Mating Type, Pathotype and RAPDs Analysis in *Didymella rabiei*, the Agent of Ascochyta Blight of Chickpea

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Eleven pathotype groups (A-K), including five not previously reported, of *Didymella rabiei* (anamorph *Ascochyta rabiei*), representing isolates of the pathogen from Ascochyta blight-affected chickpeas mainly from India, Pakistan, Spain and the USA, were characterized using 44 single-spore isolates tested against seven differential chickpea lines. Of 48 isolates tested for mating type, 58% belonged to MAT 1-1 and 42% to MAT 1-2. Thirty-nine *D. rabiei* isolates, as well as two isolates of *Ascochyta pisi* and six isolates of unrelated fungi, were analyzed using Randomly Amplified Polymorphic DNAs (RAPDs) employing five primers (P2 at 40°C, and OPA3, OPC1, OPC11 and OPC20 at 35°C). Computer cluster analysis (UPGMA / NTSYS-PC) detected a relatively low level of polymorphism among all the *D. rabiei* isolates, although at *ca* 7% dissimilarity, *ca* 10 RAPD groups [I-X] were demarcated, as well as subclustering within the larger groups. By the same criteria, the maximum dissimilarity for the whole population of *D. rabiei* isolates was *ca* 13%. No correlation was found between different RAPD groups, pathotype, or mating type of *D. rabiei*, although some evidence of clustering based on geographic origin was detected. The use of RAPDs enabled us to identify specific DNA fragments that may have a potential use as genetic markers in sexual crosses, but none which could be used as virulence markers.
KEY WORDS: *Ascochyta rabiei*; teleomorphic stage; genetic diversity; *Cicer arietinum*.

INTRODUCTION

Ascochyta blight, a rapidly spreading stem, leaf and pod necrotic disease caused by *Ascochyta rabiei* (Pass.) Labrousse [teleomorph: *Didymella rabiei* (Kovachevski) v. Arx], can devastate chickpea (*Cicer arietinum* L.), in West Asia, North Africa, South and West Europe, the Indian Subcontinent, and the Palouse region of eastern Washington and northern Idaho (USA), when cool and wet weather occurs during the growing season (9,11,19,27). The pathogen can infect all above-ground plant parts at any stage of crop development, leading to rapid tissue collapse and spreading necrotic lesions. Yield losses may reach 100% if environmental conditions, particularly rainfall, favor development of severe disease (28). Furthermore, Ascochyta blight is the main limitation for the chickpea autumn–winter sowing technology developed by ICARDA (International Centre for Agricultural Research in the Dry Areas), which otherwise has resulted in significant

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increases in yield (26,27). The use of resistant cultivars is the most effective and economical strategy for control of Ascochyta blight (19); however, its effectiveness is limited by considerable variation in pathogenicity detected in the fungal population.

Pathogenic variability in *D. rabiei* was first reported in India (32). Subsequently, five pathotypes were identified in Pakistan (22) and six in Syria and Lebanon (termed 'races' by Reddy and Kabbabeh, ref. 24). Eleven pathotypes have been reported from the Palouse region of the USA (7). Recently, work in Italy identified three virulence groups among isolates of the pathogen from that country (21). Pathotypes can be characterized by differences in the amount of disease (differential virulence) caused on a set of differential host genotypes in artificial inoculation experiments employing single-spore isolates of the pathogen. Such biological pathotyping is laborious, time-consuming and requires strict standardization of experimental conditions. In addition to variation in pathogenicity, *D. rabiei* shows considerable variability in morphological and physiological traits (4,8,32) and in phytotoxin production (1,6). Therefore, morphological, chemical and/or pathogenicity characteristics of the pathogen are not satisfactory for identification.

In recent years DNA polymorphisms have been used increasingly to complement traditional markers in the analysis of genetic identity, variability and relatedness in fungi. DNA fingerprinting with synthetic, tandem-repetitive, oligonucleotide probes, discriminated among six isolates of *D. rabiei* representative of six pathotypes from Syria (33). With this same technique, Morjane *et al.* (16) demonstrated the genetic heterogeneity of *D. rabiei* within a single chickpea field, and a clustered distribution of predominant haplotypes suggesting a rainsplash dispersal of the fungus. Analysis of DNA polymorphisms using primers of randomly chosen sequence by means of Polymerase Chain Reaction (PCR) resulting in Randomly Amplified Polymorphic DNA (RAPD), was employed by Fischer *et al.* (3). They obtained a series of unique RAPD band patterns which could be assigned to one of each of 30 different *D. rabiei* isolates from Italy, respectively, but no correlation between RAPD patterns and pathotype groups was detected.

