APPLICATION OF MOLECULAR BIOLOGY TECHNIQUES TO THE TAXONOMY AND IDENTIFICATION OF FUSARIA, PARTICULARLY *FUSARIUM OXYSPORUM* F. SP. *DIANTHI*

Barry Quinton Manicom

Submitted in partial fulfilment of the requirements for the degree

D. Sc. (Agric.)

in the Faculty of Agricultural Sciences

Department of Microbiology and Plant Pathology

University of Pretoria

PRETORIA.

November, 1988

ACKNOWLEDGEMENTS

My sincere thanks to Dr M. Bar-Joseph of the S. Tolkowsky Laboratory for Virus Research, The Volcani Centre, A.R.O., Bet Dagan, Israel, who initiated this course of investigation.

Dr A. Rosner, also of The Volcani Centre, who introduced me to the basic techniques of biotechnology.

My wife, Galia, who put up with a lot of nonsense.

Merle Becker, who did likewise, and provided superb technical aid.

Dr G. Bredell, who as Director of the C.S.F.R.I. approved the project and was instrumental in motivating for a study period in Israel.

Dr J. Terblanche, who as subsequent Director of the C.S.F.R.I. arranged for a follow up visit and lightened the load to help in the completion of the work.

Not least, Prof J. M. Kotze, my promotor, for help and encouragement.

To all, and those unnamed, my deepest gratitude.

CONTENTS

APPLICATION OF MOLECULAR BIOLOGY TECHNIQUES TO THE TAXONOMY AND IDENTIFICATION OF FUSARIA, PARTICULARLY *FUSARIUM OXYSPORUM* F. SP. *DIANTHI*

ABSTRACT

The taxonomy of *Fusarium* has long been a contentious field. Recent syntheses have brought a measure of order to classification at the species level. At the sub-specific level identification is difficult, requiring biological indexing, and knowledge of genetically isolated groups and their interrelationships is scant. This is particularly true for *F. oxysporum*, a widespread plant pathogen.

Molecular methods, ranging from protein electrophoresis to monoclonal antibodies, have been used for some decades in attempts to find identifying markers for sub-specific groups with limited success. The exponential increase in DNA technology has opened several new avenues for the investigation of these interrelationships and the utility of some was investigated using *Fusarium oxysporum* f. sp. *dianthi* as a test system.

A range of *Fusarium* species was screened for DNA restriction fragment length polymorphisms (RFLPs) by hybridisation to Southern blots of random probes cloned from an isolate of *F. oxysporum* f. sp. *dianthi*. The RFLPs derived from combinations of probes and restriction enzymes enabled differentiation at species, forma species and isolate levels, and patterns were conserved over time despite phenotypic variation. One probe hybridised only to a subset of the species *F. oxysporum*, giving a multi-band pattern, and not to isolates of the seven other *Fusarium* spp.

This probe was further used on forty-six isolates of *Fusarium* from dis-

eased carnations which were examined for RFLPs and vegetative compatibility groups (VCGs). Both RFLPs and VCGs divided the isolates into two major and three minor corresponding groups. Testing of subsets of the groups showed the major groups to be pathogenic. The two methodologies gave equivalent results for the genetic separation of populations and it is suggested that in many cases these methods will be superior to differential hosts for the establishment of taxonomic units.

Investigation of this highly specific probe found it to be derived from nuclear DNA. Subclones were explored, and the portion responsible for the multi-band pattern was sequenced. Computer searches failed to find homology with any known sequence, nor were there any features of particular note in the sequence.

It was noted that two sub-clones could be used to provide a very specific dot blot test which would determine pathogenicity of *Fusarium* isolates from diseased carnations. This technique was developed into a rapid, non-radioactive dot blot test for general use.

UITTREKSEL

Die taksonomie van *Fusarium* is lank reeds 'n kontensieuse veld. Onlangse sintese van denkrigtings het 'n mate van orde gebring in klassifikasie tot op spesie vlak. Op sub-spesie vlak is identifikasie moeilik, en vereis biologiese indeksering, en kennis van geneties ge-isoleerde groepe en hul onderlinge verwantskap is gering. Dit is veral waar vir *F. oxysporum*, 'n wydverspreide plantpatogeen.

Molekulêre metodes, wat wissel van proteïen elektroforese tot monoklonale teenliggame, is vir dekades met beperkte sukses gebruik om identifikasie-merkers vir sub-spesie groepe te vind. Die eksponensiële toename in DNA-tegnologie het verskeie nuwe rigtings ge-open vir ondersoeke na die onderlinge verwantskappe van groepe, en die bruikbaarheid van sommiges is ondersoek met *Fusarium oxysporum* f. sp. *dianthi* as toetsmodel.

Lukraak DNA peilers is van 'n isolaat van *F. oxysporum* f. sp. *dianthi* gekloneer. Hiermee is 'n reeks *Fusarium* spesies deur hibridisasie aan "Southern" kladde geselekteer vir restriksiefragment-lengte polimorfismes (RFLPs). Die RFLPs verkry met kombinasies van peilers en beperkingsensieme het dit moontlik gemaak om op spesie-, subspesie- en isolaatvlakke te onderskei. Patrone was konstant met tyd, ten spyte van fenotipiese variasie. Een peiler het slegs met 'n substel van die spesie *F. oxysporum* gehibridiseer, 'n multi-band patroon gegee, en nie met isolate van die sewe ander *Fusarium* spesies gehibridiseer nie.

Ses-en-veertig *Fusarium* isolate vanaf besmette angeliere is onderling ten opsigte van vegetatiewe verenigbare groepe (VVGs) en met hierdie peiler vir RFLPs vergelyk. Beide RFLPs en VVGs het die isolate in twee hoofgroepe en drie kleiner ooreenstemmende groepe verdeel. Toetsing van substelle van die groepe het getoon dat die hoofgroepe patogenies is. Die twee metodes het dieselfde resultate gelewer vir genetiese skeiding van bevolkings en dit word voorgestel dat hierdie metodes in

baie gevalle beter sal wees as differensiële gashere vir vestiging van taksonomiese eenhede.

Ondersoeke van hierdie hoogs spesifieke peiler het getoon dat dit afkomstig van kern-DNA is. Sub-klone van die peiler is ondersoek, en die gedeelte verantwoordelik vir die multi-band patroon se basisvolgorde is bepaal. Rekenaarondersoeke kon geen ooreenstemming met bekende volgordes of enige eienskap van spesifieke belang in hierdie volgorde vind nie.

Dit is gevind dat twee sub-klones gebruik kan word om 'n spesifieke kol-klad toets daar te stel wat sal bepaal of die *Fusarium* isolate vanaf angeliere patogenies is. Hierdie tegniek is gebruik om 'n vinnige nie-radioaktiewe kol-klad toets vir algemene gebruik te ontwikkel.

CHAPTER 1

REVIEW OF MOLECULAR METHODS USED IN THE TAXONOMY AND IDENTIFICATION OF FUNGI, PARTICULARLY *FUSARIUM*

The Fusaria consist of a widely distributed, generally soil borne fungal genus, common in cultivated crops and often comprising the major portion of the soil fungal flora (Burgess, 1981). The group is extremely diverse, having non-pathogenic and pathogenic strains, the latter naturally having received the most attention.

The importance to agriculture can scarcely be under-estimated, as various Fusaria cause diseases of (among numerous others), staple crops such as wheat, maize, sorghum and rice: major tropical crops as banana, cacao, date and oil palms: tomato, potato, vegetables and pulses of the lesser food crops: and cause problems in industrial crops such as cotton, forestry and ornamentals (Snyder, 1981).

Fusarium oxysporum, in particular, is the causal agent of a variety of vascular wilts and cortical rots of economically important crops. Booth (1971) recorded 76 wilt diseases alone due to this species, and although breeding for resistance to many of these has been successful, they continue to exact their toll wherever agriculture is practised.

For those studying this group of fungi, to quote Snyder (1981), "Perhaps the biggest problem confronting Fusarium workers today is the identification of the fungi they isolate.", a problem which remains today, *vide* "One of the problems ..... is the correct identification of each strain." (Nelson *et al.*, 1983).

THE FUNCTIONS OF TAXONOMY

The prime function of a taxonomic system is communication. The label applied to an organism must be agreed so that whether discussing traits, physiology, ecology or pathogenicity the entity involved can be known to others.

It is also desired that such a label should carry additional information, and thus like is grouped with like, and the whole arranged in an hierarchical structure which should relate to the evolutionary and genetic make-up of the organisms. This latter point has, until now, not been possible with most fungi, as they leave virtually no fossil record.

Thus if the name of an organism be known, this indicates its position in the hierarchical structure, and immediately a number of inferences may be made concerning its appearance, physiology and environmental interactions.

The Fusaria present particular difficulties in this respect. The variation of *Fusarium* in culture is well known, and sectors and patches in a colony are common. On rich media, with repeated sub-culture, there is often an irreversible progression from the mycelial "wild type" via steps to a pionnotal form (Waite & Stover, 1960). These and other variations can lead to a situation where variants of one species are morphologically indistinguishable from another species (Puhalla, 1981), which has considerably enhanced the difficulties of classical taxonomists.

The origin of pathogenic strains and the forma speciales and races thereof is unknown. No perfect stage is presently known for *F. oxysporum* although features in the imperfect stages suggest that if found, it will prove to be *Gibberella* (Snyder & Tousson, 1965). Other data, based on DNA homologies (Szeci & Dubrolovsky, 1985a & b) place *F. oxysporum* outside the *Gibberella* group. Genetic exchange and variation in nature is thus dependent on the parasexual cycle and consequent sorting out via conidia, or selection from spontaneous mutations. The immense variation and ubiquitous nature of *F. oxysporum* is an indication of the

effectiveness of these mechanisms.

Notwithstanding, there exists an underlying genetic basis for pathogenic host specific expression and the concept of forma speciales as used in current taxonomies. Bouhot (1981), using a mutagen, was able to show that saprophytic, or other forma speciales could not be mutated to attack cucurbits. The three forma attacking cucurbits however, (f. sp. *melonis, niveum and cucumerinum*) could be converted from one to the other, the implication being the existence of a higher genetic grouping (forma familialis) than the standard taxonomy of the Fusaria has allowed.

Until recently it has been impossible to address questions such as these on the genetic relationship among formae or strains of *F. oxysporum*, the ecology of strains in field situations, and the emergence of new pathogenic strains, as few suitable phenotypic markers exist.

There is thus a need for the identification of strains of *Fusarium* to confirm or realign the current taxonomy of the entire group and on a more specific level to answer questions as to the origins of forma speciales, to be able to track individual strains in the environment to determine ecological interactions, and for the rapid diagnosis of forma speciales or races attacking a particular crop.

