Identification of *Fusarium oxysporum* f. sp. *apii* on the Basis of Colony Size, Virulence, and Vegetative Compatibility

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ABSTRACT

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Fusarium oxysporum was isolated from celery roots showing symptoms of Fusarium yellows disease and from asymptomatic roots. A total of 199 isolates were classified by colony size, virulence on green celery, and heterokaryon, or vegetative, compatibility. Vegetative compatibility was assessed by pairing nitrate nonutilizing mutants. Eighty-two isolates from California, New York, and Michigan were virulent on green celery in greenhouse pathogenicity tests. These isolates were also of small colony type and vegetatively compatible with a *F. oxysporum* f. sp. *apii* race 2 tester strain. Four isolates were vegetatively compatible with *F. o.* f. sp. *apii* race 2 and of small colony type but avirulent in greenhouse pathogenicity tests. The remaining 113 isolates, primarily large colony types and avirulent on green celery, were vegetatively incompatible with *F. o.* f. sp. *apii* race 2. These data suggest that colony size and vegetative compatibility may be very useful laboratory tests for identifying *F. o.* f. sp. *apii* race 2. In our study, these tests would have correctly identified 197 of the 199 *F. oxysporum* isolates without recourse to pathogenicity tests. These laboratory tests, in conjunction with virulence tests, should allow faster, more precise identification and characterization of the *F. o.* f. sp. *apii* race 2 population.

Additional key words: anastomosis, population genetics.

Fusarium yellows disease of celery (*Apium graveolens* L. var. *dulce* (Miller) Pers.), caused by the vascular wilt pathogen *Fusarium oxysporum* Schlecht. f. sp. *apii* (R. Nelson & Sherb.) Synd. & Hans., is a major production constraint in celery-growing regions of California (11), Michigan (10), and New York (5). The disease was originally reported on yellow self-blanching celery cultivars in Michigan in 1914 (6) and reached severe proportions in those early outbreaks (18,27). With the subsequent development of resistant green celery cultivars, Fusarium yellows disease all but disappeared for many years (11). Then, outbreaks of Fusarium yellows disease on green celery cultivars again occurred in California in 1976 (20), in New York in 1980 (5), and in Michigan in 1982 (10). Currently, all commercially available celery cultivars are susceptible to this disease, although some have shown tolerance (33).

Coons and Nelson (7) first described the fungus causing Fusarium yellows disease on yellow celery cultivars. Subsequently, Schneider and Norelli (30) designated two races of *F. o.* f. sp. *apii* based on greenhouse virulence tests; race 1 was virulent only on yellow celery (cv. Golden Detroit) and race 2 was virulent on both yellow and green celery (cv. Tall Utah 52-70R). Puhalla (23) provided further evidence that the two races of *F. o.* f. sp. *apii* are genetically distinct and that it is unlikely race 2 arose as a variant of race 1.

Strains of *F. oxysporum* form a major component of the fungal flora of most cultivated soils (21), and most of these strains are morphologically indistinguishable (31,33). Isolates of *F. o.* f. sp. *apii* race 2 are commonly distinguished from the general population of *F. oxysporum* by their virulence on celery in greenhouse pathogenicity tests (32). These pathogenicity tests are difficult, time-consuming, and often inconclusive. As a result, most epidemiologic studies of Fusarium yellows disease have been limited to greenhouse studies in artificially infested soil (12,28,32). Alternative methods to expedite the identification of the pathogen that causes Fusarium yellows disease would be advantageous.

This report evaluates laboratory tests for identifying the pathogen that causes Fusarium yellows disease, *F. o.* f. sp. *apii* race 2. These tests include colony size and heterokaryon, or vegetative, compatibility and are compared to standard greenhouse virulence tests for ability to identify *F. o.* f. sp. *apii* race 2 among a population of *F. oxysporum*. A preliminary report has been published (8).