D. rabiei is heterothallic, with a bipolar, biallelic, mating incompatibility system (34). Kaiser (10) reported that fertile pseudothecia of this fungus developed on naturally infected debris from a number of chickpea-growing countries in North Africa, North America, West Asia, and East and West Europe, indicating the widespread distribution of the two mating types (MAT 1-1 and MAT 1-2) in nature. Thus, although conidia from infected seeds and infected shoot debris were originally thought to be the only source of primary inoculum in the field (19), ascospores have been reported on overwintering chickpea debris on the soil surface (17,30), and they also act as primary inoculum for the disease (31). However, the role of the teleomorphic stage in increasing genetic diversity has not been investigated in this pathogen, and McDonald *et al.* (14) have stressed that the analysis of genetic variation in sexually producing pathogen populations is an important prerequisite for understanding co-evolution in plant pathosystems.

The studies reported here were performed on isolates of the pathogen chosen to represent diverse geographic origins. We employed RAPDs using primers the sequence of which was based on known sequences, as well as random primers, to attempt to distinguish between different isolates of *D. rabiei*, and to identify any correlations with geographical origin, pathotype or mating type. In addition, data from RAPDs were used to analyze the population structure of the fungus in terms of its biodiversity.

MATERIALS AND METHODS

Fungal strains and growth conditions

Isolates were grown on 7% chickpea meal agar (CMA) medium (8). For inoculum production, monoconidial cultures were subcultured on CMA and incubated at 20°C with a 12 h photoperiod of fluorescent and near-ultraviolet light at 36 $\mu E.m^{-2}.s^{-1}$ for 10 days. To provide mycelia for DNA extraction, the isolates were subcultured in a liquid chickpea-sucrose medium and the same medium, solid, was used to obtain spore suspensions which were kept in 12% glycerol at −70°C.

Of a total of 48 available isolates of *D. rabiei* obtained from chickpea plants in nine countries, 44 were used for pathotyping in Cordoba, Spain, the remaining four isolates being untested for this character (Table 1). For RAPD analysis, 37 *D. rabiei* isolates, representing the same set of countries, were transferred to King's College, London, from Prof. R.M. Jiménez-Díaz's laboratory in Cordoba, in pieces of artificially infested, dried chickpea stems; four additional isolates were included, *viz.*, two *D. rabiei* isolates: IMI 331-919 and IMI 342-267, giving a total of 39 *D. rabiei* isolates, and two *Ascochyta pisi* Lib. isolates: IMI 137-796 and IMI 135-519, obtained from CAB-IMI (Surrey, UK). DNAs were also tested for RAPD analysis using six fungi unrelated to *D. rabiei*: *Foc*/7802 ['yellowing' pathotype] and *Foc*/8012 ['wilting' pathotype] isolates of *Fusarium oxysporum* Schlechtend.:Fr. f.sp. *ciceris* (Padwick) Matuo & Sato [*Foc*] from chickpea (13); Fm/346: *Fusarium moniliforme* J. Sheld; Af54: *Aspergillus flavus* Link:Fr.; VG10: *Verticillium longisporum* comb. nov., a near-diploid, hybrid species of *Verticillium*, recently renamed by Karapapa *et al.* (12), from oilseed rape in Germany; and V531: *V. albo-atrum* Reinke & Berthold from hop (5) (a PV2 'progressive wilt' strain from Kent, UK), totaling 47 isolates in all (Fig. 1).

Pathogenicity tests

Pathotype characterization of 44 *D. rabiei* isolates (Table 1) was carried out at Cordoba, Spain, based on the differential reactions of seven chickpea differential lines: ILC-1929, C-235, ILC-249, ICC-1903, ILC-72, ICC-3996 and ILC-3279, the susceptible local ecotype: PV-60, serving as control. Five of these lines (ILC-1929, ILC-249, ICC-1903, ICC-3996 and ILC-3279) were common with five of six lines used by Reddy and Kabbabeh (24) in their differential tests. They included F-8 which was not employed in the present study, but excluded C-235 and ILC-72 used here, which resulted in characterization of six different races of *D. rabiei* in Syria and Lebanon. Plants at the seedling stage (five–seven expanded leaves) were sprayed to incipient run-off with a 5×10^5 conidia/ml suspension from monoconidial isolates using an airbrush sprayer (Badger Airbrush Co., Illinois, USA) operated at 0.1 MPA. There were three replicated pots (four plants per pot) per isolate, incubated in moist chambers (saturated atmosphere) and darkness for 45 h. After the wetness period, plants were maintained in a growth chamber adjusted to 75–100% r.h., 20±1°C, and a 12 h photoperiod of fluorescent light of 252 $\mu E.m^{-2}.s^{-1}$. Disease severity was recorded 7 and 14 days after inoculation by means of a 1–10 rating scale (1=no symptoms; 10=dead plant), and class values were converted to percentage of affected green tissue (29). Values $\leq$45% and >45% of affected tissue were considered resistant and susceptible reactions, respectively (20).

