Vegetative Compatibility Groups of *Fusarium oxysporum* f. sp. *cepae* from Onion in Colorado

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ABSTRACT

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Nineteen isolates of *Fusarium oxysporum* f. sp. *cepae* recovered from diseased onions growing in the western, southern, and northern regions of Colorado were placed into vegetative compatibility groups (VCGs) based on pairing of complementary mutants. Pathogenic isolates from these regions were cultured on variations of potassium chlorate (1.5 or 3.0%) mutation media, potato dextrose agar (PDA), and minimal medium (MM) supplemented with L-asparagine and L-threonine. Chlorate PDA and 3% chlorate MM with L-threonine did not generate the nitrate nonutilizing (*nit*) mutants required, while MM with L-asparagine (1.5 and 3% chlorate) and MM with L-threonine (1.5% chlorate) generated complementary *nit* mutants required for compatibility pairings. Five VCGs of *F. oxysporum cepae* were identified. One VCG was present in all three regions of Colorado examined. Four VCGs were restricted to either western or eastern Colorado. Additional sampling and evaluation of a more diverse collection of *F. oxysporum cepae* isolates from other regions of onion production is needed to determine the diversity of this pathogen. Such information could assist in breeding for resistance to *F. oxysporum cepae*.

Additional keywords: Fusarium wilt, Fusarium yellows, Fusarium basal rot of onion

Fusarium basal rot of onion is caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *cepae* (H. N. Hans.) W. C. Snyder & H. N. Hans. (35). As early as 1920, researchers reported significant losses from Fusarium wilt in onion fields and storage sheds near Grand Junction and Delta, CO (26). More than 85% of the onions produced in Colorado are dry storage onions (*Allium cepae* L.), with an annual value of 40 to 50 million dollars (7). Onion production in Colorado occurs in three main growing regions that are located approximately 250 km apart. The western, southern, and northeastern regions comprise 20, 25, and 55 percent of the onion acreage in Colorado, respectively. The total area of onion production in Colorado ranges between 4,000 and 6,000 ha. Approximately 85 to 90% of the acreage is planted to onion seed, and the remaining area is planted to transplants for the early and fresh markets (33). Statewide yields of approximately 45 t/ha can be significantly reduced by adverse environmental factors or pathogens such as *F. oxysporum cepae* (13,34).

Fusarium species are some of the most important plant pathogens in the world (28). *F. oxysporum* Schlechtend. emend. W. C. Snyder & H. N. Hans., a common soilborne *Fusarium* sp. with a worldwide distribution, has no known sexual stage. As a species, this organism probably causes more economic damage than any other plant pathogen (8), and has been shown to constitute as much as 80 to 90% of the total fungal microflora in the rhizosphere of several agricultural crops (12). *F. oxysporum* is responsible for wilt and cortical rot diseases of more than 100 economically important plant hosts (4).

Seventeen years ago, Puhalla (31) 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* (29). Vegetative compatibility provides a means of characterizing variation based on the genetics of the fungus rather than on the host-pathogen interaction (8,25). Isolates in a VCG often share pathological and physiological attributes as well as geographic origins. Consequently, vegetative compatibility has been used to study the origins of and relatedness among plant pathogenic fusaria (30). Pathogenic isolates of *F. oxysporum* in the same VCG are presumed to be clones, even if they are geographically isolated from each other. The loci and al-

leles that define VCGs are presumed to be selectively neutral with respect to traits such as pathogenicity and vegetative viability (24). Research on VCGs for *F. oxysporum cepae* is lacking other than limited work by Yoo et al (39).

To determine VCGs of fungal isolates, various media augmented with potassium chlorate are used to produce nitrate nonutilizing (*nit*) mutants, which mutate at different loci associated with the nitrate-assimilation pathway. These media include potato-dextrose chlorate (2,16,20,38), potato-sucrose chlorate (6), Czapek Dox chlorate (3,27), and minimal medium chlorate (32). Bosland and Williams (5) used potato-sucrose agar, cornmeal agar with dextrose, potato-dextrose agar (PDA), and potato-carrot-dextrose agar media, each amended with potassium chlorate (11). L-asparagine typically is added to the chlorate medium to provide a source of carbon, although L-threonine (15) and L-glutamine (27) have been used.

The objective of this study was to determine the regional diversity among Colorado isolates of *F. oxysporum cepae* based on VCGs, and to identify the most effective chlorate media for generation of *nit* mutants of *F. oxysporum cepae* for VCG complementation pairings (36).