MATERIALS AND METHODS

Strains. Celery fields showing slight (no aboveground symptoms) to severe (stunting and yellowing of foliage) symptoms of Fusarium yellows disease were selected throughout the major celery-producing areas of California. Roots showing vascular discoloration were collected from several fields. All isolations were made from roots 0.5–1.0 cm in diameter. Root pieces were agitated in a sterile 1% sodium hexametaphosphate (NaHMP) solution for 10 min to free roots from soil. Samples were then put into 500-ml flasks containing 250 ml of sterile NaHMP and agitated for 15 min. This procedure was repeated three times with fresh solution. The roots were then agitated in sterile distilled water for 15 min and surface-sterilized with 95% ethanol. The vascular tissue was excised from the root, and thin (1–3 mm) cross sections were put on plates of Komada medium (16). Plates were incubated at 23 ± 2 C under an alternating 12-hr day/night regime (two Westinghouse 40W cool-white fluorescent tubes). Isolations were made from a minimum of five plants per field. A total of 47 strains of *F. oxysporum* were recovered from roots showing symptoms of Fusarium yellows disease and were used for further testing.

Celery roots showing no vascular discoloration and <0.2 cm in diameter were also collected from several fields at a depth of 10–20 cm. Samples were returned to the laboratory, stored at 4 C, and processed within 48 hr after collection. Roots were removed from soil, divided into 10 subsamples, and washed as described for roots showing vascular discoloration but were not surface-sterilized. Approximately 30 cm of root was put on each plate of Komada's selective medium and incubated as previously described. After 6–10 days, one to five colonies were randomly selected from each subsample. A total of 152 strains of *F. oxysporum* were recovered and used for further testing.

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All isolates of *F. oxysporum* were grown on water agar; either a single microconidium or a hyphal tip of each strain was then transferred to potato-dextrose agar (PDA). All isolates were identified by the methods of Nelson et al (19). Each isolate was mass-transferred to a sterilized Whatman No. 3 filter paper disk (7-cm diameter) on the surface of PDA. After 8 days, the colonized filter paper disk was removed from the PDA and allowed to air-dry in an empty sterile petri plate. The disk was then cut into 2- to 3-mm-square pieces, put into sterilized No. 2 dram screw-cap vials, and stored at 4 C.

Virulence tests. Each isolate was tested for virulence on green celery (*A. g.* var. *dulce* 'Tall Utah 52-70R'). Six-week-old plants were transplanted into 10-cm-diameter clay pots containing sterilized potting mix composed of equal proportions of peat soil, sand, and redwood soil conditioner. Plants were inoculated 5–7 days after transplanting.

A conidial suspension was prepared from 7- to 10-day-old cultures on PDA plates and used as inoculum. One milliliter of the suspension (1.0×10^6 spores/ml) was put into each of two 3-cm-deep holes near the base of the plant. Sterile water was used as a control. Plants were incubated in the greenhouse with day and night temperatures of 25–30 C and 18–25 C, respectively. All plants were fertilized three times a week with half-strength Hoagland's solution (14). After 8 wk, plants were cut longitudinally through the crown and root system and scored for disease severity on a scale of 0–5 (0 = no root discoloration, 1 = slight vascular discoloration in root tissue, 2 = extensive vascular discoloration in root tissue, 3 = vascular discoloration in crown, 4 = extensive vascular discoloration in crown, 5 = crown completely necrotic). The virulence test for each isolate was replicated on four to six plants.

Colony size. Puhalla (23) showed that strains of *F. o.* f. sp. *apii* race 2 had a significantly smaller colony diameter than other strains of *F. oxysporum* when grown on a medium containing L-sorbose (D medium). In the current study, colony size relative to a standard *F. o.* f. sp. *apii* race 2 pathogen (JA-1) was determined for all isolates using an adaptation of Puhalla's technique. Isolates were grown on PDA plates for 5 days. With a thin platinum needle, 20 stab transfers were made from the PDA to a plate of D medium (Fig. 1). Plates were incubated for 72 hr at 23 ± 2 C. The diameters of 20 colonies of each strain were then measured from the underside of the plate using a binocular dissecting microscope equipped with an ocular micrometer. Colony diameters were measured to the nearest 0.1 mm. Relative colony size was determined by dividing mean colony diameter by the mean colony diameter of isolate JA-1.

