Curr Genet (1991) 19:483–489

Current Genetics

DNA fingerprinting of *Ascochyta rabiei* with synthetic oligodeoxynucleotides

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Received February 18, 1991

Summary. The ascomycete fungus *Ascochyta rabiei*, an important pathogen of the grain legume crop chickpea (*Cicer arietinum* L.) in the Mediterranean region, has not been adequately characterized in molecular terms. We therefore used DNA fingerprinting, with synthetic oligodeoxynucleotides complementary to simple repetitive sequences, to pathotype different isolates of the fungus. Six single-spored *A. rabiei* isolates were first categorized using a host differential set of nine chickpea genotypes. Seedlings were inoculated under controlled environmental conditions, and disease severity was recorded 9 days after inoculation. DNA was extracted from in vitro-grown mycelia of the six purified fungal isolates, restricted with *Eco*RI, *Hinf*I, *Mbo*II and *Taq*I, and fingerprinted with radiolabeled $(GATA)_4$, $(GTG)_5$, $(CA)_8$, and $(TCC)_5$, respectively. High levels of polymorphism were detected with optimal enzyme/probe combinations that allow one to discriminate between the isolates. The potential of DNA fingerprinting with simple repetitive sequences can thus be expanded to the identification of fungal races and pathotypes. The characterization of the geographic distribution and genetic variability of pathotypes will facilitate the selection of suitable host cultivars to be grown in specific regions.

Key words: DNA fingerprinting – Synthetic oligodeoxynucleotides – Simple repetitive sequences – Fungal pathotypes – *Ascochyta rabiei*

Introduction

The ascomycete fungus *Ascochyta rabiei* (Pass.) attacks the economically important food legume crop chickpea (*Cicer arietinum* L.) and causes severe losses in the Mediterranean region (Nene and Reddy 1987). Traditionally, chickpea is a spring-sown crop in this region, and yield is determined by the availability of soil moisture at the onset of the dry summer period. Crop yield could be tremendously increased if the sowing time was advanced so that the vegetative development of the plants could benefit from the high winter rainfall. However, these climatic conditions favour the development of the pathogen *A. rabiei*, so that chickpea cultivars with inadequate levels of resistance to *Ascochyta* blight would be lost (Saxena and Singh 1984; Nene and Reddy 1987).

Programs for disease control and resistance breeding in chickpea depend on the reliable identification and characterization of fungal pathotypes and populations. The classical biological pathotyping technique, using a set of different host genotypes (Reddy and Kabbabeh 1985; Singh and Reddy 1990), is laborious, time-consuming, and requires strict standardization of test conditions. A reliable characterization of the genetic make-up of different strains of the pathogen, their levels of aggressiveness, their extent of variability, and their genotypic and phenotypic interaction(s) with the host plant is, therefore, very difficult to achieve by means of biological pathotyping.

The present paper introduces an innovative approach to differentiate between various *A. rabiei* isolates using a DNA fingerprinting technique that is based on the detection of hypervariable restriction fragment length polymorphisms (RFLPs) in the fungal genome. DNA fingerprinting relies on the presence of a particular class of repetitive DNA in the eukaryotic genome. This class consists of short motifs which are tandemly arranged to form long, more or less homogeneous, arrays. Depending on the length of the basic repeat units, these sequences are called minisatellites (about 15 to 35 bp; Jeffreys et al. 1985) or simple repetitive sequences (SRS; about 2 to 10 bp; Tautz and Renz 1984). SRS and minisatellite-like sequences are present in all eukaryotic genomes investigated so far (Tautz and Renz 1984; Jeffreys 1987; Epplen 1988; Weising et al. 1991). Two characteristics qualify this kind of sequence for DNA fingerprinting. First, the tandem repeats are often dispersed throughout the genome (multilocus appearance). Second, the tandemly arranged repetitions exhibit a high degree of polymor-

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phism, mainly resulting from different copy numbers of the basic motifs. Consequently, SRS and minisatellite fingerprinting have frequently been used for the analysis of vertebrate (e.g., Jeffreys et al. 1985; Georges et al. 1988), plant (e.g., Dallas 1988; Weising et al. 1989, 1991; Weising and Kahl 1990) and, most recently, fungal genomes (Braithwaite and Manners 1989; Walmsley et al. 1989; Monastyrskii et al. 1990). Genome analysis of fungi has also been performed with endogenous RFLP probes (Brown et al. 1990; Manicom et al. 1990).