MATERIALS AND METHODS

Eighteen isolates of *F. oxysporum cepae* were obtained from onions infected with Fusarium basal rot collected in the fall of 1999 in the western region (Mesa, Delta, and Montrose Counties) of Colorado. Isolates were evaluated for cultural characteristics, absence of contamination by other fungal species, and conidial morphology typical of *F. oxysporum* prior to pathogenicity testing as described below. Six additional isolates of *F. oxysporum cepae* from the cryogenic collection of H. F. Schwartz at Colorado State University also were evaluated to determine if isolates collected from onion fields in eastern (Weld County) and southern (Otero County) Colorado from 1988 to 1998 were related to the isolates from western Colorado. The *nit* mutants of these 24 isolates were generated by Swift (36) and Wickliffe (37). Of the 24 isolates, 19 produced *nit* mutants required for complementary pairings and placement into VCGs.

Yellow Sweet Spanish onions (an unknown cultivar, but susceptible to all iso-

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lates of *F. oxysporum cepae* in this study) were purchased from a grocery store. The onions were surface sterilized for 60 s with 90% methanol, the outer two scales were removed, and the cross section of each bulb was sliced aseptically to a thickness of 2 mm using a Progressive Multi-slicer (Progressive International Corp., Seattle, WA). A 10% bleach solution (0.525% sodium hypochlorite) was used to sterilize the Multi-slicer. Onion slices were placed aseptically in sterile plastic petri dishes (100 by 15 mm).

Surface-disinfected, dried tissue from the infected basal plate and roots of the individual onion samples collected in western Colorado was pulverized under aseptic conditions to produce inoculum. Sterile distilled water (5 ml) was added to each gram of pulverized tissue. A 1-ml aliquot of the resulting suspension was spread over the onion slices. *F. oxysporum* colonies that developed on onion slices at 25°C after 5 to 7 days of incubation on a lab bench under continuous fluorescent light provided evidence of isolate pathogenicity according to the methodology of Abawi and Lorbeer (1). Spores of pathogenic isolates identified as *F. oxysporum* by microscopic examination were transferred to complete medium (CM; 10) and grown for 4 days at 25°C under continuous fluorescent lights, after which hyphal tips were transferred to chlorate media.

Chlorate media consisted of minimal medium (MM) supplemented with either 1.5 or 3% potassium chlorate (KClO₃). Inadequate sectoring and lack of complementary *nit* mutants were observed for some isolates; therefore, various chlorate media were evaluated to determine which was most effective for generating complementary *nit* mutants needed to determine VCGs of the isolates of *F. oxysporum cepae*.

PDA and MM supplemented with 1.5% or 3.0% KClO₃ (wt/vol) were used as the chlorate media. MM was supplemented with L-asparagine or L-threonine at 1.6 or 2.3 g liter⁻¹, respectively. Three 1-mm³ pieces of agar containing hyphae of the isolate were placed on each 100-by-15-mm plastic disposable petri dish. Three petri dishes of each medium/chlorate level were used per isolate for a total of 18 plates per isolate. The petri dishes were placed under continuous fluorescent light at 25°C for at least 2 days. The isolates incubating on chlorate media were checked every other day for sectoring. If a colony with sparse aerial mycelium developed, a hyphal tip from the sector was transferred to a 60-by-15-mm petri plate containing MM and cultured for at least 2 days under continuous fluorescent lights at 25°C. Isolates that retained their sparse characteristics were considered *nit* mutants. Isolates that reverted to a dense aerial mycelium on MM were considered chlorate-resistant, nitrate-utilizing isolates and discarded.

Phenotyping media consisted of MM supplemented with ammonium tartrate (C₄H₁₂N₂O₆), nitrate (NO₃⁻), nitrite (NO₂⁻), and hypoxanthine (HX). Four mutants were placed on each 100-by-15-mm petri dish of phenotyping medium and incubated at 25°C under continuous fluorescent lights. Isolates were evaluated within 3 to 4 days. Colonies with abundant aerial mycelium (fluffy) were considered as having a “+” growth; colonies with sparse, transparent mycelium were considered as having a “-” growth. The *nit* mutants were characterized as *nit1* (unable to utilize nitrate), *nit3* (unable to utilize nitrate or nitrite), or NitM (unable to utilize nitrate, hypoxanthine, or uric acid) (27). One NitM and *nit1* were selected from each isolate for VCG comparison. When a NitM was not generated, a *nit3* was selected. When the combination of NitM and *nit1*, or *nit1* and *nit3*, was not generated, comparisons were not conducted. Of the 24 isolates examined in this study, 33% (8) did not generate NitM mutants.