This study was initiated to evaluate the applicability of some of the newer molecular biological techniques in addressing this problem of identification of the Fusaria, particularly *F. oxysporum*.

One should first, however, consider the current taxonomic status and biochemical techniques which have been of some use in the field of identification.

CLASSICAL (MORPHOLOGICAL) TAXONOMIC SYSTEMS

The wide phenotypic variation has been reflected in the various taxonomic structures erected for the Fusaria. The groundwork for all later systems was laid by Wollenweber & Reinking (1935). This detailed study reduced the existing 1000 or more named forms to 65 species, 55 varieties and 22 forms, grouped in 16 sections. At this time the philosophy was that all members of a species should be identical, and stress was laid on factors such colour of the stroma, septation and length and width of macrospores, now known to vary considerably from clone to clone.

The other extreme was taken by Snyder and Hansen (1940, 1941, 1945) who sought a practically useable system and reduced the group to 9 species with forma speciales and races within these defined by host reaction. The major difference in approach taken by these workers was the use of a large number of single spored isolates of each species and a concentration on similarities within a group rather than differences. The system was never formally completed but the ideas were summarised by Snyder & Tousson (1965).

Various alternative systems have been proposed (reviewed by Tousson & Nelson, 1975, and Nelson *et al.*, 1983) which take intermediate positions. Thus Messiaen & Cassini (1968) and Matuo (1972) lean toward the Snyder/Hansen system where Raillo (1950), Bilai (1970) and Joffe (1974) are somewhat intermediate and Gordon (1952; 1960), Booth (1971), and Gerlach (1970; Gerlach & Nirenberg, 1982) are heirs to the Wollenweber/Reinking system. Booth's (1971) major contributions were in recognising the value of differences in the conidiophores and conidiogenous cells and the shape of the microconidia in separating species. He also expanded and integrated the information on the perfect stages.

The most recent synthesis of ideas is that of Nelson *et al.* (1983). This has excellent descriptions and photographs of all species which have been sufficiently

well described and the identification key has broken away from the standard dichotomous system to one which can be entered with whatever information is available. This lends itself to "expert system" programming and greatly eases identification to species level.

Despite these varying schools of thought, the group *F. oxysporum* has remained reasonably homogenous. From Wollenwebers section Elegans of three subsections, containing 10 species, 18 varieties and 12 forms, the group was reduced to the species *F. oxysporum* containing 27 forma speciales in Snyders system. Booth maintains the single section Elegans of one species, one variety and 76 forma speciales, and Nelson *et al.* (1983) accept the section Elegans of one species.

From a practical point of view some form of sub-specific name will always be necessary to indicate pathogenicity and host range and it is at this level where so much confusion has entered the taxonomy due to the propensity to name each new host observation as a new forma specialis. Armstrong & Armstrong (1981) recognised 122 forma speciales and races of *F. oxysporum* based on host response. No such divisions exist for the nonpathogens which in themselves can be important in modifying disease expression, either by enhancing or suppressing disease. It is in these areas that biochemical and other markers can be most useful.

BIOLOGICAL MARKERS

Auxotrophs

Auxotrophic or antibiotic resistant mutants have long been used in genetic studies of the fungi, particularly in *Neurospora* and *Aspergillus*. Generally, these are created by violent physical or chemical means which can themselves affect the characteristic being studied. Puhalla & Spieth (1983) and Puhalla (1984a) generated colour and auxotrophic mutants with ultra-violet light, but otherwise relatively

little has been done with mutants of Fusarium (reviewed by Puhalla, 1981), until the recent introduction of vegetative compatibility grouping to the Fusaria, the use of which has developed explosively over the past few years.

Vegetative Compatibility Groups (VCGs)

A VCG is defined as a group of isolates which are able to anastomose and form heterokaryons, this being controlled by multiple incompatibility loci (at least 10 in *Gibberella fujikuroi*, Puhalla & Spieth, 1985), unrelated to the mating type locus. In 1985, Puhalla introduced a modification of an elegant technique of Cove's (1976) to studies of Fusarium using nitrate non-utilising mutants for the detection of VCGs.

Although originally suspected that VCGs may correspond to forma speciales (Puhalla, 1985), various studies on Fusaria (Bosland & Williams, 1987; Correll *et al.*, 1985; Correll *et al.*, 1987; Elmer & Stevens, 1986), have shown that although different forma speciales are not found in the same VCG, a number of VCGs may occur in a forma specialis. Though often the VCG corresponds to a race, more than one race may occur in a VCG, and Ploetz & Correll (1988) have indicated that more than one VCG can occur within a race. Puhalla (1984b) and Elmer & Stevens (1986) also found that pathogenic and non pathogenic strains could occur within a VCG.

Thus although VCGs have proved very useful in differentiating genetic groupings within the Fusaria, the initial promise of a reliable, rapid laboratory test precisely at the level of forma speciales or races has not been fulfilled.

MOLECULAR MARKERS

I do not entirely agree with Snyder & Tousson's (1965) statement that "when it becomes necessary to use electrophoresis, serology, protein analysis, the electron microscope, or pathogenicity tests in identifying fungi at the species level, something is wrong with the system."

Taxonomic systems have historically used morphological criteria for classification as the theoretical constructs and techniques for alternative approaches did not exist, and have continued to do so as morphological features are now understood to be an expression of interacting genes. Thus, if one accepts the idea of a classification system as grouping organisms on evolutionary lines, molecular methods can be definitive in confirming the correctness of a morphological taxonomic system and indicating which phenotypic characters are sufficiently stable to be used in a morphological key. Fungal identification at the species level can, and should, rely on microscopic observation, but at the sub-specific level, particularly in the Fusaria, morphological characters are variable or misleading and alternative methods become essential.

They also, as will be shown later in this work, offer the potential of precise identification to sub-specific level without the necessity for laborious biological testing as in the determination of races.

There are advantages and disadvantages to molecular methods and the choice of method will depend on the task to be accomplished. The following is intended as a review of molecular markers and their use for *Fusarium* taxonomy and identification as well as other applications.

PROTEINS

Proteins have advantages over morphological features in that they are generally an expression of a single genetic locus rather than many. They do however express only a small portion of the genome and are subject to convergent selection.

Protein patterns

The use of protein patterns has not been overly successful in the typing or classification of the Fusaria. The work done prior to 1969 is perhaps best summarised in the paper of Glynn & Reid (1969), in which they concluded that "the results do not support the current concept that this method is a useful taxonomic tool". They confirmed the results of Hall (1967) that numerous factors could affect protein patterns, as is to be expected, although under rigidly controlled conditions reproducible patterns were obtainable. However, neither in their evaluation of 13 *Fusarium* species or 33 forma speciales of *F. oxysporum* were consistent or sufficient differences found to make taxonomic decisions.

The central problem with proteins as markers is the vast number which can be generated from an organism, many of them evanescent or dependent on particular cultural conditions or growth stages. Faced with so much data only sophisticated data analysis can hope to draw meaningful conclusions. The ready availability of computers has made numerical taxonomy more accessible and some later studies on other fungi have proved useful, but for the Fusaria this methodology has fallen into disuse.

Accordingly investigators have perforce adopted strategies for selecting subsets of proteins by analysing serological markers, zymograms or isozymes.

Zymograms

Interest in zymograms arose in the 1960's and Meyer *et al.* (1964) first applied this to the Fusaria. Using a single isolate of each of eight forma speciales they found no differences in pectin methyl esterase patterns and only minor phosphatase differences but suggested that the esterase patterns could be specific to forma. They did note that carbon source affected the results and also that mutations or other isolates gave different patterns. In a later work (Meyer & Reynard, 1969) they reversed their opinion after finding that there was as much variation among isolates and repeat runs as there was between forma speciales.

Reddy & Stahman (1972) studied 13 enzymes, mostly dehydrogenases, and found five enzymes which gave distinctive patterns for each of five f. spp. of *F. oxysporum*, but again only one isolate of each was used. Scala *et al.* (1981) studied endopolygalacturonase zymograms by isoelectric focusing and found all 14 isolates comprising five f. spp of *F. oxysporum* to have the same four band pattern. Other species had different patterns ranging from 8 to 11 bands and they suggested that the method was suitable for use at species level. Ho *et al.* (1985) returned to esterase and protein patterns to try and distinguish among *F. oxysporum* f. spp. and non-pathogenic isolates, but were unable to find correlations and also noted major and inconsistent results between disc-electrophoresis and isoelectric focusing.

Immunology

The literature on serotaxonomy of the Fusaria in general is somewhat limited in extent, and much of the early work is flawed by analysis of only a few strains (Madhosingh, 1964; Morton & Dukes, 1966; Kalyanasundaram & Charudattan, 1969; Kaiser *et al.*, 1976; Abd-el-Rehim & Fadel, 1980).

Hornok (1979, 1980) introduced tandem-crossed immunoelectrophoresis to the field and was able to construct a phenogram of the species investigated which confirmed Booth's (1971) approach to the section Gibbosum rather than that of

Joffe (1974) among others. Iannelli *et al.* (1982) using double diffusion techniques were able to differentiate isolates at species, forma speciales and race levels. They stated that central to their success were two methodologies; the use of Triton X-100 for antigen extraction and cross absorption of the antiserum with an appropriate antigen. In this way they were able to obtain useful monospecific antibodies. Later this group (Iannelli *et al.* 1983) used monoclonal antibodies for the differentiation of species and forma speciales but the differences were quantitative and not qualitative as previously.

NUCLEIC ACIDS

Molecular biological techniques are rapidly gaining popularity for the typing of fungi at species and race levels. This is a logical progression as these methods deal with a direct comparison of the genome, and not as in protein analysis, a gene product.

DNA reassociation

At the species level the technique of DNA reassociation would seem to be the most appropriate. DNA when heated will separate into complementary strands (melt) and re-anneal on cooling. DNA from different species can also be combined but due to base mismatches the stability of a hybrid molecule will be compromised and it will melt at a lower temperature, generally accepted as being in the region of 1 C per 1% mismatch (King & Wilson, 1975). There are, however, several caveats. The genome is not a passive structure, randomly acquiring substitutions at a constant rate; some areas are highly conserved, others again highly unstable, all of which may create anomalous results. If too closely related, the reduction in thermal stability may not be measureable and if too much of the genome is sub-

stituted the technique is unreliable (Britten & Kohne, 1968). Thus reassociation is not suitable for very different taxa where mismatches are numerous, nor for the analysis of strains which differ too little in the nuclear DNA, e.g. races within a forma specialis. Although well established in bacteria since the early sixties, relatively few studies have been done on fungi in general (Dutta & Ojha, 1972; Jahnke & Bahnweg, 1986; Jahnke *et al.*, 1987; Vilgalys, 1988) and only Szecsi & Dobrovolsky (1985a) and Ellis (1988) have published on *Fusarium*.

