.

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