Vegetative compatibility testing. The *nit* mutants were paired in all possible combinations in two tests. Isolates were paired on 100-by-15-mm MM petri dishes in the first test and on 96-well tissue culture plates containing MM in the second test to determine the compatibility of the isolates and to classify each isolate into a VCG. A total of 21 and 25 pairs of mutants were compared in the first and second tests, respectively. The number of isolates used in the two trials was different because of the addition of isolates generated over the course of the research project and the loss of isolates due to their reversion to a wild strain.

Pairings of isolates on 100-by-15-mm petri dishes of MM was accomplished by transferring 1-mm³ pieces of MM agar containing hyphae of the mutant, as described by Correll et al. (10). Pairings of isolates in the 96-well tissue culture plates was accomplished by transferring a 1-mm³ of MM agar containing hyphae of the mutant to a 60-by-15-mm petri dish containing CM. The inoculated dishes of CM were

maintained at 25°C under fluorescent lights until the surface of each plate was covered with aerial mycelium of the mutant. A 2% solution of Tween 60 (2 ml) was added to the plate and the colony gently scraped with a sterile scalpel. Two microliters of the suspension of fungal tissue of the mutant was placed in the appropriate wells of the 96-well tissue culture plate containing MM, followed by an aliquot of the paired mutant.

Inoculated petri dishes and tissue culture plates were incubated at 25°C under continuous fluorescent lights and evaluated as early as 5 days after inoculation. Isolates were considered to be vegetatively compatible if the complementation of the *nit* mutants resulted in the formation of dense aerial growth at the zone of the anastomosing mycelia in the petri dishes, or as dense mycelial growth in the 96-cell tissue culture plates (i.e., apparent heterokaryon compatibility).

RESULTS

A total of 358 mutants of *F. oxysporum cepae* that were generated on the chlorate media were evaluated in this study (see Table 1). Media supplemented with 3% chlorate generated 185 mutants (52%), while media supplemented with 1.5% chlorate generated 173 mutants (48%). The relative frequency of mutants produced was 66, 6, and 28% for *nit1*, *nit3*, and NitM phenotypes, respectively. The 3 and 1.5% PDA chlorate media produced the greatest number of mutants (91 and 62, respectively), but the lowest percentage of NitM mutants (10 and 15%, respectively).

Of the mutants generated on the 3% chlorate MM/L-asparagine, 55% were *nit1* mutants and 43% were NitM mutants. Of the mutants generated on the 1.5% chlorate MM/L-asparagine, 50% were *nit1* and 46% were NitM mutants. The percentage of *nit1* and NitM mutants generated on the MM/L-threonine chlorate medium were 69 and 25% on 3% chlorate, respectively, and 35 and 45% on 1.5% chlorate, respectively. Of the 24 isolates, 8 (33%) did not generate NitM mutants on the different chlorate

Table 1. Generation of 358 mutants from 24 isolates of *Fusarium oxysporum* f. sp. *cepa* on 1.5 and 3.0% potassium chlorate mutation media of potato dextrose agar or of minimal media supplemented with L-asparagine or L-threonine

Medium ^a	Mutation frequency, number (%)			
	<i>nit 1</i>	<i>nit 3</i>	Nit M	Total
1.5% KClO ₃ plus:				
PDA	53 (85)	0 (0)	9 (15)	62 (17)
MM/threonine	21 (35)	12 (20)	27 (45)	59 (16)
MM/asparagine	26 (50)	2 (4)	23 (46)	52 (15)
Subtotal:	100 (28)	14 (4)	59 (16)	173 (48)
3.0% KClO ₃ plus:				
PDA	78 (86)	4 (4)	9 (10)	91 (26)
MM/threonine	26 (69)	2 (6)	9 (25)	37 (10)
MM/asparagine	31 (55)	1 (2)	25 (43)	57 (16)
Subtotal:	135 (38)	7 (2)	43 (12)	185 (52)
Total:	236 (66)	21 (6)	102 (28)	358 (100)

^a PDA = potato dextrose agar, MM = minimal medium.

media. Four isolates produced only one NitM mutant.

Through complementary pairings of Nit mutants, five VCGs were identified (Table 2). Isolates 21 and 23 were incompatible with all other isolates and were not assigned a VCG code. The three other VCGs identified were assigned VCG codes 0421, 0422, and 0423 in accordance with the Puhalla numbering system (31).