Using this approach, Szecsi & Dubrovolsky (1985a) were able to construct a phylogenetic tree using single isolates of each of 11 *Fusarium* species, all hybridised against *F. oxysporum*. It is notable, however, that the standard deviation of many of the melt temperatures exceeded the differences between species in this work. They also (Szecsi & Dubrovolsky, 1985b) used a comparison of DNA melting profiles to calculate genetic differences on the same group of fungi and arrived at a very different phylogenetic tree.

Ellis (1988) investigated isolates in the section Liseola and found that the two species proposed by Nelson *et al.* (1983), *Gibberella fujikuroi* and *G. subglutinans* gave a better reflection of DNA homologies than the four varieties of a single species proposed by Kuhlman (1982) on the basis of mating groups and morphology. It appeared in this study that 60% or greater homology defined a species.

dsRNA and Plasmids

Segmented double-stranded RNAs (dsRNAs) are common in fungi and usually associated with viruses (Day & Dodds, 1979; Ghabrail, 1980). dsRNA banding patterns have been correlated with pathogenicity in respect of the host range (Newton *et al.*, 1985) or decreased pathogenicity (Van Alfen *et al.*, 1975; Dodds, 1980). Attempts have been made to associate the patterns with fungal race or geographic origin (Hunst *et al.*, 1986) but the results have been inconclusive.

This is to be expected as one is here dealing with an independently trans-

missable agent which is not necessarily passed on to all the progeny, nor is infection likely to be consistent across a group of fungi. There may be cases where a particular pattern can act as a marker for a particular population but this cannot be expected to be stable.

Similar arguments apply to the presence of linear DNA plasmids which, too, are common in fungi and often associated with the mitochondria (Tudzynski & Esser, 1985). Kistler & Leong (1986) working with *F. oxysporum* f. sp. *conglutinans* found 1,9 kilo-base (kb) plasmids which appeared to be specific to f. sp. *conglutinans* and were different in races 1 and 5 which attack cabbage and race 2 which attacks radish. Whether the plasmids contain genes which confer host specificity or the isolates, which were distributed worldwide, are clones of the race progenitors is still under investigation.

Rubidge (1986) screened 23 strains representing 13 species of *Fusarium* but was only able to find plasmid DNA in one strain.

Restriction fragment patterns

DNA restriction enzymes recognise and cleave DNA at highly specific sequences, thus creating fragments of defined length. These fragments can be separated according to molecular size by electrophoresis in acrylamide or agarose gels and revealed with dyes such as ethidium bromide, by silver staining, or by autoradiography, if the fragments are first labelled with a radioactive tracer. An alternative is to probe the fragments using the techniques of Southern blotting and hybridisation with labelled nucleic acid. In this situation only those fragments containing a sequence complementary to the probe will be revealed.

In a seminal paper by Botstein *et al.* (1980) this technology was proposed as the basis for the creation of a genetic linkage map using these restriction fragment length polymorphisms as marker loci. The theoretical implications were rapidly taken up for agricultural species (reviewed by Beckman & Soller, 1986) and the

technique was soon applied to problems in fungal taxonomy.

Total DNA

Potentially the simplest of these methods is the restriction digest of total DNA which produces a complex pattern of bright bands against a smear of background DNA. The brighter bands are generated from multicopy sequences such as found in the nuclear ribosomal RNA coding DNA (rDNA) and mitochondrial DNA mtDNA). Klich & Mullaney (1987) were able to distinguish between two species of *Aspergillus* on this basis and Coddington *et al.* (1987) were able to separate races 1, 2 and 6 of *F. oxysporum* f. sp. *pisi.* For such a simple technique, it is surprising that it has not been put to more use.

Mitochondrial DNA

A logical progression of the idea is to investigate the restriction patterns of mitochondrial DNA as the genome is much simpler and has a number of repeat sequences. mtDNA also evolves faster than nuclear DNA, and thus is appropriate for analysis at the subspecific level. The technique is well established for animal mitochondria (reviewed by Avise & Lansman, 1983) which are in the range of 16 - 19 kilo-basepairs (kbp), but still developing for the fungi, which have widely variable mitochondria ranging in size from 19 - 176 kbp (Taylor, 1986), *Fusarium oxysporum* having a size of 46,5 kbp (Marriot *et al.*, 1984). There are some complicating factors for mtDNA in fungi in that, unlike animal mitochondria, they are not necessarily uniparentally inherited, and contain a large number of deletions and insertions giving restriction length mutations (see review by Taylor, 1986). Thus to determine evolutionary relationships it becomes necessary to map the mitochondrial genome and not work purely from fragment lengths.

Notwithstanding, Kozlowsky & Stepien (1982) successfully applied restriction length polymorphisms of mitochondrial DNA to the taxonomy of the genus

Aspergillus, finding significant divergences from the then accepted status. The methodology has been extended to other filamentous fungi (Garber & Yoder, 1984; Klimczak & Prell, 1984; Janke *et al.*, 1987).

Probe RFLPs

The third, and technically slightly more complex method of restriction polymorphism analysis is the use of probes, be they defined or random, applied to total DNA extracts. Usually the total DNA extracts are restricted with endonucleases, the fragments electrophoretically separated on an agarose gel and blotted to nitrocellulose or nylon membranes. These are then probed with radioactively labelled probes such as cloned sequences or defined DNA or RNA fragments. A pattern of bands is revealed depending on the complementary sequences and the restriction sites they contain. The resulting 'fingerprints' are highly specific and in the case of humans have recently been accepted as legal forensic evidence of identity (Jeffreys *et al.*, 1985; Marx, 1988).

Their use in fungal identification and taxonomy is as yet in its infancy but was foreshadowed in an article by Wu *et al.* (1983) on DNA polymorphisms in *Coprinus*. Garber & Yoder (1984) used a cloned mtDNA probe as an ancillary to mtDNA restriction analysis in their analysis of *Cochliobolus* populations.

Vincent *et al.* (1986) used ribosomal RNA as a probe in conjunction with mtDNA analysis in the grouping of *Histoplasma capsulatum*, a pathogen of vertebrates, into three classes. Manicom *et al.* (1987) suggested the use of random probes for the identification of strains and species in *Fusarium* and this was followed by similar studies by Kistler *et al.* (1987), also on *Fusarium*, using cloned mtDNA and rDNA as probes. The latter probes were unable to distinguish among pathotypes, but mtDNA probes could separate forma speciales. Anderson *et al.* (1987), using similar technology, were able to differentiate among the eight biological species of *Armillaria*, as defined by mating type. Magee *et al.* (1987) also used

rDNA probes for the identification of *Candida* species, and although effective, ethidium stained mtDNA digests were easier and gave the same results. Hulbert & Michelmore (1988) used a number of random probes for analysing populations of *Bremia lactucae* and were able to use the data for some genetic analysis.

Based on the literature review it was decided that RFLPs offered a very specific method of identifying isolates and it was hoped, higher level groups. Helentjaris & Gesteland (1983) had proposed the use of random probes for human RFLPs and the first task in this work was seen as determining the necessary techniques and difficulties as regards the Fusaria, and assessing the usefulness of the information obtained on identification and taxonomic groups.

REFERENCES

ABD-EL-REHIM, M. A. & FADEL, F. M., 1980. Immunoelectrophoretical differences between phytopathogenic *Fusarium* species. *Biochemical Systematics and Ecology* 8: 7-9.

ANDERSON, J. B., PETSCHE, D. M. & SMITH M. L., 1987. Restriction fragment polymorphisms in biological species of *Armillaria mellea*. *Mycologia* 79: 69-76.

ARMSTRONG, G. M. & ARMSTRONG, J. K., 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. Pages 391-399. *In:* Fusarium, Diseases, Biology, and Taxonomy. P. E. Nelson, T. A. Toussoun & R. J. Cook, Eds. The Pennsylvania State University Press, University Park.

AVISE, J. C. & LANSMAN, R. A., 1983. Polymorphism of mitochondrial DNA in populations of higher animals. Pages 147-164. *In*: Evolution of genes and proteins. M. Nei & R. K. Keohn, Eds. Sinauer, Sunderland, Mass.

BECKMANN, J. S. & SOLLER, M., 1986. Restriction fragment length polymorphisms in plant genetic improvement. Pages 197-250. *In:* Oxford surveys of plant molecular and cell biology. B. J. Miflin, Ed. Oxford Press, Oxford.

BILAI, V. I., 1970. Experimental morphogenisis in the fungi of the genus *Fusarium* and their taxonomy. *Annals of the Academy of Science of Finland A, IV Biologica* 168: 7-18.

BOOTH, C., 1971. The genus *Fusarium*. Commonwealth Mycological Institute, Kew.

BOSLAND, P. W. & WILLIAMS, P. H., 1987. An evaluation of *Fusarium oxysporum* from crucifers based on pathogenicity, isozyme polymorphism, vegatative compatibility, and geographic origin. *Canadian Journal of Botany* 65: 2067-2073.

BOTSTEIN, D., WHITE, R. L., SKOLNICK, M. & DAVIS, R. W., 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics* 32: 314-331.

BOUHOT, D., 1981. Some aspects of pathogenic potential in forma speciales and races of *Fusarium oxysporum* on Cucurbitaceae. Pages 318-326. *In:* Fusarium, Diseases, Biology, and Taxonomy. P. E. Nelson, T. A. Toussoun & R. J. Cook, Eds. The Pennsylvania State University Press, University Park.

BRITTEN, R. J. & KOHNE, D. E., 1968. Repeated sequences in DNA. *Science* 161: 529-540.

BURGESS, L. W., 1981. General ecology of the Fusaria. Pages 225-235. *In:* Fusarium, Diseases, Biology, and Taxonomy. P. E. Nelson, T. A. Toussoun & R. J. Cook, Eds. The Pennsylvania State University Press, University Park.

CODDINGTON, A., MATTHEWS, P. M., CULLIS, C. & SMITH, K. H., 1987. Restriction digest patterns of total DNA from different races of *Fusarium oxysporum* f. sp. *pisi* - an improved method of race classification. *Journal of Phytopathology* 118: 9-20.

CORRELL, J. C., PUHALLA, J. E., SCHNEIDER, R. W. & KRAFT, J. M., 1985. Differentiating races of *Fusarium oxysporum* f. sp. *pisi* based on vegetative compatibility. *Phytopathology* 75: 1347 (abs.).

CORRELL, J. C., KLITTICH, C. J. R. & LESLIE, J. F., 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77: 1640-1646.