In the present study, we applied DNA fingerprinting with oligonucleotide probes representing SRS to the analysis of different pathotypes of *A. rabiei*. Our results demonstrate (1) that the *A. rabiei* genome contains simple repetitive sequences, (2) that these sequences are polymorphic, and (3) that probes complementary to SRS allow one to discriminate between different pathotypes of this agronomically important pathogen.

Materials and methods

Plant material. Seeds of chickpea cultivars ILC (International Legume Chickpea) 190, 201, 215, 249, 482, 1929, 2956, 3279, and 5928 were obtained from the germplasm collection of ICARDA, Aleppo, Syria. For the inoculation experiments, plants were grown in germination boxes in a Conviron E7 type growth chamber and exposed to a photoperiod of 14 h per day. Light was generated by eight fluorescent tubes and two daylight bulbs. Temperature was kept at 18 °C during the night, and 24 °C during daytime.

Fungal material. Stock cultures of *A. rabiei* (Pass.) were maintained on chickpea seed meal-agar (CSMA; 4 g of ground chickpea seeds, 3 g dextrose, and 1.8 g agar/100 ml of sterile deionized water) at 18 °C and were illuminated 14 h per day.

Preparation of single-spored isolates. The existing stock cultures of the six isolates of *A. rabiei* were single-spored as follows. Stock cultures of the fungi were immersed in sterile deionized water to release the spores from the pycnidia. The resulting spore suspensions were diluted to a concentration of 3.5×10^3 spores/ml, dispersed on water agar, and incubated at 20 °C to induce germination. After 24 h, 20 × 20 mm agar pieces were transferred onto microscope slides and dissected into smaller squares (1.5 × 1.5 mm) using a multi-blade razor knife. Squares with single germinating spores were identified microscopically and transferred to fresh CSMA medium for further culture.

Inoculation of plants. Small pieces of *A. rabiei* mycelium were transferred to CSMA medium lacking agar and grown under similar light and temperature conditions as described for the fungal stock cultures. After 14 days the pycnidia were suspended in deionized water to release their spores. The resulting spore suspension was adjusted to a concentration of 1.6×10^5 spores/ml. Seven-day old chickpea seedlings were sprayed with the spore suspension. After spraying, the plants were covered with transparent plastic covers to maintain leaf moisture. Light intensity was reduced to prevent excessive heating within the germination boxes. After 36 h, plastic covers were removed. This protocol created the best conditions for infestation (Weltzien and Kaack 1984).

Screening disease severity. Readings for disease severity on single plants were taken from the 3rd to the 14th day after inoculation using the following scale:
1 = no symptoms
2 = small round tissue depression or spot
3 = elongating spot
4 = coalescent spots
5 = stem girdling
6 = stem breaking
7 = lesion growth downward from breaking point
8 = whole plant nearly dead
9 = plant dead.
For each chickpea accession, mean values were calculated from the evaluation of four individual plants. Symptom evaluation was restricted to stem lesions, since only unfolded leaves were present during the inoculation period. Leaves produced during the subsequent 12 days were not attacked by the pathogen and, therefore, were not considered in disease scoring.

Extraction of DNA. DNA was isolated from lyophilized fungal mycelia according to a modified CTAB method (Doyle and Doyle 1990; Weising et al. 1991) The lyophilized material (0.5 g) was ground to a fine powder and subsequently transferred to 15 ml of hot (60 °C) 2 × CTAB extraction buffer. After gently swirling the resulting cell lysate for 30 min, nucleic acids were isolated by extraction with an equal volume of chloroform/isoamylalcohol (24:1) followed by precipitation with an 0.6 vol of isopropanol. After centrifugation, pellets were solubilized in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8), and DNA was further purified by ultracentrifugation in CsCl/ethidium bromide followed by extraction with TE-saturated 1-butanol and ethanol precipitation.