DISCUSSION

In the 1980s, a method was proposed by Puhalla (31) in which isolates of *F. oxysporum* were identified and classified based on VCGs. This system recommended vegetatively compatible isolates be given a VCG code with the first three digits corresponding to the host specialization, or forma specialis, and one or two more digits corresponding to individual VCGs within that forma specialis. Puhalla assigned 16 specific numbers to VCGs of various formae speciales at that time. Since then, over 180 VCGs have been reported for 47 formae speciales of *F. oxysporum* (18,19). Based on the study of two Japanese isolates, Y-2 and Y-12, Yoo et al. (39) identified one VCG of *F. oxysporum* f. sp. *cepae*, the causal agent of Fusarium basal rot of onion. These two isolates were vegetatively compatible; consequently, the four-digit numerical code 0420 was assigned (19). Research in Colorado has subsequently identified five VCGs (Table 2) for this forma specialis. Three of these

VCGs (VCG 1, 2, and 3) have been tentatively numbered 0421, 0422, and 0423. Of the 19 isolates evaluated in this study, 2 were not compatible with any other isolates. Consequently, they were placed in VCG 4 and 5, but were not assigned a four-digit numerical code based on the recommendations of Kistler et al. (22) that single-member VCGs not be numbered. The final decision on assignment of a VCG code is dependent upon the VCG coordination committee. The Colorado VCGs eventually will be compared with the Japanese VCG to determine their relationship.

Isolates within a forma specialis are genetically similar, probably originating from a single pathogenic genotype or clonal origin. Kistler (21) proposed that a VCG within a population of *F. oxysporum* indicates a distinct clonal lineage. Harveson and Rush (14) found that VCGs of *F. oxysporum* f. sp. *betae* represented distinct isolated populations indigenous to their respective areas. Woo et al. (38) and Bosland and Williams (5), however, reported that the formae speciales they were investigating did not exhibit a correlation between VCG and geographical origin. Four of the Colorado VCGs were found to be limited in their geographical range.

VCG 1 was present on onion bulbs growing in two western and two eastern counties of Colorado. VCG 2 was present only in Delta County, and VCG 3 was located only in Mesa County. VCGs 4 and

5 were collected from onion bulbs in Montrose County. Table 2 provides specifics on these VCGs.

The genetic relatedness and possible evolutionary linkages among the Colorado VCGs, as well as VCGs from other onion-growing areas around the world, need to be examined. Comparisons of mitochondrial DNA, restriction fragment length polymorphism haplotypes, and other genetic fingerprinting techniques would help determine the relative genetic distances among these VCGs and their clonal relationship (17,23).

Although a more thorough study of the population of VCGs of *F. oxysporum* f. sp. *cepae* present in Colorado is required, the data at this time indicates this organism has been established in western Colorado for a longer period of time than in other Colorado onion-growing areas. The diversity of vegetative compatibility groups of *F. subglutinans* f. sp. *pini*, the causal agent of pine pitch canker, is an excellent example to further explain this assumption (9).

F. subglutinans f. sp. *pini* has been established in Florida since the mid 1970s with 45 distinct VCGs of the organism classified. On the other hand, 70% of the California population, where the organism was first reported in 1986, consists of a single VCG. A single VCG predominates in this epidemic front. The lack of diversity of the California VCG population is indicative of an asexually reproducing pathogen that has been recently introduced (9). As a population ages, the diversity increases. A more exhaustive examination of the population of *F. oxysporum* f. sp. *cepae* in Colorado may reveal a greater diversity of VCGs in northeastern or southern Colorado than has been currently found.

A better pathogenicity protocol for *F. oxysporum* f. sp. *cepae* on onion is necessary. Onion slices were used in this study to determine isolate pathogenicity. A pathogenicity protocol such as root clip inoculation might identify a set of differential cultivars that could be used to determine races of the organism. If cultivar differences were identified, differences in virulence might be found.

Complementary pairings in this study were done on both 100-by-15-mm petri dishes and 96-well tissue culture plates. Complementary pairings of *nit* mutants in petri dishes is time consuming, but these results are more distinct than those obtained with pairings in tissue culture plates. The dense aerial growth that develops is the location at which *nit* mutant colonies anastomose to form a heterokaryon. The point of actual heterokaryon formation cannot be observed when complementary pairings are made on 96-well tissue culture plates. This formation is obvious on petri dishes. In addition, the application of drops of an aliquot containing the mutant to a 96-well plate is an exacting process. Con-

Table 2. Isolate number, geographic origin (county), year the isolate was collected, and vegetative compatibility group (VCG) of 19 isolates of *Fusarium oxysporum* f. sp. *cepae* collected from diseased onion plants in Colorado