COVE, D. J., 1976. Chlorate toxicity in *Aspergillus nidulans*: the selection and characterization of chlorate resistant mutants. *Heredity* 36: 201-203.

DAY, P. R. & DODDS, J. A., 1979. Viruses of plant pathogenic fungi. Pages 202-239. *In:* Viruses and Plasmids in Fungi. L. Lemke, Ed. Marcel Dekker, New York.

DODDS, J. A., 1980. Association of Type 1 virus-like dsRNA with club-shaped particles in hypovirulent strains of *Endothia parasitica*. *Virology* 107: 1-12.

DUTTA, S. K. & OJHA, M., 1972. Relatedness between major taxonomic groups of fungi based on the measurement of DNA nucleotide sequence homology. *Molecular and General Genetics* 114: 232-240.

ELLIS, J. J., 1988. Section *Liseola* of *Fusarium*. *Mycologia* 80: 255-258.

ELMER, W. H. & STEVENS, C. T., 1986. Vegetative compatibility groupings in *Fusarium oxysporum* f. sp. *asparagi*. *Phytopathology* 76: 1114 (abs.).

GARBER, R. C. & YODER, O. C., 1984. Mitochondrial DNA of the filamentous ascomycete *Cochliobolus heterostrophus*. *Current Genetics* 8: 621-628.

GERLACH, W., 1970. Suggestions to an acceptable modern Fusarium system. *Annals of the Academy of Science of Finland A, IV Biologica* 168: 37-49.

GERLACH, W. & NIRENBERG, H., 1982. The genus *Fusarium* - A Pictorial Atlas. *Mitteilungen aus der Biologieschen Bundesanstalt fur Land-und Forstwirtschaft Berlin Dahlem* 209: 1-406.

GHABRIAL, S. A., 1980. Effects of fungal viruses on their hosts. *Annual Review of Phytopathology* 18: 441-461.

GLYNN, A. N. & REID, J., 1969. Electrophoretic patterns of soluble fungal proteins and their possible use as taxonomic criteria in the genus *Fusarium*. *Canadian Journal of Botany* 47: 1823-1831.

GORDON, W. L., 1952. The occurence of *Fusarium* species in Canada. II. Prevalence and taxonomy of *Fusarium* species in cereal seed. *Canadian Journal of Botany* 30: 209-251.

GORDON, W. L., 1960. The taxonomy and habitats of *Fusarium* species from tropical and temperate regions. *Canadian Journal of Botany* 38: 643-658.

HALL, R., 1967. Protein and catalase isoenzymes from *Fusarium solani* and their taxonomic significance. *Australian Journal of Biological Science* 20: 419-428.

HELENTJARIS, T. & GESTELAND, R., 1983. Evaluation of random cDNA clones as probes for human restriction fragment polymorphisms. *Journal of Molecular and Applied Genetics* 2: 237-247.

HO, Y. W., VARGHESE, G. & TAYLOR, G. S., 1985. Protein and esterase patterns of pathogenic *Fusarium oxysporum* f. sp. *elaedis* and *F. oxysporum* var. *redolens* from Africa and non-pathogenic *F. oxysporum* from Malaysia. *Phytopatologische Zeitschrift* 114: 301-311.

HORNOK, L., 1979. Comparison of *Fusarium acuminatum* and *Fusarium culmorum* isolates by means of tandem-crossed immunoelectrophoresis. *Antonie van Leeuwenhoek* 45: 293-302.

HORNOK, L., 1980. Serotaxonomy of *Fusarium* species of the sections Gibbosum and Discolor. *Transactions of the British mycological Society* 74: 313-319.

HULBERT, S. H. & MICHELMORE, R. W., 1988. DNA restriction fragment length polymorphism and somatic variation in the lettuce downy mildew fungus, *Bremia lactucae. Molecular Plant-microbe Interactions* 1: 17-24.

HUNST, P. L., LATTERELL, F. M. & ROSSI, A. E., 1986. Variation in double-stranded RNA from isolates of *Pyricularia oryzae. Phytopathology* 76: 674-678.

IANNELLI, D., CAPPARELLI, R., CRISTINZIO, G., MARZIANO, F., SCALA, F. & NOVIELLO, C., 1982. Serological differentiation among forma speciales and physiological races of *Fusarium oxysporum. Mycologia* 74: 313-319.

IANNELLI, D., CAPPARELLI, R., MARZIANO, F., SCALA, F. & NOVIELLO, C., 1983. Production of hybridomas secreting monoclonal antibodies to the genus *Fusarium. Mycotaxon* 17: 523-532.

JAHNKE, K. D. & BAHNWEG, G., 1986. Assessing natural relationships in the Basidiomycetes by DNA analysis. *Transactions of the British mycological Society* 87: 175-191.

JAHNKE, K. D., BAHNWEG, G. & WORRALL, J. J., 1987. Species delimitation of the *Armillaria mellea* complex by analysis of nuclear and mitochondrial DNAs. *Transactions of the British mycological Society* 88: 572-575.

JEFFREYS, A. J., WILSON, V. & THEIN, S. L., 1985. Individual-specific 'fingerprints' of human DNA. *Nature* 316: 76-79.

JOFFE, A. Z., 1974. A modern system of *Fusarium* taxomony. *Mycopathologia et Mycologia Applicata* 53: 201-218.

KAISER, S. A. K. M. & SEN GUPTA, P. K., 1976. Serological and electrophoretic studies of three formae speciales of *Fusarium oxysporum. Transactions of the British mycological Society* 67: 33-37.

KALYANASUNDARAM, R. & CHARUDATTAN, R., 1969. Serological studies in the genus *Fusarium*: A comparison of strains. *Phytopathologische Zeitschrift* 64: 28-31.

KING, M. C. & WILSON, A. C., 1975. Evolution at two levels. Molecular similarities and biological differences between humans and chimpanzees. *Science* 188: 107-116.

KISTLER, H. C. & LEONG, S. A., 1986. Linear plasmid DNA in the plant pathogenic fungus *Fusarium oxysporum* f. sp. *conglutinans. Journal of Bacteriology* 167: 587-593.

KISTLER, H. C., BOSLAND, P. W., BENNY, U., LEONG, S. & WILLIAMS, P. H., 1987. Relatedness of strains of *Fusarium oxysporum* from crucifers measured by examination of mitochondrial and ribosomal DNA. *Phytopathology* 77: 1289-1293.

KLICH, M. A. & MULLANEY, E. J., 1987. DNA restriction enzyme fragment polymorphism as a tool for rapid differentiation of *Aspergillus flavus* from *Aspergillus oryzae*. *Experimental Mycology* 11: 170-175.

KLIMCZAK, L. J. & PRELL, H. H., 1984. Isolation and characterization of mitochondrial DNA of the oomycetous fungus *Phytophthora infestans*. *Current Genetics* 8: 323-326.

KOSLOWSKI, M. & STEPIEN, P. P., 1982. Restriction enzyme analysis of mitochondrial DNA of members of the genus *Aspergillus* as an aid in taxonomy. *Journal of General Microbiology* 128: 471-476.

KUHLMAN, E. G., 1982. Varieties of *Gibberella fujikuroi* with anamorphs in *Fusarium* section *Liseola*. *Mycologia* 74: 759-768.

MADHOSINGH, C., 1964. A serological comparison of three *Fusarium* species. *Canadian Journal of Botany* 42: 1143-1146.

MAGEE, B. B., D'SOUSA, T. M. & MAGEE, P. T., 1987. Strain and species identification by restriction fragment length polymorphisms and ribosomal DNA repeat of *Candida* species. *Journal of Bacteriology* 169: 1639-1643.

MANICOM, B. Q., BAR-JOSEPH, M., ROSNER, A., VIGODSKY-HAAS, H. & KOTZE, J.M., 1987. Potential applications of random DNA probes and restriction fragment length polymorphisims in the taxonomy of the Fusaria. *Phytopathology* 77: 669-672.

MARRIOT, A. C., ARCHER, S. A. & BUCK, K. W., 1984. Mitochondrial DNA of *Fusarium oxysporum* is a 46.5 kilobase pair circular molecule. *Journal of General Microbiology* 130: 3001-3008.

MARX, J. L., 1988. DNA fingerprinting takes the witness stand. *Science* 240: 1616-1618.

MATUO, T., 1972. Taxonomic studies of phytopathogenic fusaria in Japan. *Review of Plant Protection Research* 5: 34-45.

MESSIAEN, C. M. & CASSINI, R., 1968. Recherches sur les fusarioses IV. La systematique des *Fusarium*. *Annales de Epiphytes* 19: 387-454.

MEYER, J. A., GARBER, E. D. & SHAEFFER, S. G., 1964. Genetics of phytopathogenic fungi. XII. Detection of esterases and phosphatases in culture filtrates of *Fusarium oxysporum* and *F. xylaroides* by starch-gel electrophoresis. *Botanical Gazette* 125: 298-300.

MEYER, J. A. & REYNARD, J. L., 1969. Protein and esterase patterns of two forma speciales of *Fusarium oxysporum*. *Phytopathology* 59: 1409-1411.

MORTON, D. J. & DUKES, P. D., 1966. Serological differentiation of race 1 from race 2 of *Fusarium oxysporum* f. sp. *lycopersici*. *Plant Disease Reporter* 50: 444-445.

NELSON, P. E., TOUSSON, T. A. & MARASAS, W. F. O., 1983. *Fusarium* species. An illustrated manual for identification. The Pennsylvania State University Press, University Park.

NEWTON, A. C., CATEN, C. E. & JOHNSON, R., 1985. Variation for isozymes and double-stranded RNA among isolates of *Puccinia striformis* and two other cereal rusts. *Plant Pathology* 34: 235-247.

PLOETZ, R. C. & CORRELL, J. C., 1988. Vegetative compatibility among races of *Fusarium oxysporum* f. sp. *cubense*. *Plant Disease* 72: 325-328.

PUHALLA, J. E., 1981. Genetic considerations of the genus *Fusarium*. Pages 291-305. *In:* Fusarium: Diseases, Biology, and Taxonomy. P. E. Nelson, T. A. Tousson & R. J. Cook, Eds. The Pennsylvania State University Press, University Park.

PUHALLA, J. E., 1984a. A visual indicator of heterokaryosis in *Fusarium oxysporum* from celery. *Canadian Journal of Botany* 62: 540-545.

PUHALLA, J. E., 1984b. Races of *Fusarium oxysporum* f. sp. *apii* in California and their genetic interrelationships. *Canadian Journal of Botany* 62: 546-550.

PUHALLA, J. E., 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatability. *Canadian Journal of Botany* 63: 179-183.

