Use of Genetic Fingerprinting and Random Amplified Polymorphic DNA to Characterize Pathotypes of *Fusarium oxysporum* f. sp. *ciceris* Infecting Chickpea

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ABSTRACT

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Isolates of *Fusarium oxysporum* f. sp. *ciceris* induce either yellowing or wilt syndromes in chickpea and can be characterized into seven races by means of biological typing. DNA from 63 isolates of *F. o. ciceris* and from 11 isolates of other fungi was amplified by the random amplified polymorphic DNA (RAPD) technique by using the polymerase chain reaction with single primers. The primers used were based either on known ribosomal DNA sequences from *Penicillium hordei* or on sequencing primers. The amplification products were analyzed for polymorphisms by gel electrophoresis to determine whether pathotypes and/or races could be distinguished at the molecular level. UPGMA (unweighted paired group method with arithmetic averages) cluster analysis and principal coordinate analysis were used to divide the *F. o. ciceris* isolates into two distinct clusters that correlated with the pathotypes causing the yellowing or wilt disease syndrome of chickpea. These clusters were clearly distinct from other *F. oxysporum* formae speciales and from other chickpea pathogens such as *F. eumartii*, *F. solani*, and *Ascochyta rabiei*. Nineteen of the *F. o. ciceris* isolates, which had previously been characterized with regard to race by chickpea differential lines, were used initially to identify yellowing or wilt pathotype-associated DNA markers. One isolate that had previously been characterized as a yellowing-inducing isolate was subsequently shown to be a misclassified nonpathogenic isolate. Another 22 isolates of *F. o. ciceris* were subsequently correctly identified as to disease pathotype in a "blind trial." In a further trial, twenty-one previously uncharacterized (by host inoculation) isolates were identified as yellowing- or wilt-inducing pathotypes by RAPD analysis, and these identifications were later confirmed in all cases by chickpea differential line tests under controlled environmental conditions.

Fusarium wilt, induced by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato, is a major constraint to chickpea (*Cicer arietinum* L.) cultivation in most of the chickpea-growing regions in the world (20). Estimated yield losses of 10% have been reported from India (27) and Spain (31). Isolates of the pathogen fall into two types according to whether they induce yellowing or wilt syndromes. Both syndromes arise as a result of vascular infections (31), and both pathotypes show varying degrees of pathogenicity toward chickpea lines (13,31). Seven races of *F. o. ciceris* have been identified by their differential interactions with chickpea lines (10,13,31). Races 1, 2, 3, and 4 were first described in India (10) and races 0, 5, and 6 in Spain (13). Race 0 isolates cause the yellowing syndrome in the absence of wilting symptoms, whereas isolates of races 5 and 6, like those from India, cause the typical Fusarial wilt and necrosis (10). Race 0 is believed to be widespread in the Mediterranean basin (12). Races 0, 1, 5, and 6 have recently been observed in California and Spain (11), where race 1 isolates have been divided into subgroups on the basis of further chickpea differential line tests. Race 1A isolates are wilt-inducing isolates, whereas isolates from races 1B and 1C induce the yellowing syndrome in the absence of wilting symptoms (11).

Differential line tests can take at least 40 days to complete; therefore, it would be desirable to have a more rapid, cheaper, and less labor-intensive method of distinguishing these pathotypes. Restriction fragment length polymorphism (RFLP) analyses in which mitochondrial, ribosomal, or total DNA were used have provided more rapid methods in a wide variety of species (4,6,32). Alternatively, digested genomic DNA may be probed with random genomic clones (16), simple repeat oligonucleotides (34), or M13 phage DNA (24).

Methods involving Southern hybridizations and/or cloning are, however, relatively labor intensive and costly. The recent development of random amplification of polymorphic DNA (RAPD) (36) or arbitrarily primed polymerase chain reaction (PCR) (35) has allowed the rapid generation of reliable, reproducible DNA fragments or fingerprints in a wide variety of species, including several fungi (5,7,8,17).