Mating type

Forty-eight *D. rabiei* isolates were characterized for mating type in Cordoba (Table 1). Stem pieces from healthy chickpea plants, cv. Blanco Lechoso, were cut into 6-cm-long fragments and sterilized with propylene oxide. Sterilized fragments were dipped for 30 min in a conidial suspension containing a mixture of 10^5 conidia/ml of the unknown isolate, and either of the two mating-type tester isolates of *D. rabiei* (30,34) (ATCC-76501, MAT 1-1; and ATCC-76502, MAT 1-2). Infected stem pieces were incubated under moist conditions at 8°C in the dark for 40–50 days. Sexual compatibility was determined by the development of mature pseudothecia of the fungus and discharge of viable ascospores on water agar (18,30).

DNA extractions

DNA extraction was based on the method of Raeder and Broda (23) adapted to micro-scale conditions. All extraction, subsequent RAPDs and computer analyses were performed in London.

RAPDs

The reaction conditions for PCR using a TR2 (Hybaid) Thermocycler were: 200 μM dNTPs, 0.5 μM single primer, 50 ng template DNA, and 2 Units Taq DNA polymerase (Promega) in a 50 μl reaction. There were 30 cycles of the following: denaturation for 1 min at 94°C, annealing for 1 min at 35–45°C (depending on the primer), and extension for 3 min at 72°C, followed by a final extension step of 6 min at 72°C to complete DNA synthesis. Products were visualized under UV light on a 1.8% agarose gel after electrophoresis at 1–3 Vcm^{-1}. The primers used initially were: [1] P2 (5'-3' CACCGCCCCAAAATGGCCAC); [2] P6 (GTCCTCAGTCCCCCAATCCC) from the intergenic spacer region (IGS) of the ribosomal RNA complex of *Penicillium hordei* Stolk; [3] KS (CGAGGTCGACGGTATCG) derived from the pBluescript^{TM} (Stratagene) sequencing primer; and [4] 40 synthetic 10-mer oligonucleotides corresponding to OPA and OPC primer sets (Operon). Primers P2, P6 and KS were used initially because in previous research they generated DNA bands which are specific for the 'yellowing' and 'wilting' pathotypes of *Foc* (13). Finally, five primers were selected based upon the resulting maximum number of discriminating DNA bands: P2, OPA3, OPC1, OPC11 and OPC20.

Computer analysis of data

Data from RAPDs were analyzed using the UPGMA method with Jaccard's similarity coefficient (2), which disregards negative matches between pairs of isolates and provides a more accurate picture of relatedness between isolates. The analysis was performed using the NTSYS-PC software package (25). The data were collated and converted into binary form on the basis that 1 was ascribed to an isolate if a band of a specific molecular weight was present, and 0 if it was absent.

RESULTS

Pathogenic variability

The disease reactions of seven chickpea differential lines to 44 selected, monoconidial isolates of *D. rabiei* indicated that the isolates could be classified into 11 pathotype groups, *viz.*, A to K (Tables 1, 2). In Table 1, isolates of *D. rabiei* were also classified into races, tentatively, by comparing disease reactions in our study with those obtained by Reddy and Kabbabeh on a set of six differential lines (24). However, we used only five of these six differential lines. Group A, characterized by a low level of virulence, included 19 isolates and was the largest and most widely distributed group. Isolate 9185 (group B), which was pathogenic to C-235, could be considered alternatively as a variant within group A, rather than being placed in a separate group. Group C included seven isolates from Spain, three from the USA and one from Pakistan that were pathogenic to ILC-1929. Isolates 8930 and 9109 (group D), 9183 (group E), and 8967, 8970, 9008 and 9186 (group F) all closely resemble group C, except for an increased virulence to one of the three differential chickpea lines: C-235, ILC-249 and ILC-1903, respectively (Table 1).