Isolate	County ^a	Year	VCG ^b	VCG code ^c
22	Montrose	1999	1	0421
25	Delta	1999	1	0421
27	Delta	1999	1	0421
28	Delta	1999	1	0421
FOC 1	Weld	1992	1	0421
FOC 4	Otero	1988	1	0421
FOC 8	Delta	1993	1	0421
FOC 201A	Weld	1998	1	0421
FOC 201D	Weld	1998	1	0421
FOC 204A	Weld	1998	1	0421
31	Delta	1999	2	0422
32	Delta	1999	2	0422
41	Mesa	1999	3	0423
42	Mesa	1999	3	0423
47	Mesa	1999	3	0423
48	Mesa	1999	3	0423
50	Mesa	1999	3	0423
21	Montrose	1999	4	not assigned
23	Montrose	1999	5	not assigned

^a Regions of onion production in Colorado: northeastern (Weld County), southeastern (Otero County), and western (Delta, Mesa, and Montrose Counties).

^b Potato-dextrose chlorate agar (CPDA) and chlorate minimal medium (CMM) were used to generate nit mutants. CMM was supplemented with L-asparagine or L-threonine at 1.6 or 2.3 g liter⁻¹. All media were supplemented with 1.5 or 3.0% KClO₃ (wt/vol). Nit mutants were paired in all possible combinations to determine the compatibility and VCG classification of each isolate. Complementation pairings were made between *nit1* and NitM mutants, or *nit1* and *nit3* mutants, based on availability. Isolates were considered to be vegetatively compatible if the complementation of the *nit* mutants resulted in the formation of a heterokaryon with dense aerial growth.

^c Vegetatively compatible isolates are given a VCG code consisting of a four- or five-digit number, with the first three digits corresponding to host specialization, or forma specialis, and the following digits corresponding to individual VCGs within that forma specialis.

tamination of neighboring wells is a possibility that might result in false positive readings.

Puhalla (31) and Puhalla and Spieth (32) report isolates of *F. oxysporum* and *F. moniliforme* sectored when cultured on MM containing 1.5% KClO₃ (MMC). *F. oxysporum* f. sp. *niveum* mutants are easily obtained on PDA containing 1.5% KClO₃ (16). *F. subglutinans* f. sp. *pini* isolates from California, however, are not restricted on 1.5% MMC, but chlorate-resistant sectors are readily recovered from these isolates on 3% MMC. In contrast, the Florida isolates are readily recovered on 1.5% MMC (9). According to Woo et al. (38), the relative frequencies of *nit* mutants of *F. oxysporum* f. sp. *phaseoli* varied based on the chlorate media used. The *nit* mutants recovered from PDA supplemented with 1.5% potassium chlorate were as follows: *nit1* = 60 to 86%, *nit3* = 4 to 23 %, and NitM = 0 to 15%. On MM supplemented with 1.5% potassium chlorate, the following percentages of mutants were recovered: *nit1* = 58 to 62%, *nit3* = 5 to 25%, and NitM = 0 to 25%. Bosland and Williams (5) used potato-sucrose (PS), cornmeal agar with dextrose (CMDA), PDA, and potato-carrot-dextrose agar (PCDA) media to generate mutants. Each was supplemented with potassium chlorate at 20 g liter⁻¹. Their research indicated that 2% chlorate PDA and CMDA generated more sectors than chlorate PCDA.

To determine which chlorate media were most effective in generating mutants from Colorado isolates of *F. oxysporum cepae*, PDA, MM supplemented with L-asparagine at 1.6 g liter⁻¹ of distilled water, and MM supplemented with L-threonine at 2.3 g liter⁻¹ of distilled water were tested. Each was further supplemented with KClO₃ at 1.5 and 3% (wt/vol). A total of 358 mutants were generated. In all, 33% of the isolates did not generate NitM mutants. An additional 16% produced only one NitM mutant each, and 6% of the mutants were *nit3*.

Chlorate PDA and 3% chlorate MM with L-threonine did not generate the *nit* mutants required, whereas MM with L-asparagine (1.5 and 3% chlorate) and MM with L-threonine (1.5% chlorate) generated the necessary complementary *nit* mutants. The latter two chlorate media should be considered for use when generating *nit* mutants of *F. oxysporum* f. sp. *cepae*.

Transferring mutants to DM resulted in more reversion to the wild-type growth than when mutants were grown on MM (*unpublished data*). MM will be used in future trials when maintaining mutants of this pathogen in culture.

Isolates of the five Colorado VCGs reported in this article are maintained in the cryogenic collection of Howard F. Schwartz at Colorado State University. Representatives of each will be submitted

for storage at internationally recognized fungal culture collections upon request by the VCG coordination committee and henceforth will be available for comparison by other researchers.

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