These techniques are based on PCR (25), but instead of two specific primers, short single primers of arbitrary or random base sequence, with over 50% G+C content, are used to amplify genomic DNA under low stringency annealing conditions. Only the sequences that have proximal priming sites in the correct orientation will be amplified; and because of the low stringency, some mismatch annealing may occur between primer and template, giving rise to further products. As well as base sequence changes, length polymorphisms can arise because of insertions, deletions, substitutions, or inversions, either at or between the priming sites. Secondary structure may also affect amplification products (3).

The purpose of this study was to use RAPDs to distinguish between isolates of different pathotypes and, where possible, different races of *F. o. ciceris* and to use this information to provide a rapid method for the diagnosis of the pathotype and/or race type.

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MATERIALS AND METHODS

Fungal strains. The 74 isolates used in this study, together with the source and race classification of each, are listed in Table 1. The isolation and maintenance of cultures was as described in Nelson et al (19). Single-spore cultures were isolated and subcultured on potato-dextrose agar at 25 C with a photoperiod of 16 h of light and 8 h of dark. Spore suspensions from single-spore colonies were used to inoculate flasks containing 50 ml of potato-dextrose broth, which were incubated for 4 days at 25 C on an orbital shaker at 125 rpm. Mycelia from cultures on potato-dextrose broth were vacuum filtered, lyophilized, and subsequently stored at −20 C. DNA extraction from lyophilized material was by the large-scale method of Bainbridge et al (1).

Pathogenicity tests. Pathotype and race classifications of *F. o. ciceris* isolates were based on the differential reactions of 10 chickpea lines in replicated tests as previously described (10,11,13).

Primers. The primers used in this study are listed in Table 2. Primer KS is derived from the multiple cloning site of the phagemid pBluescript (Stratagene, Cambridge, U.K.). Primers P2 and P6 are derived from conserved and semiconserved sequences of the intergenic spacer (IGS) region of the ribosomal RNA gene complexes of *Penicillium hordei* Stolk and *Aspergillus fumigatus* Fresen. and were originally part of an analysis to amplify species-specific DNA for detection purposes (22,29). Primers P2 and P6 were synthesized at the Molecular Biology Unit, King's College, London.

Amplification conditions. Conditions were modified slightly from those of Williams et al (36). All reactions (100 μl) consisted of 0.5 μM of primer, 200 μM of deoxynucleotide triphosphates, 10 μl of 10× reaction buffer, 2 U of *Taq* DNA polymerase (Promega, Southampton, U.K.), 1.5 mM $MgCl_2$, and 100 ng of fungal DNA. Reactions were performed on a TR2 thermal cycler (Hybaid Ltd., Teddington, U.K.).

A hot-start PCR procedure was used for amplification. The conditions were as follows: denaturation at 94 C for 5 min followed by a "hold" step at 72 C to allow addition of *Taq* DNA polymerase; 30 cycles of 1 min at the appropriate annealing temperature (Table 2); extension for 3 min at 72 C; and denaturation for 1 min at 94 C. The final cycle consisted of 1 min of annealing followed by 6 min at 72 C to produce fully double-stranded fragments. A 20-μl volume of each reaction was analyzed on a 2% agarose (Sigma, St. Louis, MO) gel run at 1.5 V cm^{-1} overnight in 1× Tris-acetate–EDTA buffer (40 mM Tris-acetate and 1 mM EDTA) and stained with ethidium bromide (26). The DNA marker used for electrophoresis was the 1-kb ladder (GIBCO-BRL, Inchinnen,

TABLE 1. Isolates used in this study with reference number, origin, and race as determined by differential reactions of chickpea lines (D.C.) and classification of pathotype by random amplified polymorphic DNA (RAPD) analysis