Restriction of DNA, agarose gel electrophoresis and gel-drying. DNA was digested with *Eco*RI, *Hinf*I, *Taq*I or *Mbo*II (according to supplier's instructions; six units of enzyme per µg DNA) and electrophoresed on 1.0% agarose gels in TAE buffer (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA; pH 7.8). The gels were stained with ethidium bromide, photographed, and then dried on a vacuum gel dryer.

Probe labeling, hybridization and autoradiography. The dried gels were denatured, neutralized and hybridized to ^{32}P-endlabelled oligodeoxynucleotides essentially as described by Ali et al. (1986) and Schäfer et al. (1988). Temperatures of hybridization and stringent washing steps were 35 °C for $(GATA)_4$, 43 °C for $(CA)_8$, and 45 °C for $(TCC)_5$ and $(GTG)_5$. One and the same gel was successively used for different hybridization probes. Before reprobing, probes were stripped off the gel by washing in 5 mM EDTA at 60 °C (2 × 15 min).

Results

Pathogenicity of different A. rabiei isolates

A host differential set was used to characterize the six different *A. rabiei* isolates according to the disease severity they cause on inoculated chickpea plants. Even though there was some intracultivar variation in response to the fungus, the six isolates could be grouped into different pathotypes (Fig. 1). Notwithstanding its differential aggressiveness towards different chickpea cultivars, isolate No. 3 was consistently the least aggressive fungus. However, in combination with susceptible cultivars (e.g., ILC 201, ILC 1929) it caused severe disease symptoms. Isolates No. 1 and No. 5 can also be included in this "weak" pathogenicity group. Isolates No. 2, 4 and 6 were generally more aggressive, with isolate No. 6 being the most destructive on most cultivars. In conclusion, the following order of aggressiveness was obtained: isolate No. 6 > 4 > 2 > 1 > 5 > 3.

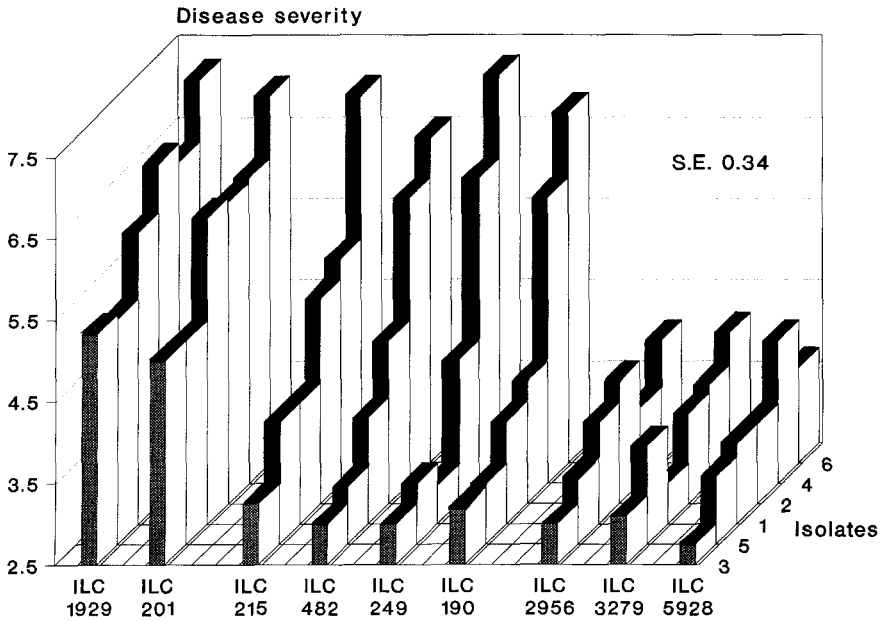

Fig. 1. Host-pathogen interactions between nine selected chickpea genotypes and six single-spored isolates of *A. rabiei*. Disease severity was recorded for single plants 9 days after inoculation using a scale from 1 to 9 (see Materials and methods section). Each value represents a mean of four replicates. S.E., standard error

DNA fingerprinting of different A. rabiei isolates

DNA was isolated from in vitro-grown mycelia of the six different *A. rabiei* isolates, digested with *Eco*RI, *Hin*fI, *Taq*I, or *Mbo*II, electrophoresed, and in-gel hybridized to oligonucleotide probes complementary to simple doublet, triplet or quadruplet repeat sequences. The results are summarized in Figs. 2 and 3. Though all motifs are obviously present in the genome of all *Ascochyta* isolates, their organization and relative abundance appear to be somewhat different. Whereas $(GATA)_4$ tracts occur less frequently and appear to be concentrated at one or two predominant loci (Fig. 2, upper panel), the motifs $(GTG)_5$, $(CA)_8$, and $(TCC)_5$ are more abundant (Fig. 2, lower panel; Fig. 3) and exhibit more complex hybridization patterns.