PUHALLA, J. E. & SPIETH, P. T., 1983. Heterokaryosis in *Fusarium moniliforme*. *Experimental Mycology* 7: 328-335.

PUHALLA, J. E. & SPIETH, P. T., 1985. A comparison of heterokaryosis and vegetative incompatibility among varieties of *Gibberella fujikuroi (Fusarium moniliforme)*. *Experimental Mycology* 9: 39-47.

REDDY, M. N. & STAHMAN, M. A., 1972. Isozyme patterns of *Fusarium* species and their significance in taxonomy. *Phytopathologische Zeitschrift* 74: 115-125.

RIALLO, A., 1950. Griby roda *Fusarium*. State Publication, Moskva.

RUBIDGE, T., 1986. Survey of *Fusarium* species for plasmid-like DNA and some evidence for its occurrence in a strain of *F. merismoides*. *Transactions of the British mycological Society* 87: 463-466.

SCALA, F., CRISTINZIO, G., MARZIANO, F. & NOVIELLO, C., 1981. Endopolygalacturonase zymograms of *Fusarium* species. *Transactions of the British mycological Society* 77: 587-591.

SNYDER, W. C., 1981. Introduction. Pages 1-8. *In:* Fusarium: Diseases, Biology, and Taxonomy. P. E. Nelson, T. A. Tousson & R. J. Cook, Eds. The Pennsylvania State University Press, University Park.

SNYDER, W. C. & HANSEN, H. N., 1940. The species concept in *Fusarium*. *American Journal of Botany* 27: 64-67.

SNYDER, W. C. & HANSEN, H. N., 1941. The species concept in *Fusarium* with reference to section Martiella. *American Journal of Botany* 28: 738-742.

SNYDER, W. C. & HANSEN, H. N., 1945. The species concept in *Fusarium* with reference to Discolor and other sections. *American Journal of Botany* 32: 657-666.

SNYDER, W. C. & TOUSSON, T. A., 1965. Current status of taxonomy in *Fusarium* species and their perfect stages. *Phytopathology* 75: 833-837.

SZECSI, A. & DOBROVOLSKY, A., 1985a. Phylogenetic relationships among *Fusarium* species measured by DNA reassociation. *Mycopathologica* 89: 89-94.

SZECSI, A. & DOBROVOLSKY, A., 1985b. Genetic distance in fungus genus *Fusarium* measured by comparative computor analysis of DNA thermal denaturation profiles. *Mycopathologica* 89: 95-100.

TAYLOR, J. W., 1986. Fungal evolutionary biology and mitochondrial DNA. *Experimental Mycology* 10: 259-269.

TOUSSON, T. A. & NELSON, P. E., 1975. Variation and speciation in Fusaria. *Annual Review of Phytopathology* 13: 71-82.

TUDZYNSKI, P. & ESSER, K., 1985. Mitochondrial DNA for gene cloning in eukaryotes. Pages 403-416. *In:* Gene manipulations in fungi. J. W. Bennet & L. L. Lasure, Eds. Academic Press, New York.

VAN ALFEN, N. K., JANES, R. A., ANAGNOSTAKIS, S. L. & DAY, P. R., 1975. Chestnut blight: biological control by transmissible hypovirulance in *Endothia parasitica. Science* 189: 890-891.

VILGALYS, R., 1988. Genetic relatedness among anastomosis groups in *Rhizoctonia* as measured by DNA/DNA hybridization. *Phytopathology* 78: 698-702.

VINCENT, R. D., GOEWERT, R., GOLDMAN, W. E., KOBAYASHI, G. S., LAMBOWITZ, A. M. & MEDOFF, G., 1986. Classification of *Histoplasma capsulatum* isolates by restriction fragment polymorphisms. *Journal of Bacteriology* 165: 813-818.

WAITE, B. H. & STOVER, R. H., 1960. Studies on *Fusarium* wilt of bananas. VI. Variability and the cultivar concept in *Fusarium oxysporum* f. *cubense. Canadian Journal of Botany* 38: 985-994.

WOLLENWEBER, H. W. & REINKING, O. A., 1935. Die Fusarien, ihre Beschreiben, Schadwirkung und Bekampfung. Paul Parey, Berlin.

WU, M. M. J., CASSIDY, J. R. & PUKKILA, P.J., 1983. Polymorphisms in DNA of *Coprinus cinereus*. *Current Genetics* 7: 385-392.

CHAPTER 2

POTENTIAL APPLICATIONS OF RANDOM DNA PROBES AND RESTRICTION FRAGMENT LENGTH POLYMORPHISMS IN THE TAXONOMY OF THE FUSARIA

INTRODUCTION

The genus *Fusarium* comprises a wide and heterogeneous group of fungi, many of considerable importance in industry, as food contaminants, and as pathogens in agriculture. Taxonomically, the species are notorious for their variability, especially in culture, to the extent that different species are morphologically identical (Puhalla, 1981). Furthermore, within a species, *sensu* Snyder and Hansen (1940), forma speciales are only identifiable in terms of host specificity.

Attempts have been made to classify *Fusarium* on the basis of soluble protein electrophoretic patterns (Glyn & Reid, 1969), zymograms (Scala *et al.*, 1981), immunoelectrograms (Abd-el-Rahim & Fadel, 1980), and monoclonal antibody reactions (Iannelli *et al.*, 1983), with partial success. Restriction fragment length

---

This chapter has been previously published as:

MANICOM, B. Q., BAR-JOSEPH, M., ROSNER, A., VIGODSKY-HAAS, H. & KOTZE, J.M., 1987. Potential applications of random DNA probes and restriction fragment length polymorphisims in the taxonomy of the Fusaria. *Phytopathology* 77: 669-672.

polymorphisms (RFLPs) provide a powerful technique for the determination of molecular variation at the DNA level.

Polymorphic markers have applications, among others, in taxonomy and in identifying marker loci of biological significance (Beckmann & Soller, 1983), and they are becoming widely used for genetic analysis and linkage mapping (Botstein *et al.*, 1980). This study essays to evaluate the application of RFLP methodology as a tool for addressing some of the problems of *Fusarium* taxonomy.

MATERIALS AND METHODS

Fungal cultures

Fusarium isolates were either newly isolated or cultures were obtained from the collection of Dr. H. Vigodsky-Haas, Volcani Centre, Israel. Original sources and maintenance media are listed in Table 1. All cultures were single spored and stocks were maintained on carnation leaf agar (CLA) (Fisher *et al.*, 1982).

Preparation of DNA

Spore suspensions from CLA plates were used to inoculate cellophane-covered potato-dextrose agar (PDA) plates. Mycelium was collected after 7-10 days growth at room temperature, lyophilized, and stored at -20 C. Various methods of DNA preparation were tested (Garber & Yoder, 1983; Garber & Yoder, 1984; Murray & Thompson, 1980; Specht *et al.*, 1982), and the following modification of the method of Murray and Thompson (1980) was selected. Lyophilized mycelium (0,5 g) was ground with a mortar and pestle in the presence of sand and added to 15 ml of hot extraction buffer (1% cetyltrimethylammonium bromide [CTAB], 50 mM tris-HCl, 0,7 M NaCl, 10 mM EDTA, pH 8,0) and kept at 65 C for 10 min. The

suspension was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged at 12.000g for 10 min at room temperature. The upper aqueous phase was tranferred to a clean tube, 0,1 volume of extraction buffer containing 10% CTAB was added, and the extraction was repeated.

The upper phase was mixed with an equal volume of 1% CTAB, 50 mM tris-HCl (pH 8,0) and 10 mM EDTA to precipitate nucleic acids and then incubated at room temperature for 30 min. The DNA was pelleted at 3.000g for 5 min, washed twice by centrifugation with a large volume of cold 70% ethanol/0,3 M sodium acetate (pH 5,5), and taken up in 700 µl of TE buffer (10 mM tris-HCl, 1 mM EDTA, pH 8,0). The preparation was digested with 100 µg of DNAse-free RNAse for an hour at 37 C and then with 10 µg self-digested proteinase K for an hour at 37 C. Proteinaceous material was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and then with chloroform/isoamyl alcohol (24:1). The DNA was precipitated with 0,1 volume 3 M sodium acetate and 0,6 volume isopropanol, taken up in TE, adjusted spectrophotometrically to 1 µg/ml, and stored at -20 C.

Construction of a DNA library

Standard protocols were used throughout (Maniatis *et al.*, 1982). Total DNA of isolate 1 of *Fusarium oxysporum* f. sp. *dianthi* (Prill. & Del.) was digested with Hind III and ligated into the Hind III site of pBR 322 that was used to transform *Escherichia coli* MM 294. Plasmid DNA containing *Fusarium* DNA inserts was prepared from ampicillin-resistant, tetracycline-susceptible clones by the alkaline lysis method (Maniatis *et al.*, 1980).

Southern blots and hybridization

Approximately 8 µg per lane of total DNA was digested overnight with 40

units of restriction enzyme according to manufacturer's (Boehringer) recommendations, and the DNA was separated on 1% agarose gels at 0,75 V/cm until the bromophenol blue dye front had moved 14 cm. The methods of Meinkoth and Wahl (1983) were used for blotting and hybridization. Plasmid probes were labelled with $dCT^{32}P$ to a specific activity of approximately 10^8 dpm/µg by nick translation as recommended for the Amersham kit N. 5000. The hybridization mix contained 50% formamide and hybridization was at 42 C overnight. Blots were washed for 2 X 15 min in 1 X SSPE (0,15 M NaCl, 0,01 M NaH_2PO_4, 0,001 M EDTA, pH 7,4), 0.1% SDS at room temperature and 2 X 15 min in 0,1 X SSPE, 0,1 % SDS at 42 C. Autoradiography was for 18 hr to several days at -70 C with intensifying screens.

RESULTS

DNA preparations

The alternative methods of DNA preparation tested (Garber & Yoder, 1983 & 1984; Specht *et al.*, 1982) resulted in *Fusarium* DNA preparations with heavy polysaccharide contamination. The method as described is rapid and yields in excess of 1 mg of DNA per gram of dried mycelium, of a size greater than the 23 kilobase-pair (kbp) lambda marker with an $A_{260/280}$ of 1,8-2,0.

Ethidium-stained gels

Bands of repetitive DNA were faintly visible with several hexanucleotide-recognizing restriction enzymes. Eco R1 produced two distinct bands, at 3,2 and 4,2 kb, respectively, with 10 isolates of *F. oxysporum* of widely different cultural morphology and comprising four different forma speciales. The 3,2 kb band was also common to an isolate of each of the eight other species of the Snyder and

Hansen classification of the Fusaria, each of which had different pattern of fainter bands (data not shown).