Isolate number	Origin	Race by D.C.	Pathotype by RAPDs
Fusarium oxysporum f. sp. *ciceris*			
7802	Spain	0	Yellowing
7952	Spain	0	Yellowing
8207	Spain	0	Yellowing
8250[a]	Spain	...[b]	Nonpathogenic
8310	Spain	0	Yellowing
8401	Spain	0	Yellowing
7932	Spain	0	Yellowing
7982	Spain	0	Yellowing
82108	Spain	0	Yellowing
8733	Spain	0	Yellowing
USA 3-1(JG62)	United States	1B	Yellowing
1987-W17	United States	1C	Yellowing
8012	Spain	5	Wilt
8408	Spain	5	Wilt
7989	India	1A	Wilt
8605	India	2	Wilt
8606	India	3	Wilt
8607	India	4	Wilt
8272	Spain	6	Wilt
1987-W6-1	United States	5	Wilt
8317	Spain	0	Yellowing (W)[c]
8413	Spain	0	Yellowing (W)
8415	Spain	0	Yellowing (W)
82113	Spain	0	Yellowing (W)
82115	Spain	0	Yellowing (W)
1987B	United States	0	Yellowing (W)
9018(JG62)	Spain	0	Yellowing (W)
9018(PV1)	Spain	0	Yellowing (W)
90111(JG62)	Spain	0	Yellowing (W)
8717	Spain	0	Yellowing (W)
8257	Spain	5	Wilt (W)
8508	Spain	5	Wilt (W)
9026(JG62)	Spain	6	Wilt (W)
9093(PV1)	Spain	6	Wilt (W)
USA 1-1(JG62)	United States	5	Wilt (W)
USA 1633-2R1	United States	1A	Wilt (W)
1992R1N	India	1A	Wilt (W)
1992R2N	India	2	Wilt (W)
1992R3N	India	3	Wilt (W)
F. o. ciceris (*continued*)			
1992R4N	India	4	Wilt (W)
1987T	United States	6	Wilt (W)
8720	Spain	6	Wilt (W)
8601	Spain	0	Yellowing (U)[d]
8602	Spain	0	Yellowing (U)
8604	Spain	0	Yellowing (U)
8901	Spain	0	Yellowing (U)
8911	Spain	0	Yellowing (U)
8912	Spain	0	Yellowing (U)
8913(PV13)	Spain	0	Yellowing (U)
8914(PV13)	Spain	0	Yellowing (U)
91114	Spain	0	Yellowing (U)
91105	Spain	0	Yellowing (U)
91100	Spain	0	Yellowing (U)
91108	Spain	0	Yellowing (U)
9198	Spain	0	Yellowing (U)
8924	Spain	6	Wilt (U)
8905	Spain	6	Wilt (U)
9164	Spain	6	Wilt (U)
9168	Morocco	1A	Wilt (U)
9166	Morocco	1A	Wilt (U)
9020	Spain	6	Wilt (U)
9023	Spain	6	Wilt (U)
9035	Spain	5	Wilt (U)
F. o. melonis			
FOM VAL	Spain	...	...
1168[e]	Cyprus	...	...
1173[e]	Cyprus	...	...
1176[e]	Cyprus	...	...
1178[e]	Cyprus	...	...
1225[e]	Cyprus	...	...
F. o. niveum			
1167[e]	Cyprus	...	...
8527	Spain	...	...
F. solani			
8013	Spain	...	...
F. eumartii			
8017	Spain	...	...
Ascochyta rabiei			
8503	Spain	...	...

[a] Originally classified as race 0, yellowing-inducing (2).
[b] Tests not performed.
[c] Pathotype was withheld in a blind trial.
[d] Pathotype was assessed by RAPD analysis prior to host differential tests.
[e] From the culture collection of C. A. Poullis, Department of Agriculture, Nicosia, Cyprus.

Scotland).