Recovery of nitrate nonutilizing mutants. Nitrate nonutilizing mutants of *F. oxysporum* can be readily generated without the use of a mutagen (24). For this method, mass transfers (2-mm^3 PDA blocks) of the fungus were placed on potato-sucrose agar containing 1.5% potassium chlorate ($KClO_3$). Initially, the colonies were greatly restricted, but after 5–10 days, fast-growing sectors emerged from the restricted colonies. These sectors were transferred to a minimal medium that contained $NaNO_3$ as the sole nitrogen source (24). Those sectors producing thin expansive growth on minimal medium were considered to be nitrate nonutilizing mutants. At least one nitrate nonutilizing mutant was recovered from each of the strains used in this study.

Test for vegetative compatibility. Certain nitrate nonutilizing mutants paired on minimal medium complement each other (24). Complementation is evident by dense aerial mycelium where the two thin nitrate nonutilizing mutant colonies contact (Fig. 2). Complementary nitrate nonutilizing mutants (nitA and nitB) of *F. o.* f. sp. *apii* race 1 (JA-11) and *F. o.* f. sp. *apii* race 2 (JA-3), developed earlier by Puhalla (24), were used as testers in all pairings. An additional nitrate nonutilizing mutant, produced during the course of this study and designated C-42 nitC, also was used as an *F. o.* f. sp. *apii* race 2 tester.

At least one nitrate nonutilizing mutant from each isolate of *F. oxysporum* was paired with all five of the tester nitrate nonutilizing mutants. Two nitrate nonutilizing mutants were placed 1.5 cm apart in the center of a 9-cm-diameter petri plate containing minimal medium, and the plate was incubated at room temperature (23 ± 2 C). Heterokaryon formation was scored 7, 14, and, in some instances, 21 days later on a scale of 0–2 (0 = no reaction, 1 = little or no aerial mycelium, and 2 = abundant aerial mycelium) (Fig. 2). All pairings were repeated at least once.

Tester nitrate nonutilizing mutants showing positive heterokaryon formation were assayed for cross-feeding by physically separating the paired nitrate nonutilizing mutants with a strip of sterilized cellophane. Cellophane prevents hyphal contact but does not prevent the passage of small molecules (cross-feeding). Therefore, a dense aerial growth even when the nitrate nonutilizing mutants are separated by cellophane would indicate cross-feeding.

RESULTS

In greenhouse pathogenicity tests the mean virulence ratings among controls ranged from 0.5 to 1.2. Therefore, only strains giving mean virulence reactions ⩾1.5 were considered virulent.

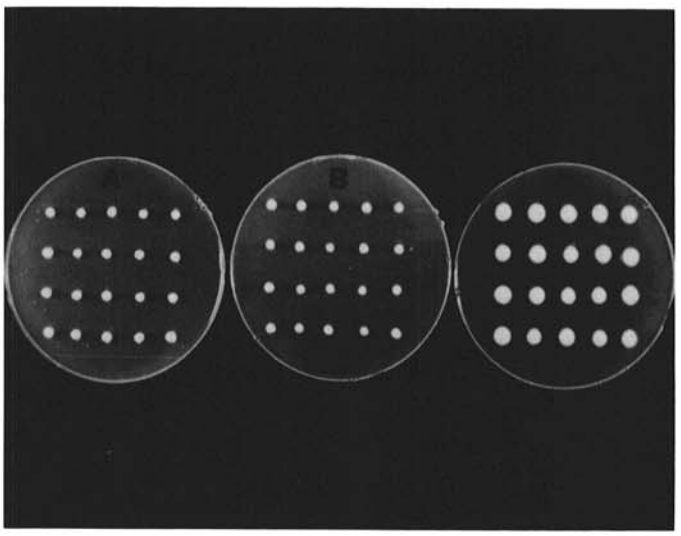

Fig. 1. Colonies of *Fusarium oxysporum* on sorbose medium (D medium) incubated for 72 hr at 23 ± 2 C. **A,** *F. o.* f. sp. *apii* race 2 standard, JA-1. **B,** Typical *F. o.* f. sp. *apii* race 2 strain, C-42. **C,** Typical nonpathogenic strain, A-47.