TABLE 1. Pathotype (race[z]) groups characterized by testing seven differential chickpea lines separately against each of 44 isolates of *Didymella rabiei*

Differential chickpea lines							Pathotype group (race)	Number of isolates
ILC 1929	C 235	ILC 249	ICC 1903	ILC 72	ICC 3996	ILC 3279		
R[y]	R	R	R	R	R	R	A	19
R	S	R	R	R	R	R	B	1
S	R	R	R	R	R	R	C (1?)	11
S	S	R	R	R	R	R	D (2?)	2
S	R	S	R	R	R	R	E	1
S	R	R	S	R	R	R	F (3a)	4
S	S	R	S	R	R	R	G (3b)	2
S	S	R	R	R	S	R	H	1
S	S	S	R	R	R	R	I	1
S	R	S	S	R	R	R	J (4a)	1
S	S	S	S	S	R	R	K (4b)	1

[z]Race identifications (1–4, in parentheses) were designated using data for reactions of five race differential chickpea lines (ILC-1929, ILC-249, ICC-1903, ICC-3996 and ILC-3279) common to this study, and to those employed by Reddy and Kabbabeh (24) for *D. rabiei* isolates from Syria and Lebanon; susceptible local ecotype PV-60 served as control; pathotype groups A, B, E, H and I could not be assigned to race.

[y]R=Resistant, ≤45% of green tissue affected. S=Susceptible, >45% of green tissue affected.

Two of the isolates from Spain (9037, 9039), one from Pakistan (9007), and one from the USA (8627), were characterized as groups G, H and I, respectively. Isolates included in these groups were pathogenic to lines ILC-1929 and C-235, and additionally to ICC-1903 (group G), ICC-3996 (group H) or ILC-249 (group I), respectively.

Isolate 9163 from India, and 8948 from Spain, constituted groups J and K, respectively. Isolate 8948 was the most virulent of all those tested, and only ICC-3996 and ILC-3279 showed a resistant reaction to it.

TABLE 2. *Didymella rabiei* isolates (n=total 48, ranked by isolate ref. no.), geographical origin, pathotype group and mating type

Isolate no.	Origin	Pathotype group[z]	Mating type[y] (MAT)
8600	USA	C	1-1
8609	USA	C	1-2
8627	USA	I	1-1
8629	USA	C	1-2
8805	Spain	n.t.	1-2
8808	Spain	A	1-1
8811	Spain	C	1-1
8908	Spain	C	1-1
8930	Spain	D	1-2
8948	Spain	K	1-1
8961	Spain	C	1-2
8967	Pakistan	F	1-1
8969	Pakistan	A	1-1
8970	Pakistan	F	1-1
9001	Spain	A	1-2
9003	Pakistan	A	1-1
9004	Pakistan	A	1-1
9007	Pakistan	H	1-1
9008	Pakistan	F	1-1
9009	Pakistan	A	1-1
9011	Spain	n.t.	1-2
9016	Spain	n.t.	1-2
9017	Spain	n.t.	1-2
9032	Spain	C	1-1
9037	Spain	G	1-2
9038	Spain	C	1-1
9039	Spain	G	1-1
9108	Morocco	A	1-2
9109	Morocco	D	1-2
9113	USA	A	1-2
9114	USA	A	1-1
9115	Pakistan	A	1-1
9117	Turkey	A	1-1
9118	Pakistan	A	1-1
9119	Pakistan	C	1-1
9123	USA	A	1-2
9139	Italy	A	1-2
9142	Greece	A	1-2
9144	Greece	A	1-2
9161	India	A	1-1
9163	India	J	1-1
9167	Spain	C	1-2
9172	Spain	C	1-2
9174	Spain	A	1-2
9183	India	E	1-1
9185	India	B	1-1
9186	India	F	1-1
9188	France	A	1-1

[z] Pathotype groups (n=11, A–K) characterized by reactions of seven differential chickpea lines (Table 1) and susceptible local ecotype PV-60 as control, inoculated separately with 44 monoconidial isolates. Groups A, B, E, H and I not previously described.

[y] By crossing each of 48 isolates separately with two testers to distinguish between MAT 1-1 and MAT 1-2 (34, see text).

n.t., not tested.