Analysis of RAPD bands. RAPDs generated by single primer PCR were used to compare relatedness of isolates. For each isolate, a data record was constructed in which each band of a particular molecular weight, as generated by each primer, was represented as either being present, "1," or absent, "0." A binary matrix combined all the data records for all 74 isolates employed in this investigation from all three primers. The numerical taxonomy software package NTSYS 1.80 (23) was used to order the isolates by an unweighted paired group method with arithmetic averages (UPGMA) based on Jaccard's similarity coefficient (28). A principal coordinate analysis was performed by the PCOORDA routine, and the results of the first three coordinates were plotted three dimensionally to show the relatedness of the isolates.

RESULTS

Pathotype analysis. The race classifications for the *F. o. ciceris* isolates are given in Table 1. DNA isolated from the 74 fungal isolates was analyzed for RAPD patterns with the three primers. Sixty-three isolates of *F. o. ciceris* were analyzed along with representative isolates of *F. o. melonis* W. C. Snyder & H. N. Hans., *F. o. niveum* (E. F. Sm.) W. C. Snyder & H. N. Hans., *F. solani* (Mart.) Sacc., *F. eumartii* C. Carpenter, and *Ascochyta rabiei* (Pass.) Labrousse. The primers used in this study are given in Table 2 together with the percentage of G+C content and annealing temperatures. All reactions were repeated at least three times and always included negative controls (no template DNA). No amplified bands were observed in any of the control reactions.

Twenty informative amplified DNA bands were generated from isolates of *F. o. ciceris* employing primer P2; but with the inclusion of other species and formae speciales, this number rose to 46 (Fig. 1). Primer P6 generated 35 informative bands in total, and primer KS generated 27 overall. The five other pBluescript primers (SK, T3, T7, −20, and Reverse) were assessed for their suitability in generating RAPDs. Only a few nonpolymorphic products were amplified (data not shown), and these were not used further. Only bands between 0.2 and 4.0 kb that were consistently amplified in replicate experiments were included in the computer analysis. Variation in intensity was observed with some bands in replicate experiments, but this had no bearing upon the computer analysis. The only factor considered was the presence or absence of any particular band.

TABLE 2. Primers used in this study, annealing temperatures, and the number of informative random amplified polymorphic DNA (RAPD) bands generated for all isolates

Primer	Sequence 5′-3′	Annealing temperature (C)	RAPD bands
KS	CGAGGTCGACGGTATCG	40	27
P2	CACCGCCCCAAAATGGCCAC	45	46
P6	GTCCTCAGTCCCCCAATCCC	45	35

Amplification with primer P2 gave rise to a band of 1.3 kb (Fig. 1) in all race 0 isolates (which cause the yellowing syndrome), except isolate 8250 (not shown). In addition, a band of 0.55 kb was found to be present in all *F. oxysporum* isolates but was absent in *F. solani, F. eumartii,* and *A. rabiei* (Fig. 1). With primer KS (Fig. 2), two bands of approximately 1.6 kb were observed in wilt-inducing isolates (races 1A, 2, 3, 4, 5, and 6) of *F. o. ciceris,* but only one was seen in the yellowing-inducing isolates of this forma specialis (except isolate 8250, which had no bands of this size). Primer P6 generated a band of 1.05 kb in the yellowing-inducing isolates (except isolate 8250), which was absent in all wilt-inducing isolates (Fig. 3). No bands specific to geographical origin were observed. A few polymorphic bands specific to individual isolates were observed, most notably the 1.15-kb band generated from *F. o. ciceris* isolate 7802 with primer KS (Fig. 2).

UPGMA analysis of the RAPDs separated the isolates into three distinct clusters (Fig. 4). All the isolates of *F. o. ciceris* races 0, 1B, and 1C constituted one cluster. Isolates 3-1(JG62) and 1987-W17 (races 1B and 1C) were grouped together within the yellowing-inducing isolate cluster. Isolate 8250 was very dissimilar to all other *F. o. ciceris* isolates examined. All the wilt-inducing isolates of *F. o. ciceris* formed another cluster, consisting of races 1A, 2, 3, 4, 5, and 6. Eleven other isolates in this study, including isolates of *F. o. melonis, F. o. niveum, F. solani, F. eumartii,* and *A. rabiei*, formed a loose, dissimilar third grouping, distinct from *F. o. ciceris. A. rabiei* was the most dissimilar of all.