Genetic differences between the isolates, resulting in different fingerprint patterns, were obtained with all probes. The discriminatory potential of a probe, however, is strongly dependent on the enzyme. $(GTG)_5$, for example, is very informative in combination with *Taq*I, but much less so with either *Eco*RI, *Hin*fI, or *Mbo*II (Fig. 2, lower panel). Generally, the six-base cutter *Eco*RI gave more uniform restriction patterns as compared to the four- and five-base cutters. The molecular weight of the fragments in this enzyme/probe combination is too high to allow easy and reproducible discrimination between the isolates on 1.0% agarose gels. Restriction with *Hin*fI, *Taq*I, and *Mbo*II, on the other hand, allowed the detection of polymorphic sites with almost all probes, and thus the production of highly informative fingerprints. Even the relatively uncomplicated patterns obtained with the $(GATA)_4$ probe allow the conclusion that isolates 1, 2, 3 and 4 are definitely different, whereas isolates 3 and 5, and 4 and 6, respectively, are similar, if not identical (Fig. 2, upper panel). This result is corroborated by all other probe/enzyme combinations. A noteworthy exception is the pattern obtained with *Taq*I/$(GTG)_5$ that additionally permits the detection of differences between isolates 4 and 6 (Fig. 2, lower panel, indicated by arrows). These differences however, did, not occur upon re-digestion of the DNA samples using much higher enzyme concentrations (15 Units/μg DNA) and may, therefore, be the consequence of a "hidden partial" digest (Nürnberg and Epplen 1989). In view of this phenomenon, it is absolutely required to reexamine unexpected banding patterns (e.g., differences occurring with one enzyme only) using extraordinarily high enzyme concentrations and/or repeated addition of the restriction enzyme.

To examine the conservation of SRS hybridization patterns during tissue culture, we repeated our experiments with mycelia that were harvested after several rounds of in vitro-subculturing. So far, we have not observed any changes in isolate-specific banding patterns (data not shown).

Discussion

Though biological pathotyping using a differential host genotype set allows a rough estimate of the pathogenic potential of *A. rabiei* towards chickpea (*C. arietinum*), this technique suffers from several disadvantages. First, reproducibility is poor. This is illustrated by the fact that different investigations lead to a different ordering of aggressiveness of the same six *Ascochyta* isolates: $6 > 5 > 4 > 3 > 2 > 1$, as reported by Reddy and Kabbabeh (1985); $6 > 4 > 2 > 1 > 5 > 3$, as documented in the present study. Such differences may be explained by, e.g., the use of different sets of host cultivars, the influence of plant age, different environmental conditions, or mutations. Even under controlled conditions, however, individual differences from host to host and from pathogen to pathogen make reproducibility at least difficult. Second, the expense is enormous, even if one only considers