Hybridization patterns with selected probes

In initial tests, plasmid minipreparations (Maniatis *et al.*, 1982) of 60 random recombinant clones were screened by electrophoresis and eight arbitrary choices with inserts ranging from 600 to 4.000 base pairs (bp) were further analyzed for restriction patterns with seven restriction enzymes recognizing hexanucleotide sites. Four of these plasmids containing inserts with multiple restriction sites were selected, labelled by nick translation and hybridized to blots of four *F. oxysporum* isolates (f. sp. *dianthi*, isolates 1 and 2; f. sp. *lycopersici* race 1 and f. sp. *gladioli*, isolate 2).

As shown in the Southern blots depicted in Figure 1, each of the four cloned probes hybridized to fragments of the expected size in Hind III digests of the source isolate. This fragment was identical over the four isolates for probe A3 (950 bp) but polymorphic for probe B2 (1.550 bp), enabling a differentiation between f. sp. *dianthi* and the other two forma speciales. Probe E8 (760 bp) separated the two isolates of f. sp. *dianthi* by means of an additional band in isolate 1. Probe D4 (3.400 bp) gave multiple bands that were highly polymorphic among the three forma speciales but identical for the two f. sp. *dianthi* isolates (Fig. 1; a band at 3,1 kb has not reproduced well - *cf.* Fig. 2).

When the fungal DNA was digested with Eco R1, further polymorphisms were evident. Probe A3 now distinguished between the two f. sp. *dianthi* isolates on the basis of an extra band, as did probe E8, which in addition separated f. sp. *dianthi* from f. spp. *lycopersici* and *gladioli*. Probe B2 also distinguished between the latter two. Probe D4 gave the same results as before, although the band patterns differed. When tested against Hind III digestion blots of isolates from each of the eight Fusarium species of the Snyder and Hansen classification, probe D4

reacted only with a subset of members of the species *oxysporum* (Fig. 2). The RFLP pattern obtained for isolate 3 of f. sp. *dianthi* (Fig. 2, lane j) was identical to that obtained for isolates 1 and 2, although it had been isolated from the same field more that 7 yr previously and had mutated in culture to a highly pigmented pionnotal form (Waite & Stover, 1960). Results were consistent over three batches of *Fusarium* DNA preparations and digestions. A Bam H1 digestion of the set of isolates shown in Figure 2 probed with D4 also gave useful polymorphisms within the *F. oxysporum* species shown while giving a single band for the other species (data not shown).

DISCUSSION

Ethidium staining of restriction digests offers the possibility of a rapid confirmation of species identification, although a large collection of isolates would have to be surveyed to establish this. It is, however, difficult to visualize repetitive bands on the smear obtained with a digest of total DNA. For this methodology it would be better to use the less complex mitochondrial DNA, as has been done with other fungi (Collins & Lambowitz, 1983; Garber & Yoder, 1984). Hybridization patterns, on the other hand, were highly effective, and by using a limited set of probes and restriction enzymes we were able to distinguish isolates at species, forma speciales, race and isolate levels (Figs 1 and 2). The RFPL patterns were also conserved over time and despite morphological differences in culture.

The hybridization patterns and signal strengths of probes A3, B2, and E8 are indicative of sequences with one or a few copies per genome, whereas the multiple banding of probe D4 is of the type associated with satellite DNA, repetitive DNA, transposable elements, or gene family hybridization patterns. Previous DNA reassociation experiments on various fungi have shown that repetitive DNA ranges

from 2-3% in *Aspergillus nidulans* (Eidam) Wint. (Timberlake, 1978) to 21-23% in *F. graminearum* Schwabe (Szecsi, 1981). The sequence organization of repetitive DNA in the fungi has been found to be of a very long interspersion pattern (Hudspeth *et al.*, 1977; Kramlauf & Marzluf, 1979) and most is believed to code for ribosomal RNA (Timberlake, 1978).

If probe D4 is recognizing repetitive ribosomal coding DNA of nuclear or mitochondrial origin, one would expect these sequences to be conserved throughout the Fusaria. A similar argument would apply to conserved gene families such as that for tubulin (Lewis *et al.*, 1985). However, when hybridized to a range of *Fusarium* species, probe D4 reacted only with a subset of the species *oxysporum*. It is suggested that D4 is recognizing a component of a gene family particular to a group within the species *oxysporum*, with obvious implications for the taxonomy of this species.

Based on the evidence presented here, RFPLs show the potential of being a useful diagnostic tool in the study of *Fusarium* taxomony. If anything, short probes such as those used here are too specific, and although this in itself has applications in race typing, for example, we would have preferred to find a probe that recognizes a species in its entirety, a not insurmountable problem given the very few probes herein tested. Many isolates must still be screened to establish constant restriction patterns within a group before the method can be applied practically. Probes such as D4, with its multiple-banding pattern and specificity for a group, are seen as being particularly useful in that particular bands could indicate membership in a group and polymorphisms enable finer distinctions to be made within the group.

TABLE 1. List of *Fusarium* isolates and their sources.

Species	Code	[a]Source
F. episphaeria	E15	1
F. lateritium	L112	1
F. moniliforme	M125	1
	MRC3235	5
	MRC2293	5
F. nivale	N13	1
F. oxysporum	MRC3239	5
f. sp. *dianthi* isol. 1	SS1	2
f. sp. *dianthi* isol. 2	SS2	2
f. sp. *dianthi* isol. 3	J	3
f. sp. *gladioli* isol. 1	S3	3
f. sp. *gladioli* isol. 2	S6	3
f. sp. *lycopersici* race 1	TR1	4
f. sp. *lycopersici* race 2	TR2	4
f. sp. *cubense* race 1	NJ	6
f. sp. *cubense* race 4	TM	6
F. rigidiusculum	R37	1
	MRC3224	5
F. roseum var. *graminearum*	GR132	1
	MRC2556	5
	MRC3234	5
var. *culmorum*	MRC2626	5
var. *gibbosum*	MRC3278	5
	MRC3312	5
F. solani	S133	1
	MRC3237	5
F. tricinctum	T415	1
	MRC3295	5

[a]1 = Fusarium Workshop held at University of Minnesota, St. Paul, 1981, via H. Vigodsky-Haas; 2 = field isolates, Israel, 1984; 3 = H. Vigodsky-Haas, Volcani Centre, Israel, 1978; 4 = J. Katan, Hebrew University, Israel; 5 = W.O. Marasas, NRIND, South Africa; 6 = field isolates, South Africa, 1982. Isolates J, S3, S6, TR1 and TR2 had been stored on potato-dextrose agar, all others on carnation leaf agar (see Materials and Methods).

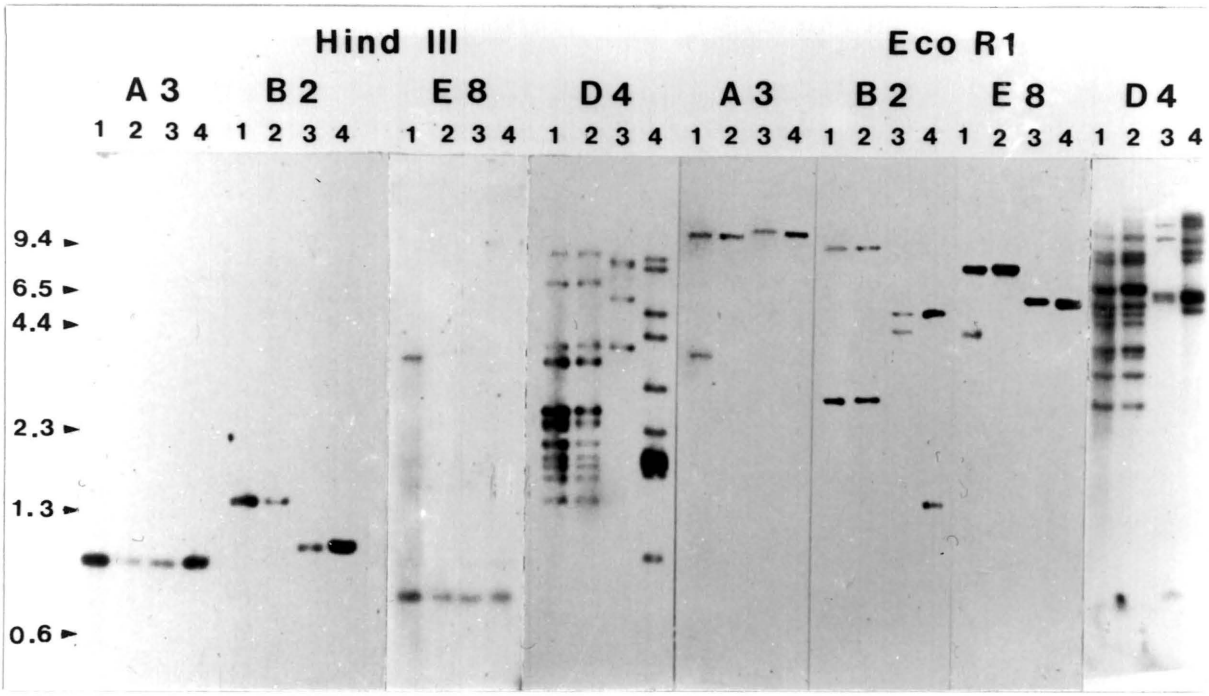


FIG. 1. Composite autoradiogram of restriction fragment length polymorphisms of Southern blots of four isolates of *Fusarium oxysporum* digested with Hind III or Eco R1, run for 14cm on 1% agarose gels, and hybridised to four random recombinant DNA probes derived from f. sp. *dianthi*. 1 = f. sp. *dianthi* isolate 1, 2 = f. sp. *dianthi* isolate 2, 3 = f. sp. *lycopersici* race 1, and 4 = f. sp. *gladioli* isolate 2.

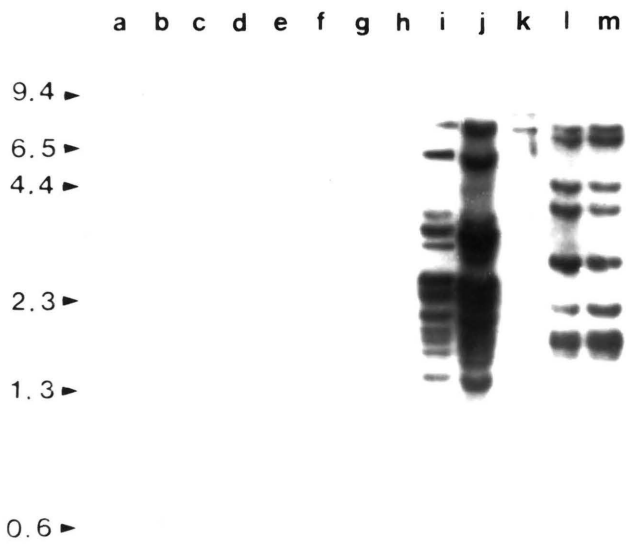


FIG. 2. Composite autoradiogram of restriction fragment length polymorphisms of Hind III digest of *Fusarium* species probed with clone D4. a = T415, b = M125, c = R37, d = S133, e = E15, f = N13, g = L112, h = GR132, i = SS2, j = J, k = TR2, l = S6, and m = S3. Also tested and found negative were MRC 3295, MRC2293, MRC3224, MRC3237, MRC2556, MRC3234, MRC2626, MRC3278, MRC3312, MRC3239, NJ, and TM.