Principal coordinate analysis of the RAPD data (Fig. 5) also separated the two different pathotypes (i.e., wilting and yellowing) of *F. o. ciceris* into two distinct groups; only isolate 8250 was not included. All the other isolates in the study were clearly distinct from the two major groups. In addition, isolates 8605 and 1992R2N (race 2), 8606 and 1992R3N (race 3), and 8607 and 1992R4N (race 4), all from India, formed a distinct subgroup

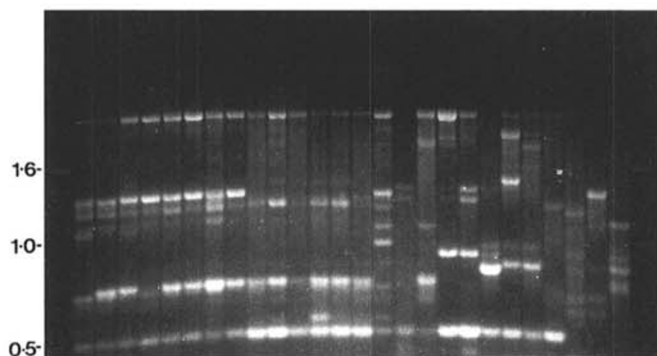

Fig 1. Random amplified polymorphic DNA analysis with primer P2 (5′-CACCGCCCCAAAATGGCCAC-3′). DNA was fractionated on a 2% agarose gel and stained with ethidium bromide. Numbers on the side are sizes in kilobase pairs. Lanes 1 and 28 are the 1-kb DNA ladder (GIBCO-BRL). *Fusarium oxysporum* f. sp. *ciceris* isolates (race and pathotype are given in parentheses): lane 2, 7932 (0, Y); lane 3, 7982 (0, Y); lane 4, 8733 (0, Y); lane 5, 82108 (0, Y); lane 6, 1987-B (0, Y); lane 7, 9018(JG62) (0, Y); lane 8, 9018(PV1) (0, Y); lane 9, 90111 (0, Y); lane 10, 9020 (6, W); lane 11, 9023 (6, W); lane 12, 9035 (5, W); lane 13, 9164 (6, W); lane 14, 9166 (1A, W); lane 15, 9168 (1A, W); lane 16, 9198 (0, Y). *F. o. melonis* isolates: lane 17, FOM VAL; lane 18, 1168; lane 19, 1173; lane 20, 1176; lane 21, 1178; lane 22, 1225. *F. o. niveum* isolates: lane 23, 1167; lane 24, 8527. Lane 25, *F. eumartii* 8013; lane 26, *F. solani* 8017; and lane 27, *Ascochyta rabiei* 8503.