TABLE 3. RAPD groups (I–X) for 39 *Didymella rabiei* isolates, along with mating type, pathotype group and geographic origin. Cluster analysis was performed using UPGMA with Jaccard's similarity coefficient, employing the NTSYS-PC numerical taxonomy and multivariant analysis system

Isolate no.	RAPDs group	Mating type (MAT)	Pathotype group	Geographic origin
8600	I	1-1	C	USA
9186	I	1-1	F	India
9188	I	1-1	A	France
9183	I	1-1	E	India
9109	I	1-2	D	Morocco
8908	I	1-1	C	Spain
9017	II	1-2	n.t.	Spain
9016	II	1-2	n.t.	Spain
9032	II	1-1	C	Spain
8805	II	1-2	n.t.	Spain
9172	II	1-2	C	Spain
8503	II	n.t.	n.t.	Spain
9008	III	1-1	F	Pakistan
9115	III	1-1	A	Pakistan
9123	III	1-2	A	USA
8967	III	1-1	F	Pakistan
9038	III	1-1	C	Spain
9009	III	1-1	A	Pakistan
9144	III	1-2	A	Greece
9001	III	1-2	A	Spain
9011	III	1-2	n.t.	Spain
9007	III	1-1	H	Pakistan
331-919	III	n.t.	n.t.	Pakistan
9163	III	1-1	J	India
8627	IV	1-1	I	USA
8930	IV	1-2	D	Spain
8629	V	1-2	C	USA
8811	V	1-1	C	Spain
8808	V	1-1	A	Spain
9161	V	1-1	A	India
342-267	V	n.t.	n.t.	India
8961	V	1-2	C	Spain
9108	VI	1-2	A	Morocco
8609	VII	1-2	C	USA
9142	VIII	1-2	A	Greece
9139	IX	1-2	A	Italy
9039	X	1-1	G	Spain
9117	X	1-1	A	Turkey
9037	X	1-2	G	Spain

n.t., not tested. The three *D. rabiei* isolates 8503, 331-919 and 342-267 included here in the RAPD cluster analysis were not listed in Table 2 and are additional ones. For pathotype group and distinction between MAT 1-1 and MAT 1-2 mating types, see text.

Mating type

Of the 48 isolates of *D. rabiei* tested for mating type, 58% belonged to MAT 1-1, and 42% to MAT 1-2 (Table 2). Both mating types were found in Spain and the USA. Of the 23 isolates from other countries, 78% belonged to MAT 1-1 and 22% to MAT 1-2. All isolates from India (five) and Pakistan (11) were exclusively of MAT 1-1. Isolates from Greece (two), Italy (one) and Morocco (two) were all of MAT 1-2.

TABLE 4. Primers employed in final RAPDs analysis for 39 *Didymella rabiei* isolates[z], aith the resulting differential band patterns and discriminating band sizes obtained

Primer	Number of bands	Differential pattern	Discriminating band size (bp)
P2	10-11	Presence/absence of 1 band	2100
OPA3	8	1 band at 6 different positions	1550-1880
OPC1	7	1 band at 2 different positions	830 or 870
OPC11	12-16	1-5 bands at 8 different positions	680-1035
OPC20	10-14	1-4 bands at 5 different positions	800-940

[z] See Table 3 for details of isolates.

RAPDs

A total of 39 *D. rabiei* isolates, 16 from Spain and 23 from other countries (which included three additional isolates: 8503 [transferred to King's College, London from the Institute of Sustainable Agriculture in Cordoba], and IMI 331-919 and IMI 342-267 – not listed in Table 2, but included in Table 3 and Fig.1), as well as two *A. pisi* isolates, were employed here. In preliminary tests, RAPDs were obtained using different single primers (P2 and P6 20-mers and the KS 17-mer) and a variety of conditions.

Reproducible band patterns were obtained at annealing temperatures of 37° and 40°C using 15 different DNAs corresponding to isolates belonging to the two mating types, and to three of the 11 different pathotypes (*i.e.*, groups C , F and G) of *D. rabiei*. In addition, 40 synthetic 10-mer oligonucleotides (Operon OPA and OPC sets) were screened (as primers) at 35°C using four *D. rabiei* DNAs, representing the two mating types and the same three pathotypes (see above). A selection of primers was made using those that resulted in clear differences in the band pattern obtained with different isolates. The analysis was repeated to test reproducibility and extended to 18 further *D. rabiei* isolates and one *A. pisi* isolate.