REFERENCES

ABD-EL-REHIM, M. A. & FADEL, F. M., 1980. Immunoelectrophoretical differences between phytopathogenic *Fusarium* species. *Biochemical Systematics and Ecology* 8: 7-9.

BECKMANN, J. S. & SOLLER, M., 1983. Restriction fragment length polymorphisms in genetic improvement: Methodologies, mapping and costs. *Theoretical and Applied Genetics* 67: 35-43.

BOTSTEIN, D., WHITE, R. L., SKOLNICK, M. & DAVIS, R. W., 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics* 32: 314-331.

COLLINS, R. A. & LAMBOWITZ, A. M., 1983. Structural variations and optional introns in the mitochondrial DNAs of Neurospora strains isolated from nature. *Plasmid* 9: 53-70.

FISHER, N. L., BURGESS, L. W., TOUSSON, T. A. & NELSON, P. E., 1982. Carnation leaves as a substrate for preserving cultures of *Fusarium* species. *Phytopathology* 72: 151-153.

GARBER, R. C. & YODER, O. C., 1983. Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal and plasmid components. *Analytical Biochemistry* 135: 416-422.

GARBER, R. C. & YODER, O. C., 1984. Mitochondrial DNA of the filamentous ascomycete *Cochliobolus heterostrophus. Current Genetics* 8: 621-628.

GLYNN, A. N. & REID, J., 1969. Electrophoretic patterns of soluble fungal proteins and their possible use as taxonomic criteria in the genus *Fusarium. Canadian Journal of Botany* 47: 1823-1831.

HUDSPETH, M. E. S., TIMBERLAKE, W. E. & GOLDBERG, R. B., 1977. DNA sequence organisation in the water mold *Achyla*. *Proceedings of the National Academy of Science of the USA* 74: 4332-4336.

IANNELLI, D., CAPPARELLI, R., MARZIANO, F., SCALA, F. & NOVIELLO, C., 1983. Production of hybridomas secreting monoclonal antibodies to the genus *Fusarium*. *Mycotaxon* 17: 523-532.

KRAMLAUF, R. & MARZLUF, G. A., 1979. Characterization of the sequence complexity and organization of the *Neurospora crassa* genome. *Biochemistry* 18: 3705-3713.

LEWIS, S. A., GILMARTIN, M. E., HALL, J. L. & COWAN, N. J., 1985. Three expressed sequences within the human B-tubulin multigene family each define a distinct isotype. *Journal of Molecular Biology* 182: 11-20.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J., 1982. Molecular cloning, a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

MEINKOTH, J. & WAHL, G., 1984. Hybridization of nucleic acids immobilized on solid supports. *Analytical Biochemistry* 138: 267-284.

MURRAY, M. G. & THOMPSON, W. F., 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* 8: 4321- 4325.

PUHALLA, J. E., 1981. Genetic considerations of the genus *Fusarium*. Pages 291-305. *In*: Fusarium: Diseases, Biology and Taxonomy. P. E. Nelson, T. A. Tousson & R. J. Cook, Eds. Pennsylvania State University Press, University Park.

SCALA, F., CRISTINZIO, G., MARZIANO, F. & NOVIELLO, C., 1981. Endopolygalacturonase zymograms of *Fusarium* species. *Transactions of the British mycological Society* 77: 587-591.

SNYDER, W. C. & HANSEN, H. N., 1940. The species concept in *Fusarium* with reference to the section Martiella. *American Journal of Botany* 27: 64-67.

SPECHT, C. A., DiRUSSO, C. C., NOVOTNY, C. P. & ULLRICH, R. C., 1982. A method for extracting high molecular weight deoxyribonucleic acid from fungi. *Analytical Biochemistry* 119: 158-163.

SZECSI, A., 1981. Kinetic complexity and repetitiveness of *Fusarium graminearum* nuclear DNA. *Experimental Mycology* 5: 322-329.

TIMBERLAKE, W. E., 1978. Low repetitive DNA content in *Aspergillus nidulans*. *Science* 202: 973-975.

WAITE, B. H. & STOVER, R. H., 1960. Studies on *Fusarium* wilt of bananas. VI. Variability and the cultivar concept in *Fusarium oxysporum* f. *cubense*. *Canadian Journal of Botany* 38: 985-994.

CHAPTER 3

AN RFLP PROBE RELATING VEGETATIVE COMPATIBILITY GROUPS AND PATHOGENICITY IN *FUSARIUM OXYSPORUM* F. SP. *DIANTHI*

INTRODUCTION

Fusarium oxysporum f. sp. *dianthi* is an important fungal pathogen of cultivated species of carnations causing a serious wilt disease. Eight races have been reported within this forma specialis by Garibaldi (1983), but most of these are confined to the French and Italian Riviera. Race 2, however, to which only partial resistance exists, is found worldwide and is the only race currently known in Israel.

Isolation of a *F. oxysporum* from diseased carnation is usually regarded as presumptive evidence for the isolate being f. sp. *dianthi* although this is not necessarily true. While it is relatively easy to identify *Fusarium* to species level, the confirmation of forma specialis requires pathogenicity tests, which are time consuming, and if large numbers are involved, require an extensive infrastructure. Race determinations further increase the experimental requirements due to the need for differential hosts.

Methods for the rapid identification of pathogenic isolates would thus be an advantage in such situations as a plant disease clinic or in the application of phytosanitary regulations. Recently two powerful techniques have been developed

---

This chapter has been accepted, with revision, by *Phytopathology* as of March, 1989.

which can be used for the rapid classification of fungal strains and isolates, these being the use of restriction fragment length polymorphisms (RFLP's) (Beckmann & Soller, 1986; Manicom *et al.*, 1987; Kistler *et al.*, 1987) and vegetative compatibility groups (VCG's) (Puhalla, 1985; Correll *et al.*, 1987).

We report here on the use and interrelationship of VCG's and RFLP's for the determination of genetic groups and their relation to pathogenicity for a collection of isolates of *F. oxysporum* obtained from diseased carnations.

MATERIALS AND METHODS

Fungal isolates

Forty-six *Fusarium* isolates from diseased carnation plants were supplied under code by Dr Ruth Cohen of the Plant Disease Clinic, ARO, Bet Dagan. Most of these were *Fusarium oxysporum*, but no particular effort was made to exclude other Fusaria recovered from the plants as we wished to gain an idea of the specificity of the technique. All were collected from material submitted to the clinic during 1985 and early 1986 and were single spored and maintained on carnation leaf agar. The sources and codes are given in Table 1, as are the identifications and VCGs, which were only determined after the RFLP analyses.

DNA extraction

For the first part of this study the previously described CTAB method (Manicom *et al.*, 1987) was used. Later the method of Raeder and Broda (1985) which is faster, more convenient and gave identical RFLP's was adopted as the standard, with the addition of a final precipitation step using 0,25 vol. of 7,5% ammonium acetate and 2 vol. ethanol. This was found to enhance the digestibility of many isolates.

RFLP probe

Cloning of the random probe used in this study (probe D4) has been previously described (Manicom *et al.*, 1987). The probe consisted of an approximately 3400 base pair (bp), random, Hind III restricted fragment, from a partial digest of a total DNA extract of *Fusarium oxysporum* f. sp. *dianthi*, cloned into pBR322.

Restriction, blotting and hybridisation

The methods as previously described (Manicom *et al.*, 1987) based on those of Meinkoth and Wahl (1984) were used throughout. Total DNA of the isolates was digested with Hind III according to manufacturer's instructions and the restriction digests were electrophoretically resolved in 1% agarose gels. The DNA was blotted to nitrocellulose or nylon membranes, probed with nick-translated probe D4 and autoradiographed, all as previously described.

Pathogenicity tests

A subset of isolates was drawn from each of the major RFLP groups for pathogenicity testing (See Table 2). Fungi to be tested were grown for 8 days on PDA plates. These were then homogenized in 200ml water with short bursts of an Ultraturrax and counts of spores and pieces of mycelium containing intact cells were made with a haemocytometer. The concentration was adjusted to approximately 7×10^6 propagules per ml.

Rooted cuttings of the carnation cultivars, Barbi, White Royalette, Tony and Red Baron were obtained from a commercial nursery. These are all of the "Miniature" group. Red Baron is resistant to Race 1 of *Fusarium oxysporum* f. sp. *dianthi*, susceptible to Race 2 and all the others are susceptible to both races (Garibaldi, 1977). Eight of each cultivar were planted in a row in 40 X 30 X 15cm containers in a sterile commercial potting mix (peat/sponge/volcanic tuf, 3:1:1). After planting, 200 mls of inoculum was poured into shallow trenches between the

rows of cuttings. The plants were maintained in a glasshouse at 27-30 C and container positions were rotated every few days. Plants were scored weekly from the third week after planting according to the 0 = healthy, 4 = dead, disease index scale of Baayen and De Maat (1987).

After the final score at 42 days, reisolations were made from the stem just above the crown onto PDA containing 250 mg per litre of chloramphenicol. The RFLPs of these reisolated fungi were subsequently determined according to the methods described above.

Vegetative compatibility grouping

The slightly modified methods of Puhalla (1985) were used for the determination of VCGs. Initially *nit* mutants were generated on commercial Potato-dextrose-agar, as opposed to the sucrose medium of Puhalla, with either 1,5% or 3,5% $KClO_3$, as many isolates were relatively insensitive to chlorate. Where suitable mutants were not obtained the alternate mineral salts based medium was used.

Mutants from each of the isolates used in the pathogenicity tests were paired with each other in all possible combinations to find complementing pairs. Two complementing mutants were then chosen from each isolate and all possible comparisons made among the 16 isolates used in the pathogenicity studies. Three mutants from each of the VCGs thus determined were designated as test strains and paired with two complementing mutants from each of all the other *F. oxysporum* isolates in this study.

Those *F.oxysporum* not reacting with the probe were in addition paired in all combinations with each other. We were unable to generate mutants from isolate 32 even at 60 g/L of $KClO_3$. This is not unknown (Correll *et al.*, 1986; Bosland & Williams, 1987).