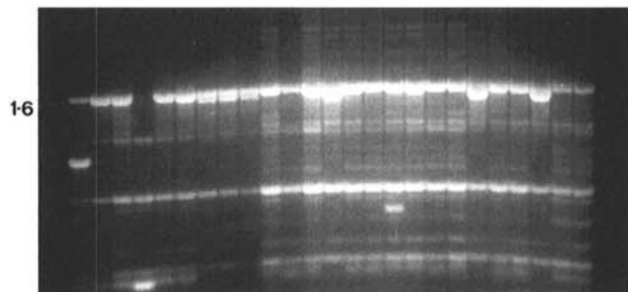

Fig 2. Random amplified polymorphic DNA analysis with primer KS (5′-CGAGGTCGACGGTATCG-3′). DNA was fractionated on a 2% agarose gel and stained with ethidium bromide. Number on the side is the size in kilobase pairs. Lanes 1 and 28 are the 1-kb DNA ladder (GIBCO-BRL). *Fusarium oxysporum* f. sp. *ciceris* isolates (race and pathotype are given in parentheses): lane 2, 7802 (0, Y); lane 3, 7952 (0, Y); lane 4, 8207 (0, Y); lane 5, 8250 (nonpathogenic); lane 6, 8310 (0, Y); lane 7, 8401 (0, Y); lane 8, 8012 (5, W); lane 9, 8408 (5, W); lane 10, 8257 (5, W); lane 11, 8317 (0, Y); lane 12, 8413 (0, Y); lane 13, 8415 (0, Y); lane 14, 8508 (5, W); lane 15, 82113 (0, Y); lane 16, 82115 (0, Y); lane 17, 8601 (0, Y); lane 18, 8602 (0, Y); lane 19, 8604 (0, Y); lane 20, 8901 (0, Y); lane 21, 8905 (6, W); lane 22, 8911 (0, Y); lane 23, 8912 (0, Y); lane 24, 8924 (6, W); lane 25, 8913 (0, Y); lane 26, 8914 (0, Y); and lane 27, control.

within the wilt-inducing isolates. Isolates 3-1(JG62) and 1987-W17 (races 1B and 1C) could be distinguished from the other yellowing-inducing isolates (Fig. 5).

Twenty-two isolates of *F. o. ciceris* (labeled "W" in Table 1) were assigned to putative pathotypes in a blind trial in London. The pathotypes of these isolates had previously been characterized in Cordoba by the differential reactions of chickpea lines, but the results had been withheld. For example, isolates 82113 and 82115, which exhibited very similar RAPDs to yellowing-inducing isolates with the three primers being used, were correctly assigned to this group. Similarly, isolates 8257, 8905, and 8924 had similar RAPDs to wilt-inducing isolates of *F. o. ciceris* and were correctly assigned. Twenty-one previously uncharacterized isolates (labeled "U" in Table 1) were each putatively assigned a pathotype in London by RAPDs alone prior to subsequent chickpea differential line tests in Cordoba, which in every case proved the pathotype assignment based upon RAPD data to be correct.

DISCUSSION

The aim of this study was to determine whether races or pathotypes of *F. o. ciceris* could be distinguished by RAPD analysis. Using single primers in a PCR-based reaction, we found it possible to separate isolates of *F. o. ciceris* into one of two groups (Figs. 4 and 5). These groupings correlated well with the disease-inducing characteristics of isolates (pathotypes) as previously determined by pathogenicity tests with chickpea differential lines. It was also possible to place previously untested isolates into one of the two groups by RAPD analysis alone, the reliability of this approach having been demonstrated in the blind trials. Isolates 8717, 91100, 91105, 91108, 91114, and 9198 had been tentatively assigned to the yellowing-inducing group of *F. o. ciceris*, and 8720, 9020, 9023, 9035, 9164, 9166, and 9168 appeared to be wilt-inducing isolates. These placements were subsequently shown to be correct in every case by pathogenicity tests with chickpea differential lines.

Cluster analysis of the RAPD data placed the *F. o. ciceris* isolates into two groups (pathotypes) clearly distinct from other formae speciales and from other fungi examined. The similarity values of 11–18% found between *F. solani* and *F. oxysporum* isolates in this study (Fig. 4) are compatible with that of 20% between *F. solani* and *F. oxysporum* found by Szécsi and Dobrovolsky (30), who used DNA thermal denaturation profiles.