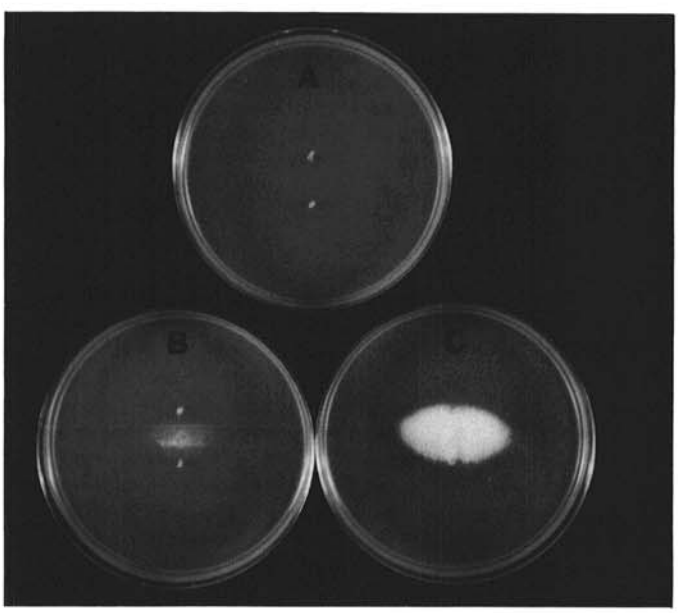

Fig. 2. Pairing reactions of nitrate nonutilizing (nit) mutants of *F. o.* f. sp. *apii* race 2 strain C-42 after 7 days. **A,** NitC and nitC, reaction = 0 (no reaction). **B,** NitA and nitB, reaction = 1 (little or no aerial mycelium). **C,** NitB and nitC, reaction = 2 (abundant aerial mycelium).

Forty-six of the 47 strains isolated from diseased roots and 55 of the 152 asymptomatic root colonizers were virulent in greenhouse pathogenicity tests.

The *F. o.* f. sp. *apii* race 2 standard (JA-1) had a mean colony diameter of 3.3 mm after 72 hr (standard deviation ± 0.3 mm). Colony size (relative to JA-1) among the strains tested ranged from 0.8 to 2.3 (Table 1). The standard deviation in colony diameter for all of the strains tested ranged from 0.2 to 0.5 mm.

Complementary nitrate nonutilizing mutants within *F. o.* f. sp. *apii* race 1 or within *F. o.* f. sp. *apii* race 2 showed evidence of heterokaryon formation after 7 days. *F. o.* f. sp. *apii* race 1 was not vegetatively compatible with *F. o.* f. sp. *apii* race 2. NitA and nitB of strain JA-3 (*F. o.* f. sp. *apii* race 2) reacted with each other, whereas nitC reacted with both nitA and nitB. The nitC tester consistently gave the earliest and strongest reaction.

There was no evidence of cross-feeding between complementary tester nitrate nonutilizing mutants. Dense growth developed only if the hyphae of the nitrate nonutilizing mutants were in physical contact (Fig. 3). Where cellophane separated two complementary nitrate nonutilizing mutants, no heavy growth (aerial mycelium) was observed. Heavy growth was visible only after the mycelium grew around the cellophane and made contact.

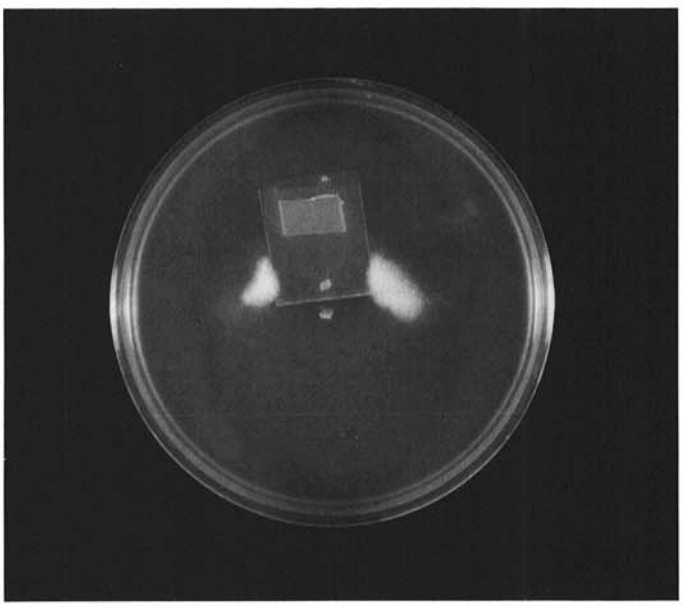

Fig. 3. Cellophane test for cross-feeding between nitB and nitC of strain C-42. Dense aerial growth develops only after mycelium has grown around cellophane.