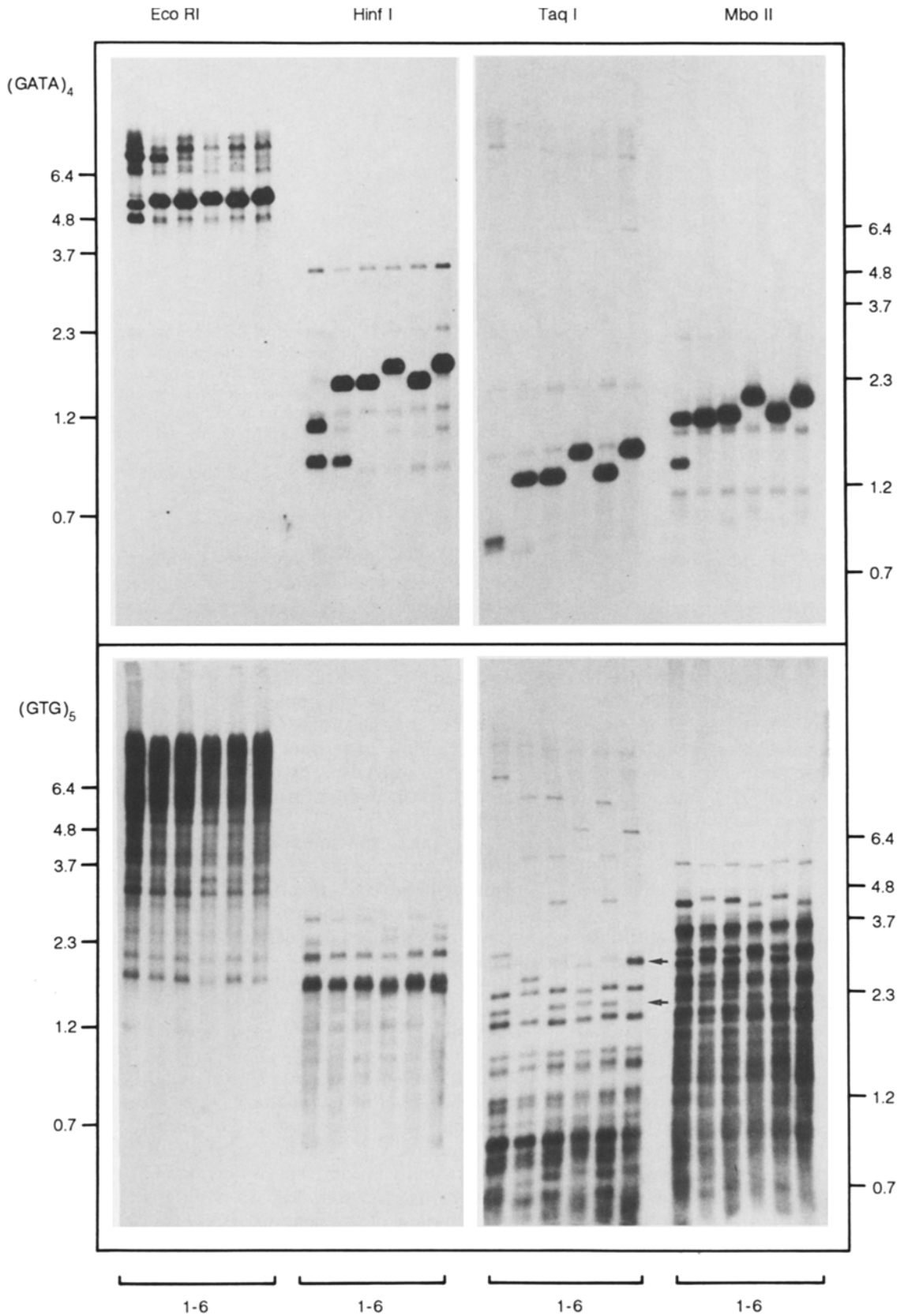

Fig. 2. DNA fingerprinting of *A. rabiei* isolates with synthetic oligodeoxynucleotides. Total DNA was isolated from lyophilized mycelia derived from single-spored cultures. After digestion with *Eco*RI, *Hin*fI, *Taq*I or *Mbo*II, the restriction fragments were electrophoresed in 1% agarose gels (4.5 µg per lane). The gels were dried and hybridized to the radiolabeled oligonucleotides $(GATA)_4$ and $(GTG)_5$, respectively. *Lanes 1–6* (from left to right), DNA from fungal isolates No. 1, 2, 3, 4, 5 and 6. Positions of molecular weight markers are indicated, in kilobases, at the left-hand side for the gel with *Eco*RI- and *Hin*fI-digested DNA, at the right-hand side for the gel with *Taq*I- and *Mbo*II-digested DNA