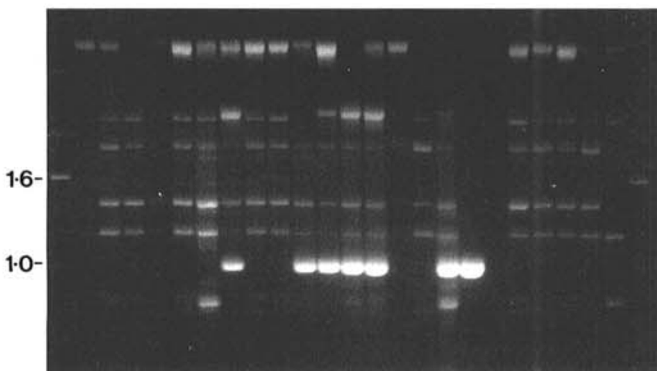

Fig 3. Random amplified polymorphic DNA analysis with primer P6 (5′-GTCCTCAGTCCCCCAATCCC-3′). DNA was fractionated on a 2% agarose gel and stained with ethidium bromide. Numbers on the side are sizes in kilobase pairs. Lanes 1 and 25 are the 1-kb DNA ladder (GIBCO-BRL). *Fusarium oxysporum* f. sp. *ciceris* isolates (race and pathotype are given in parentheses): lane 2, 8272 (6, W); lane 3, 9026 (6, W); lane 4, 9093 (6, W); lane 5,1-1(JG62) (5, W); lane 6, 1987-W6-1 (5, W); lane 7, 1633-2R1 (1A, W); lane 8, 8717 (0, Y); lane 9, 8720 (6, W); lane 10, 1987-T (6, W); lane 11, 91100 (0, Y); lane 12, 91105 (0, Y); lane 13, 91108 (0, Y); lane 14, 91114 (0, Y); lane 15, 7989 (1A, W); lane 16, 1992R1N (1A, W); lane 17, 3-1(JG62) (1B, Y); lane 18, 1987-W17 (1C, Y); lane 19, 8605 (2, W); lane 20, 1992R2N (2, W); lane 21, 8606 (3, W); lane 22, 1992R3N (3, W); lane 23, 8607 (4, W); and lane 24, 1992R4N (4, W).

Yellowing-inducing isolates (races 0, 1B, and 1C) appear to form a more homogeneous group than do the wilt-inducing isolates (races 1A, 2, 3, 4, 5, and 6).

Race 0 isolates, found mainly in Spain and the Mediterranean region, may well have become recently adapted to infect the local *kabuli* variety of chickpeas. In contrast, nearly all *desi* chickpea cultivars (the Indian subcontinent variety) are resistant to race 0 isolates (13,14). The relative similarity between the two groups of isolates within *F. o. ciceris*, together with the lack of polymorphisms within the mitochondrial DNA of races of *F. o. ciceris* (21), probably rules out the possibility of two different ancestors for this forma specialis.

One isolate, 8250, appeared to be quite dissimilar to all other *F. o. ciceris* isolates (Figs. 4 and 5). When originally tested for pathogenicity, this isolate was classified as a race 0 yellowing isolate (2), but it was among the least virulent of all race 0 isolates tested, with disease index scores significantly lower than other race 0 isolates. This isolate was first cultured in 1982 and was maintained on potato-dextrose agar for at least 6 yr by routine mass culture before being maintained on sterile sand. However, a recent repeat pathogenicity test by standardized procedures (13), with isolate 8250 on susceptible chickpea lines over a period of 45 days, revealed neither yellowing symptoms nor infection of the plant at all (Jiménez-Díaz et al, *unpublished results*), and therefore this isolate at present is clearly nonpathogenic to chickpea cultivars. It is possible that a loss in pathogenicity can arise as a result of long periods of mass culture, as has been observed in several *Fusaria* (19), but the repeat pathogenicity tests coupled with the absence of RAPD markers for yellowing-inducing isolates suggest that this isolate may have been originally misclassified.

With RAPD analysis, it was also possible to distinguish *F. o. ciceris* from other formae speciales, other *Fusarium* species, and *A. rabiei*. Because *F. solani* and *F. eumartii* are also soilborne pathogens of chickpea and often occur together with *F. o. ciceris* (12,31), an analysis that can clearly distinguish between fungi likely to be present in the same host or environment is obviously an advantage.