TABLE 1. Relative colony size of isolates of *Fusarium oxysporum* from celery

Relative colony size[a]	Isolates tested[b] (no.)	Isolates compatible with *F. o.* f. sp. *apii* race 2[b] (no./% of total)
0.8	8	8/100
0.9	22	22/100
1.0	21	21/100
1.1	25	22/88
1.2	17	8/47
1.3	18	4/22
1.4	9	1/11
1.5	30	0
1.6	8	0
1.7	9	0
1.8	13	0
1.9	9	0
2.0	3	0
2.1	3	0
2.2	3	0
2.3	1	0

[a] Colony diameter relative to that of isolate JA-1 of *F. o.* f. sp. *apii* race 2.
[b] Based on vegetative compatibility with isolate C-42 nitC of *F. o.* f. sp. *apii* race 2.

A total of 120 isolates had a relative colony size ≤1.4. Eighty-six of these small colony types were vegetatively compatible with *F. o.* f. sp. *apii* race 2, and 82 of these were virulent on celery in greenhouse pathogenicity tests. No isolates collected were vegetatively compatible with *F. o.* f. sp. *apii* race 1.

None of the 79 isolates of large colony type were vegetatively compatible with *F. o.* f. sp. *apii* race 2. However, 21 gave virulent reactions in greenhouse pathogenicity tests.

All isolates with a relative colony size ≤1.0 were vegetatively compatible with *F. o.* f. sp. *apii* race 2, whereas all those with a relative colony size ≥1.5 were incompatible (Table 1). The relative colony size between 1.1 and 1.4 included both vegetatively compatible and vegetatively incompatible isolates. The percentage of isolates compatible with *F. o.* f. sp. *apii* race 2, however, dropped off sharply as relative colony size increased from 1.1 to 1.4 (Table 1).

The isolates tested fell into six of eight possible categories based on relative colony size, heterokaryon formation with *F. o.* f. sp. *apii* race 2, and virulence on green celery (Table 2). Eighty-two of the 86 isolates in vegetative compatibility group *F. o.* f. sp. *apii* race 2 were virulent on green celery and were small colony types; the other four were also small colony types but were avirulent in greenhouse pathogenicity tests.

A total of 113 isolates colonizing symptomless celery roots were vegetatively incompatible with *F. o.* f. sp. *apii* race 2. Sixty (53%) of these isolates were both large colony types and avirulent on green celery, and 32 (28%) were small colony types (relative size between 1.1 and 1.4) and avirulent. Nineteen isolates (17%) were large colony types and virulent on green celery, although 15 were only weakly virulent (≥1.5 and ≤2.0). The remaining two isolates were small colony types and virulent on green celery.

DISCUSSION

Identifying formae speciales and races of *F. oxysporum* based solely on greenhouse virulence tests is often inconclusive and misleading. Results from virulence tests vary with environmental conditions, methods of inoculation, and host age (12,17). Such factors can influence symptom expression of many Fusarium vascular wilt diseases. A case in point is that of pea wilt, caused by *F. oxysporum* Schlecht. f. sp. *pisi* (van Hall) Snyd. & Hans. As many as 11 races of *F. o.* f. sp. *pisi* were initially described by Armstrong and Armstrong (4) on the basis of virulence reactions in differential pea cultivars. Later, however, Kraft and Haglund (17), using standardized inoculation procedures, reduced the number of races to two.