Five primers (P2 at 40°C, and OPA3, OPC1, OPC11 and OPC20 at 35°C) were finally selected, and the RAPDs analysis included DNA from all 39 isolates of *D. rabiei* (Table 3), from the two *A. pisi* isolates, as well as DNAs from the six unrelated fungi included (see Materials and Methods), totaling 47 isolates in all (Fig. 1). The five primers selected gave consistent and reproducible results with different PCR machines, and with DNAs extracted from different cultures, and at different times, from the same isolate. Some of the differential RAPD bands, obtained when five different primers were used (Table 4), consistently clustered certain *D. rabiei* isolates together. However, none of the

differential bands correlated with pathotype, or mating type. Nevertheless, clear and strong polymorphic bands could be detected by RAPDs, and more particularly, the 870 bp and 830 bp bands generated using OPC1 (Table 4) could be very useful as markers in future studies of segregation after genetic crosses in *D. rabiei*.

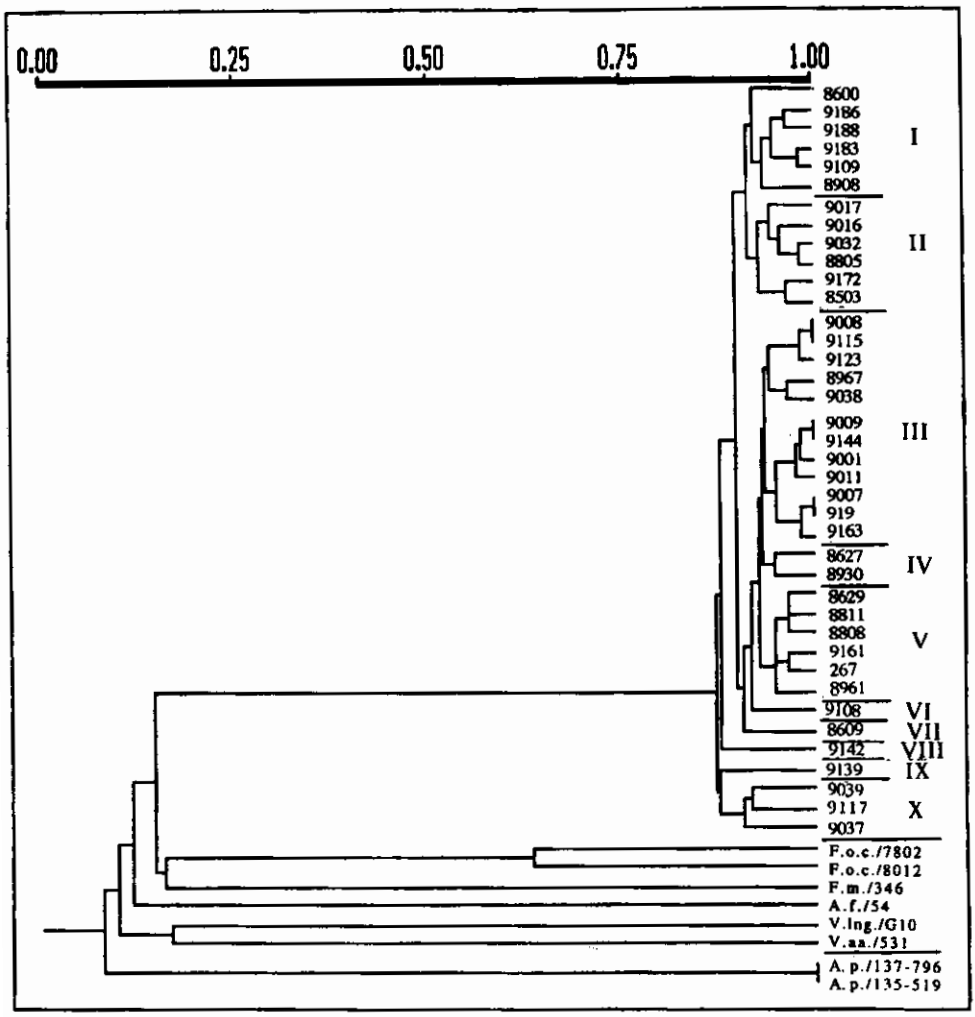

Fig. 1. Dendrogram produced from cluster analysis based on 172 RAPD bands for a total of 47 isolates including 39 isolates of *Didymella rabiei* (ranked in same order, *i.e.*, 8600–9037, groups I–X, as shown in Table 3), plus two of *A. pisi* (A.p./) and six unrelated fungi: F.o.c./7802 ['yellowing' pathotype] and F.o.c./8012 ['wilting' pathotype] isolates of *Fusarium oxysporum* f.sp. *ciceris* from chickpea; F.m./346, *Fusarium moniliforme*; A.f./54, *Aspergillus flavus;* V.Ing./G10, *Verticillium longisporum* comb. nov. (12) from oilseed rape; V.aa./531, *V. albo-atrum* from hop (a PV2 'progressive wilt' strain). Cluster analysis was performed using UPGMA with Jaccard's similarity coefficient, employing the NTSYS-PC numerical taxonomy and multivariant analysis system (numbers from 0.00–1.00 represent percent similarity).