The above findings show that RAPD analysis provides a rapid method of differentiating between isolates of the yellowing- and wilt-inducing pathotypes of *F. o. ciceris* and between nonpathogenic isolates, other formae speciales, and other fungi. The distinguishing bands were reproducible in repeated experiments. In replicate amplifications, variation in the intensity of some of the bands was observed, but the number of bands did not change. This variation may be the result of minor differences in the quality of the DNA preparations. Variation in intensity between isolates could arise as a result of greater homology between the primer and the annealing sites in some isolates, resulting in more efficient amplification. Variation in intensity may also be the result of differences in copy number of the target sites or even variations in temperature in the wells of the thermal cycler.

Two of the primers (IGS P2 and P6) were selected from repetitive DNA sequences. Variation in the IGS of the ribosomal DNA complex region has been observed in other fungi (33) and may be responsible for some of the differences observed, although in comparisons of other formae speciales of *F. oxysporum* no variation in ribosomal DNA RFLPs has been observed (15). In addition, use of primers derived from the IGS region of *P. hordei* does not necessarily mean that the PCR products in the present study have been generated from the IGS region, but this can not be verified without further study that includes the use of homologous ribosomal DNA probes and/or the sequencing of the RAPD bands in question. Further studies will also be required to establish the basis for polymorphisms generated by the pBluescript primer KS. It is possible that the products may be derived from coding regions of the genome or from regulatory elements, such as enhancers or promoters, although no significant homology has been found between the primers used and known filamentous fungal regulatory elements (9; A. Kelly, *unpublished data*). Although the primers distinguished between the two pathotypes of *F. o. ciceris* on the basis of common bands, it

Fig. 4. Dendrogram derived from random amplified polymorphic DNA analysis of *Fusarium oxysporum* f. sp. *ciceris* and other fungi by UPGMA (unweighted paired group method with arithmetic averages). The top scale is the percentage of similarity by Jaccard's similarity coefficient. Isolates are as given in Table 1. * = Isolate 8250. Cophenetic correlation $r = 0.98$.

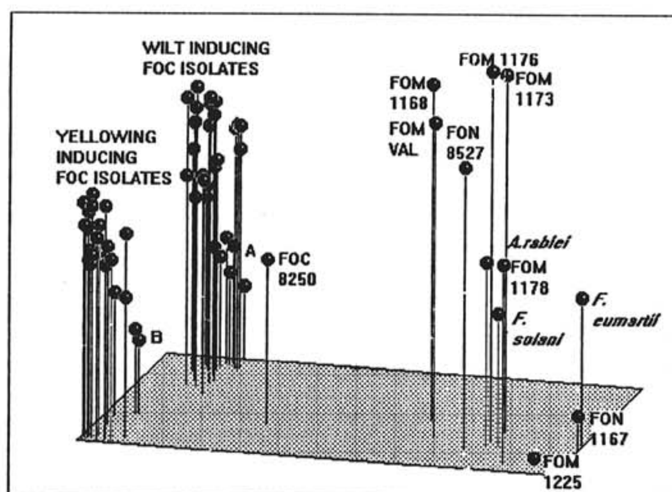

Fig. 5. Three-dimensional display generated by NTSYS of principal coordinates based on 108 random amplified polymorphic DNA bands generated by primers KS, P2, and P6. The relatedness of 63 isolates of *Fusarium oxysporum* f. sp. *ciceris* and 11 other fungal isolates is shown.

is not known to what degree such bands are homologous; it is known only that they are approximately the same size. The DNA bands specific to both of the disease types of *F. o. ciceris* (i.e., wilting and yellowing) have been cloned and sequenced (A. Kelly, B. W. Bainbridge, and J. B. Heale, *unpublished data*) and will be assessed as to their suitability for the in planta or soil detection of this pathogen, either by hybridization or in a specific PCR amplification, as has been achieved for some species of *Verticillium* (18).

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