Variation in the results of greenhouse pathogenicity tests was observed in our study of *F. o.* f. sp. *apii*. For example, some celery plants inoculated with the pathogen showed severe disease

TABLE 2. Isolates of *Fusarium oxysporum* from celery classified by vegetative compatibility, relative colony size, and virulence

Number of isolates	Vegetative compatibility with *F. o.* f. sp. *apii* race 2[a]	Relative colony size ≤1.4[b]	Disease severity ≥1.5[c]
82	+	+	+
4	+	+	−
0	+	−	+
0	+	−	−
60	−	−	−
32	−	+	−
19	−	−	+
2	−	+	+

[a] + = Vegetatively compatible and − = vegetatively incompatible with isolate C-42 nitC.
[b] + = Small and − = large colony diameter relative to that of isolate JA-1.
[c] Cultivar Tall Utah 52-70R plants scored on a scale of 0–5 (0 = no root discoloration, 5 = crown completely necrotic); + = ≥1.5 and virulent, − = <1.5 and avirulent.

symptoms (virulence reaction >3.0) while others inoculated with the same isolate showed no symptoms. Other workers have reported similar findings (23). Moreover, the pathogenicity tests were conducted over a 6-mo period, and seasonal fluctuations in temperature may have influenced symptom expression. Although various methods, e.g., incorporating colonized barley straw into soil (29), may improve the efficacy of virulence tests, the logistics would be prohibitive with a large number of isolates.

Two laboratory tests may be more useful than virulence tests in identifying isolates of *F. o.* f. sp. *apii* race 2. In the first test, colonies of the fungus are grown on a sorbose-containing medium (23) and their diameters are measured relative to a standard *F. o.* f. sp. *apii* race 2 (JA-1) strain. The second test assesses the ability of strains to anastomose and form a heterokaryon (vegetative compatibility). Heterokaryon, or vegetative, compatibility among strains of *F. oxysporum* can be tested by the use of nitrate nonutilizing mutants (24).

Puhalla (23) suggested that all strains of *F. o.* f. sp. *apii* race 2 may have a characteristically small colony size and be vegetatively compatible and thus belong to the same vegetative compatibility group (VCG). This conclusion, however, was based on a small sample of isolates from diseased tissue only. To further test Puhalla's hypothesis, a large collection of strains of *F. oxysporum* were isolated both from celery roots with Fusarium yellows disease and from symptomless celery roots. Root samples were collected from all of the major celery-producing regions of California. Pathogenic isolates from New York and Michigan also were included. All 47 of the isolates collected from celery plants showing symptoms of Fusarium yellows disease from California, Michigan, and New York were vegetatively compatible and therefore in the same VCG. These same 47 isolates also formed characteristically small colony types on sorbose medium, and 46 were virulent in greenhouse pathogenicity tests. Thirty-nine of the 152 strains from apparently healthy celery roots also were vegetatively compatible with *F. o.* f. sp. *apii* race 2 and had the characteristic small colony size, and all but three were virulent in greenhouse pathogenicity tests. These data suggest a strong correlation among virulence, small colony size, and vegetative compatibility in a large sample of *F. o.* f. sp. *apii* race 2 strains.

F. oxysporum is apparently a very efficient colonizer of celery roots, with symptomless roots having up to 2.3 colonies per centimeter of root. Of the 152 asymptomatic root colonizers, 113 were not vegetatively compatible with *F. o.* f. sp. *apii* race 2. The largest group of these isolates (53%) included large colony types that were avirulent on green celery. These strains represent the parasitic but nonpathogenic portion of the population of *F. oxysporum* on celery roots. A portion of this population may be responsible for suppressing infection by the pathogen (28).

Thirty-two isolates from asymptomatic roots were avirulent and incompatible with *F. o.* f. sp. *apii* race 2 but of small colony size. Although these isolates were considered small colony types, their colony diameters were near the upper cutoff value of 1.4. The upper cutoff value for small colony type is somewhat arbitrary; very few *F. o.* f. sp. *apii* race 2 isolates had a relative colony size >1.0, and the probability of an isolate being *F. o.* f. sp. *apii* race 2 decreased very quickly as relative colony size increased from 1.1 to 1.4. The assignment of the isolates to small colony types differs somewhat from the data presented by Puhalla (23) but may reflect more inherent variability with our method of size determination. In addition, we used a larger collection of *F. oxysporum* not associated with diseased celery tissue.

Nineteen of the asymptomatic root colonizers were virulent on celery in greenhouse pathogenicity tests but had large colonies and were not vegetatively compatible with *F. o.* f. sp. *apii* race 2. Although none of these isolates was associated with diseased celery tissue under field conditions, some were able to invade the vascular tissue and were mildly virulent under greenhouse conditions. Vascular infection of "nonhosts" by *F. oxysporum* has been demonstrated by others (3,13,15).