Of the 39 *D. rabiei* isolates tested finally, only three apparent pairs (9008/9115, 9009/9144, 9007/919), all in the large RAPD group III, shared one of three different RAPD DNA band patterns, respectively. However, there was either contrary, or insufficient, data to infer clonality within these apparent pairs, and this possibility can probably be disregarded here given the widespread sampling procedures employed. The 33 other isolates of the same pathogen produced 33 uniquely different patterns to varying degrees (Fig. 1).

Computer analysis of RAPDs data

A binary data file was generated for a total of 172 RAPD bands from the 47 isolates (including partially related and unrelated fungi) included in the final analysis, using data derived from the use of the five selected primers (Table 4). The results are presented in Figure 1 as a dendrogram. The two *A. pisi* isolates grouped at a slightly greater distance from those of *D. rabiei* than isolates belonging to different genera, such as *Fusarium* or *Aspergillus*. As expected, the two *Foc* isolates were grouped together with the *F. moniliforme* isolate, but the isolate of the latter species was clearly separated from them. The two isolates producing wilt (*Foc*/8012) and yellowing (*Foc*/7802) symptoms, respectively, in chickpea, were much more heterogeneous (*ca* 36% dissimilarity) when compared with the maximum level of dissimilarity (*ca* 13%) detected here in the entire *D. rabiei* population. A relatively homogeneous population of *D. rabiei* was observed, but *ca* ten RAPD clusters/groups (I-X, with some evidence of subclusters in the larger groups: I, II, III and V) were detectable using the arbitrary figure of *ca* 7% dissimilarity (Fig. 1).

DISCUSSION

Pathogenic variability among *D. rabiei* isolates has been described in different countries (7,19,21,22,24,32). Nevertheless, the occurrence of large differences in the experimental procedures and chickpea differential lines employed makes it difficult to compare results from these various studies. Reddy and Kabbabeh (24) were able to distinguish six races within a group of 50 chickpea isolates from Lebanon and Syria on a set of six differential chickpea lines. If disease reactions in our study are compared with their data on the set of five differential lines common in both studies (Materials and Methods), our pathotype groups C and D could be designated race 1 and 2, respectively; groups F and G as race 3a and 3b, respectively; and groups J and K as race 4a and 4b, respectively. However, groups A, B, E, H and I detected here appear to represent pathotypes not previously described (21,24). This is most likely to be due to variation in the sets of differential host cultivars employed as compared with those in the present study.

The distribution of mating types suggested by our results agrees in most cases with that recently shown by Kaiser (10) using a larger sample of isolates. Thus, it appears that MAT 1-1 is the prevalent mating type in India and Pakistan. Isolates of *D. rabiei* from Greece, Italy and Morocco were all of MAT 1-2; however, no conclusion can be drawn regarding mating type prevalence in those countries because of the low number of isolates from them used in the study. Although results for mating type suggest that there could be a slight positive selection in some areas for survival of one mating type over the other, much more evidence is needed if we are to substantiate this conclusion. Although conidia from infected seeds and shoot debris were thought to be the only source of primary inoculum in the field (19), the ascospore-producing sexual stage has now been described in several countries, and there are reports on overwintering on chickpea

debris (10,17) and of ascospores also acting as primary inoculum (30,31). The role of the teleomorphic stage here in increasing genetic diversity leading to novel pathotypes has not yet been investigated in this pathogen, and McDonald *et al.* (14) have stressed that the analysis of genetic variation in plant–pathogen populations is an important prerequisite for understanding co-evolution in plant pathosystems. Because conidia of *D. rabiei* are dispersed by rainsplash in the field (19), it is likely that clusters of identical haplotypes (clones) will develop at infection foci in the dense monoculture of the host (16). Morjane *et al.* (16) employed oligonucleotide (complementary to simple repetitive sequences) hybridization after endonuclease restriction, to detect genetic diversity among 50 *D. rabiei* isolates from four well-separated locations in a single chickpea field in Tunisia. Twelve different haplotypes were found at various frequencies around the field: seven were confined to one location, four at two, one at three, and none at all four locations. In some cases more than one haplotype was obtained from the same lesion of a single host plant. The haplotypes were distributed across the field in a fine-scaled mosaic pattern of genetically uniform clones. A similar situation was also found by McDonald and Martínez (15) for *Septoria tritici* Roberge in Desmaz when studying isolates from a single wheat field; this suggested a genetically diverse founder population serving as primary inoculum in the field.