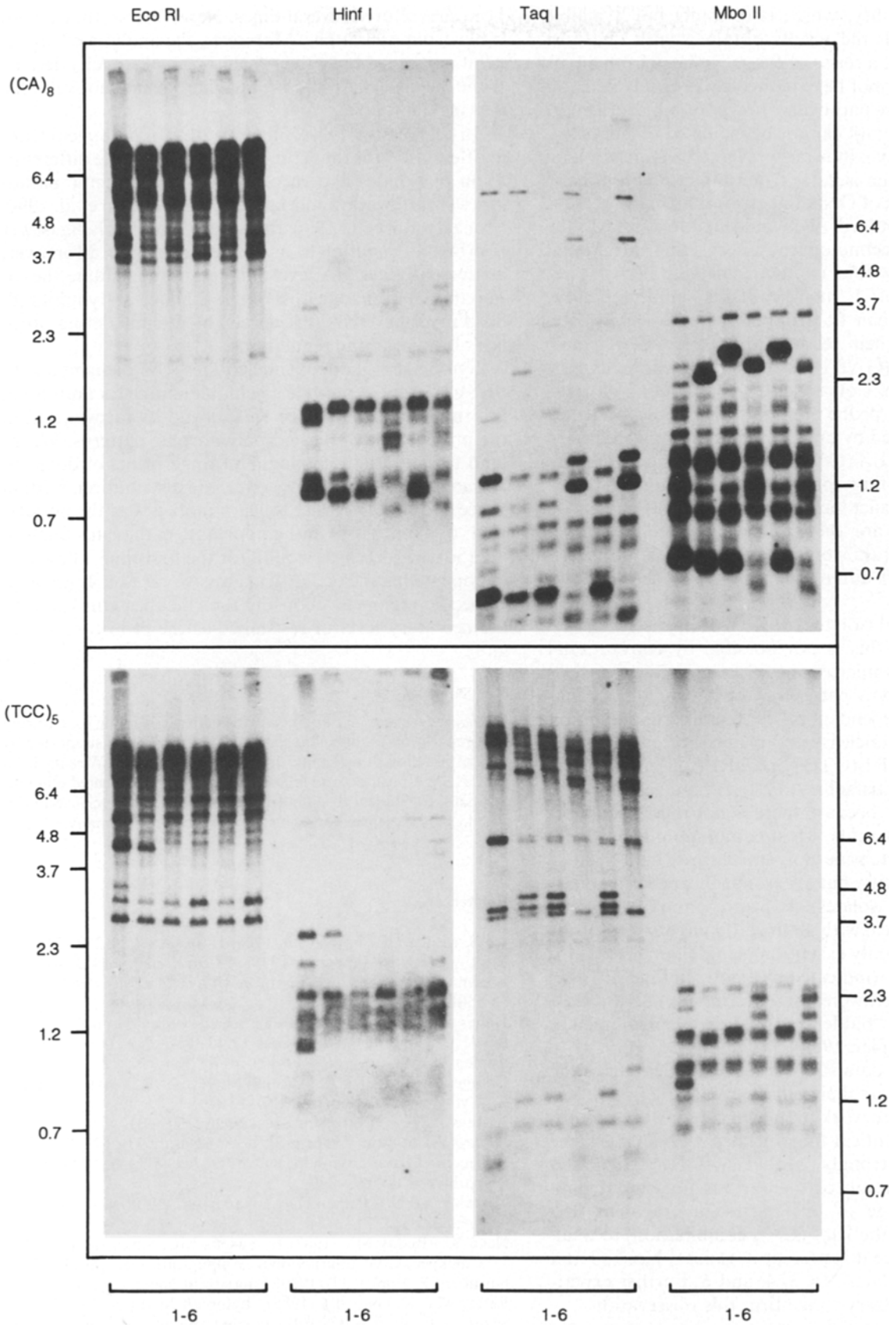

Fig. 3. DNA fingerprinting of *A. rabiei* isolates with the synthetic oligodeoxynucleotides $(CA)_8$ and $(TCC)_5$. For explanations see legend to Fig. 2

the phytotron facility, which is certainly not available everywhere. Third, the results obtained with one host differential set and a series of fungal isolates from a particular country cannot be exploited by researchers in another country, since pathogenic fungi are subject to quarantine regulations and cannot be exported or imported. These obstacles have, thus far, prevented any meaningful comparison between isolates from different countries.