Two isolates gave virulent reactions and were small colony types but were not vegetatively compatible with *F. o.* f. sp. *apii* race 2. These two isolates may not be *F. o.* f. sp. *apii* race 2; more likely they represent a small portion of the *F. o.* f. sp. *apii* race 2 population incapable of forming a heterokaryon. Puhalla (23) also reported such an isolate in his studies of *F. o.* f. sp. *apii*.

Four isolates were compatible with *F. o.* f. sp. *apii* race 2 and were small colony types but were avirulent in greenhouse pathogenicity tests. Three were recovered from asymptomatic celery roots and one from a root showing symptoms of Fusarium yellows disease. These isolates either represent an avirulent portion of the *F. o.* f. sp. *apii* race 2 population or are attentuated strains of *F. o.* f. sp. *apii* race 2.

The ability to differentiate formae speciales and races of *F. oxysporum* has, in the past, relied on virulence. As a result, the use of vegetative compatibility and colony size to distinguish races of *F. o.* f. sp. *apii* may seem artificial. We can assume, however, that a strain's virulence, vegetative compatibility, and growth rate are all under genetic control. For example, work with other fungi has shown that vegetative compatibility is determined by several gene loci (2,22,26). This mechanism controlling vegetative compatibility may result in the genetic isolation of strains within a population (9,25). The constant association of such genetic traits as vegetative compatibility and growth rates, in turn, reflects a combination of specific genes. If an organism is capable of sexual reproduction and thus meiosis, we would expect such gene combinations to be continually made and broken over time. However, for an organism like *F. oxysporum* that apparently does not undergo sexual reproduction, these gene combinations, once made, are fixed. Puhalla (24) has discussed how such combinations could arise and lead to the association of seemingly unrelated traits. Thus, the demonstration of only a few genetic traits in an asexual organism may be sufficient to identify its entire genome. Vegetative compatibility within *F. o.* f. sp. *apii* race 2 and small colony size indicate that a specific genotype is present—a genotype that also contains those genes governing virulence to celery.

On the basis of our data, the use of vegetative compatibility and relative colony size would have correctly identified 197 of 199 isolates of *F. oxysporum* without recourse to pathogenicity tests. These two laboratory tests would provide a rapid and easy method for *F. o.* f. sp. *apii* race 2 determination in California. However, neither the colony size test nor the vegetative compatibility test would reflect any difference in virulence within the population.

Care should be taken in selecting the nitrate nonutilizing mutant testers. A large number (64%) of *F. o.* f. sp. *apii* race 2 isolates did not pair with either of the two original complementary nitrate nonutilizing mutants of *F. o.* f. sp. *apii* race 2 (nitA and nitB). However, all of these isolates did pair with an additional *F. o.* f. sp. *apii* race 2 nitC mutant. This may not be peculiar to this particular VCG. Therefore, extra time and effort may be required to recover strong tester nitrate nonutilizing mutants within a VCG. Once good tester nitrate nonutilizing mutants are available within a VCG, only one nitrate nonutilizing mutant should be needed for any subsequent compatibility tests.

Studies of the epidemiology and of the dynamics of root colonization of *F. oxysporum* lag behind those of other soilborne diseases, owing, in part, to the difficulty in distinguishing pathogenic and nonpathogenic populations. Because *F. oxysporum* is such a major component of the fungal flora in almost all cultivated soils, a rapid laboratory test to enumerate pathogenic populations would make it possible to quantify soil populations more accurately. The relative colony size test and pairing test should prove useful in rapidly quantifying soil and root populations of the Fusarium yellows disease pathogen. These laboratory tests, in conjunction with virulence tests, will allow more precise identification and characterization of the population of *F. o.* f. sp. *apii* race 2. Similar tests should also be useful in working with other formae speciales.

In addition to identifying the pathogenic portion of the population, preliminary data indicate that vegetative compatibility also may be a way of identifying strains within the omnipresent nonpathogenic population of *F. oxysporum* (8). Vegetative compatibility groups that distinguish strains of effective nonpathogenic competitors (1,28) would be extremely useful in quantifying this important segment of the population.

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