In our study we took no account of such local population variability, but we made the assumption that the majority of the 39 isolates of *D. rabiei* employed here more likely represented commonly occurring haplotypes than rare haplotypes. Thus, our data reflect diversity between populations in different countries and not local population diversity. Fischer *et al.* (3) reported that phylogenetic (PAUP) analysis of RAPDs data, derived from the use of three decamer primers and 30 isolates of *D. rabiei* from infected chickpeas at different sites in Italy, resulted in 30 uniquely different RAPD band patterns, but no correlation between RAPD patterns and the six separate pathogenic groups was observed. Nevertheless, they concluded that such unique bands characteristic of particular isolates could be used to re-identify particular strains of the pathogen. This would certainly be possible using the clearly discriminating bands we obtained here (Table 4).

The 39 isolates of *D. rabiei* were much less heterogeneous than the two isolates of *Foc* representative of the 'yellowing' and 'wilting' pathotypes, respectively. These two isolates grouped at a smaller distance from those of *D. rabiei* than isolates of other fungal species in the study. In previous research using a large number of isolates of *Foc* and primers P2, P6 and KS, there was *ca* 43% dissimilarity between the two pathotypes as compared with *ca* 36% dissimilarity among the isolates of *Foc* in this study. It is possible that the higher level of dissimilarity shown here for isolates of *Foc* than for those of *D. rabiei*, obtained at the same time under identical conditions, may be explained by genetic changes being selected in the pathotypes of *Foc* during the prolonged biotrophic phase. By comparison, after an early stage of infection, *D. rabiei* is involved in an extended necrotrophic phase within the dying and dead host tissues, thus minimizing the time period for the selection and accumulation of genetic changes in the pathogen which are directly involved in the interactions between the living host and pathogen. Clearly, a study of diversity comparing several different biotrophic and necrotrophic plant pathogenic fungi would be required to determine whether there is any validity in this suggestion, although contributions to diversity coming from genetic recombination *via* the sexual stage would be an additional, complicating factor.

The present study indicated *ca* ten RAPD clusters/subgroups at *ca* 7% dissimilarity in the tested *D. rabiei* inter-country populations, with some evidence of further subclusters within some of the largest clusters. Morjane *et al.* (16), using oligonucleotide hybridization (see above), calculated that the genetic distance (using cluster analysis and UPGMA) between their *D. rabiei* isolates in a single field was between 0.05 (5%) and 0.22 (22%). These findings reflect the reports for *D. rabiei* describing variability in traits such as morphology (4,8), pathogenicity (7,21,22,24) and phytotoxins (1,6). Nevertheless, none of the RAPD clusters/groups we detected correlated with pathotype or mating type, thus agreeing with the molecular studies of this pathogen by Weising *et al.* (33), Fischer *et al.* (3) and Morjane *et al.* (16). Certain groups of isolates apparently related to geographic origin were evident, however, in the present study (Table 3); thus, all six isolates from group II are from Spain (but other Spanish isolates are also placed within groups III, IV, V and X). Whereas all six isolates from Pakistan that were investigated by RAPDs are confined to group III, the five Indian isolates are found in groups I, III and V, and the five USA isolates are distributed within five groups, I, III, IV, V and VII, respectively. These apparent differences among groups of isolates between certain countries may also partly reflect the non-random way in which isolates were collected.

Finally, useful information regarding diversity and strain characterization in *D. rabiei* has been gained by RAPD analysis, which can be utilized in future studies employing genetic analysis *via* sexual crosses. However, neither in this study nor in those reported above involving different approaches, has any DNA fragment been identified that has a detectable function in pathogenesis. Fischer *et al.* (3) on the basis of their RAPDs investigation involving *D. rabiei*, have calculated that several hundred primers would be necessary to detect pathogenicity-correlated genomic differences with a reasonable probability of success, thus limiting the use of this approach in future studies.

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