With the advent of DNA fingerprinting, many of these problems can be solved. We would like to illustrate the potential of this technology by interpreting the present results which allow four main conclusions:

(1) The genome of *A. rabiei* contains simple repetitive sequences. These can be grouped into two main categories based on their relative abundance. The simple quadruplet repeat $(GATA)_4$ is only sparsely present in the *Ascochyta* genome. Consequently, the banding patterns obtained with this probe are relatively simple, and they are largely produced by one single polymorphic locus. In contrast, the $(GTG)_5$, $(CA)_8$ and $(TCC)_5$ tracts are more abundant, and the fingerprints are, hence, more complex. Since neither the exact location of the various repeats on the chromosomes nor their function(s), if any, are yet known, we will refrain from hypothesizing about the relative abundance of different simple repeat sequences in the fungal genome.

(2) Different fungal isolates can be readily discriminated by fingerprinting; this is not possible by conventional techniques such as microscopy, electron microscopy, or evaluation of growth patterns. Convenient discrimination is, however, dependent on a favourable combination of restriction endonuclease and probe. For example, the combinations *Eco*RI/$(GTG)_5$, *Eco*RI/$(CA)_8$, or *Hin*fI/$(GTG)_5$ are not satisfactory for the characterization of this fungal species, because there is not much polymorphism in the fingerprints. Most combinations, however, not only detect extensive polymorphisms, with various levels of informativity, but also some unexpected similarities. For example, isolates No. 3 and 5, and No. 4 and 6 are perfectly identical with all the different enzyme/probe combinations, the only exception being *Taq*I/$(GTG)_5$ for isolates No. 4 and 6 (indicated by arrows in Fig. 2). These differences, however, were not reproducible and are most probably due to a "hidden partial" digest phenomenon (Nürnberg and Epplen 1989).

(3) The fingerprint data support our classification of the isolates according to their aggressiveness (Fig. 1). Isolates No. 3 and 5 are weak pathogens on most host cultivars, and share identical fingerprint patterns. The same holds true for the strongly pathogenic isolates No. 4 and 6. Careful examination of the various fingerprint data suggests the linkage of one particular fragment (the 2.0 kb fragment in the *Taq*I/$(CA)_8$ combination) to weak aggressiveness, since it is present in isolates No. 1, 3 and 5 but absent in isolates No. 2, 4, and 6. Further experiments will be necessary to confirm this observation.

(4) The mutation rate of most of the repeat sequences used in this study seems to be moderate, so that the DNA fingerprint patterns are relatively stable. For example, isolates No. 4 and 6 have been separated from each other for several years, were single-spored 1 year ago and have been subcultured several times. Nevertheless, they do not differ from each other. Moreover, the analysis of fingerprint patterns during the subculturing of mycelia derived from the six *Ascochyta* isolates has so far, not revealed any instability.

In perspective, DNA fingerprinting of pathogenic fungi offers itself for the reproducible and reliable differentiation of isolates and races, maybe even specific pathotypes (Braithwaite and Manners 1989; Brown et al. 1990; Monastyrskii et al. 1990; this study). The resolving power is probably superior to any other technique. Moreover, analysis at the DNA level will certainly facilitate the international exchange of biological material (lyophilized, dead mycelia; DNA; dried gels) by circumventing problems of quarantine regulations.

Synthetic oligodeoxynucleotides complementary to SRS are likely to provide a valuable source for additional informative probes, since all four motifs investigated in the present study revealed polymorphic patterns. We intend to establish a catalogue of fingerprints of different *A. rabiei* races and their geographic distribution, both in space and time, in order to learn more about the population dynamics of this important pathogen. Such a "pathotype forecast" would, for the first time, allow one to cope with a particularly aggressive race of *A. rabiei* in a specific region by choosing the chickpea cultivar with the necessary level of resistance as the crop for this region.

Acknowledgements. We appreciate the help of Sieglinde Kost during the preparation of this manuscript. D. Kaemmer was supported by a FAZIT fellowship (Frankfurt am Main) und K. Weising by a DECHEMA postdoctoral fellowship. The simple repeated oligonucleotides are subject to patent applications. Commercial inquiries should be directed to Fresenius AG, Oberursel, Germany.

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Communicated by F. K. Zimmermann