Species identification

The methods and identification keys of Nelson *et al.* (1983) were used to identify the species of the isolates.

RESULTS

Of the 46 isolates investigated, 29 gave a complex pattern of bands (D4-14) when a Hind III digest of total DNA was probed with D4. These included the intense band at approx. 3000 bp and a faint band at approx. 300 bp (not reproduced in Fig. 1 to avoid overexposing the other bands), corresponding to the probe itself. Nine isolates gave a single band pattern (D4-01). This heavy band, centred around 4000 bp appeared to be composed of three closely spaced bands but was not clearly resolved, even on extended runs. Six isolates did not react with the probe at all, of which two, no's 24 and 47, were later found to be *F. moniliforme.* One (No. 32) gave two closely spaced bands, the lower of which corresponded to the upper part of the D4-01 pattern plus an additional lower and faint upper bands, and No. 23 gave a two band pattern, the bands of which did not correspond with those in any other patterns (Fig. 1 and Table 1).

All the Fusaria with a D4-14 pattern belonged to a single VCG as did all those with the D4-01 pattern. In line with Puhalla's (1985) coding system these are labelled VCG 0021 and VCG 0022 respectively but are not to be seen as indicating races. Isolates with other RFLP patterns were not compatible with each other or with VCG groups 0211 or 0022. The vegetative compatibility groups (VCG's) corresponded exactly with the RFLP patterns (Table 1) as did pathogenicity of those isolates tested (Table 2).

Contingency tables were constructed from the pathogenicity data and analysed by Pearsons' Chi square. Cultivars differed significantly in susceptibility

in the order: White Royalette > Tony = Red Baron > Barbi ($P > 0.01$). Isolate 21 was significantly less aggressive than the others ($P > 0.001$), and isolates SS1, 3 and 42 were more aggressive than the remainder ($P > 0.01$). However, overall the two VCGs did not differ in pathogenicity. There was no separation of groups by locality or nursery, and in some cases both groups were isolated from the same material.

Isolates in groups D4-14 and D4-01 were recovered from inoculated plants and proved to have the same RFLP pattern on retesting. The isolates which did not react with the probe and were non-pathogenic could not be recovered from the plants except for isolate 23.

DISCUSSION

Interesting, is the correspondence of RFLP groups and VCGs. A VCG is defined as a group of isolates which are able to anastomose and form heterokaryons among each other, and not with those outside the group, this being controlled by multiple incompatibility loci (Correll *et al.*, 1987). It was originally suspected that VCG's may correspond with forma speciales (Puhalla, 1985). Subsequent studies found that for *F. oxysporum* f. sp. *apii*, VCGs corresponded to races (Correll *et al.*, 1986; Ireland & Lacy, 1986) as was the situation with *F. oxysporum* f. sp. *vasinfectum* (Katan & Katan, 1988).
However, more than one race, as determined by differential hosts, can occur within a VCG (Correll *et al.*, 1985; Bosland & Williams, 1987).

The data of Ploetz & Correll (1988) for *F. oxysporum* f. sp. *cubense*, on the other hand, shows that a race can comprise more than one VCG. Only race 2 of *F. oxysporum* f. sp. *dianthi* is known in Israel and the reaction of Red Baron confirms that race 1 was not amongst the test set. Thus the results here show a similar

situation in that one race has two VCGs. However, it cannot be discounted that a complete set of differential hosts, or a host yet to be found, may not resolve the two groups found here into races.

Little is known of the random probe used. It is derived from the nuclear DNA and a 387 bp portion giving the multiband pattern has been sequenced, but a computor search of the EMBL and GENBANK data bases has not shown it to be related to any currently known sequence. Nor are there any particularly unusual features which might give a clue to its function (data not given) (Queen & Korn, 1984). Until such information is available one cannot draw conclusions as to a link between the probe and one or more of the nitrate reductase system genes, vegetative incompatabilty loci and pathogenicity. Rather, the RFLP patterns are here seen as separate evidence for a genetic distance between the two groups.

We see no reason to modify the current use of forma speciales and races in *F. oxysporum* nomenclature, as it is of such practical value. However, Armstrong & Armstrong (1975) have reviewed some of the difficulties in assigning a name to an isolate based on pathogenicity tests. Among others, some races have a wide host range, host cultivars with the same name can have differing resistance genes, and environmental conditions and host age can affect pathogenicity, as others, also, have found (Pound & Fowler, 1953; Kraft & Hagland, 1978; Hart & Endo, 1981). The pathogenicity results in this study too, show a range in agressiveness which could have led to the false declaration of races (Table 2).

There is a need for a timely revision and consolidation of the many forma speciales and races (Armstrong & Armstrong, 1981) of the *Fusarium* section Elegans using the objective techniques of RFLPs and VCGs as indicators of genetic difference. Bosland & Williams (1987) have laid the groundwork with their study f. sp. *conglutinans* (Wr.) Snyd. & Hans.

The peculiar reaction of this probe also opens the way for a rapid pathogenicity test. Although only 12 isolates were tested, the correspondence of

pathogenicity, RFLP pattern and VCGs leads us to believe that this relationship holds throughout f. sp *dianthi*. Only isolate 23, and perhaps isolate 32, as its pathogenicity is unknown, would have given a false pathogenic reaction on a simple dot blot test, assuming always that the fusaria were isolated from carnations, as it is known that this probe also reacts with *F. gladioli* and *F. lycopersici* (Manicom *et al.*, 1987).

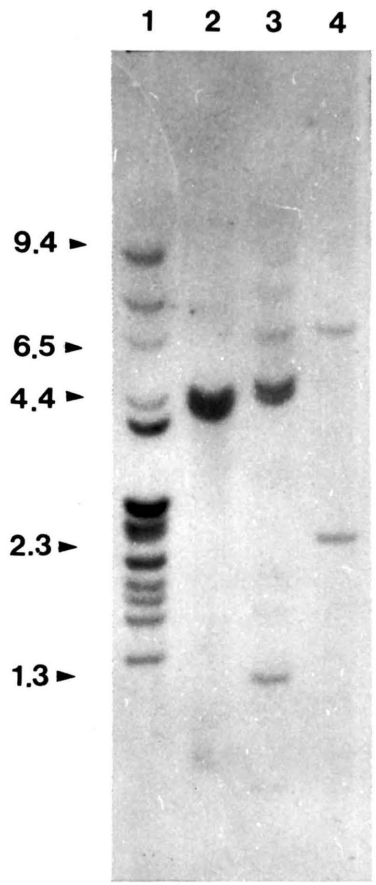


FIG. 1. RFLP patterns of representative isolates of the four groups. Total DNA digested with Hind III and probed with D4. 1 = D4-14 pattern (isolate 30), 2 = D4-01 pattern (isolate 42), 3 = isolate 32, 4 = isolate 23.

TABLE 1. List of *Fusarium* isolates used, codes and original codes, source cultivar, RFLP and VCG groups and species.

Code	Cohen code	Host cultivar	RFLP pattern	VCG group	Species
1	22/2	P[1]	D4-12	0021	Fox[2]
2	24/2	CR	NIL		Fox
3	26/2	CR	D4-12	0021	Fox
4	31/2	CR	D4-12	0021	Fox
5	197/2	WR	D4-01	0022	Fox
6	198/1/2	P	D4-01	0022	Fox
7	199/2/2	R	D4-01	0022	Fox
8	213/2	M	D4-12	0021	Fox
10	216/2	TA	D4-12	0021	Fox
11	217/2	M	D4-12	0021	Fox
12	/4	M	D4-12	0021	Fox
13	218/2	TA	D4-12	0021	Fox
14	/4	TA	D4-12	0021	Fox
15	230/2	T	D4-12	0021	Fox
16	/4	T	D4-12	0021	Fox
17	231/2	L	D4-12	0021	Fox
18	232/2	R	D4-12	0021	Fox
19	233/2	P	D4-12	0021	Fox
20	/4	P	D4-12	0021	Fox
21	/6	P	D4-01	0022	Fox
22	/8	P	D4-12	0021	Fox
23	1181/1/2	NA	TWO		Fox
24	/2/2	NA	NIL		Mon
25	1194/1	NA	D4-12	0021	Fox
26	/1/2	NA	D4-12	0021	Fox
28	/2	NA	D4-12	0021	Fox
30	/3	NA	D4-12	0021	Fox
31	1401/1	CR	D4-12	0021	Fox
32	/2	CR	1+1	ND	Fox
33	1402/1	CR	D4-12	0021	Fox
34	1403/1	CR	D4-12	0021	Fox
35	/3	CR	D4-12	0021	Fox
36	1404/3	CR	D4-12	0021	Fox
37	1405/1	CR	D4-12	0021	Fox
38	1406/1	R	D4-01	0022	Fox
39	/2	R	NIL		Fox
40	1407/1	CR	D4-01	0022	Fox
42	1447/1	CR	D4-01	0022	Fox
44	1448/1	CR	D4-01	0022	Fox
45	/2	CR	D4-01	0022	Fox
46	1449/1	CR	D4-12	0021	Fox
47	1450/1	CR	NIL		Mon
48	/2	CR	NIL		Fox
49	1451/1	CR	D4-12	0021	Fox
50	1474/1	CR	NIL		Fox
51	SS1	NA	D4-12	0021	Fox

[1]P = Pepito, CR = Cerise Royalette, WR = White Royalette, R = Rony, T = Tony, TA = Tanga, M = Ministar, NA = Not available.

[2]Fox = *F. oxysporum*, Mon = *F. moniliforme*.

All collected in 85/86.

TABLE 2. Results of pathogenicity tests on four carnation cultivars with isolates from various RFLP groups. Total disease score of 8 plants on a scale of 0 = healthy, 4 = dead after 42 days (max = 32).

RFLP group	Isolate code	Cultivar B[1]	WR	T	RB	Mean
D4-12	SS1	24	30	25	24	25.8
	3	19	30	26	30	26.3
	16	11	25	15	21	18.0
	46	12	29	21	14	19.0
	Mean	16.5	28.5	21.8	22.3	22.3
D4-01	21	3	15	2	6	6.5
	40	11	21	28	19	19.8
	42	16	32	23	30	25.3
	44	10	24	18	21	18.3
	Mean	10.0	23.0	17.8	19.0	17.4
Cultivar mean		13.3	25.8	19.8	20.6	
NIL	2	0	0	0	0	
	24	0	0	0	0	
	50	0	3	1	2	
TWO	23	0	0	0	0	
CONTROL		0	0	0	0	

[1]B = Barbi, WR = White Royalette, T = Tony, RB = Red Baron.

REFERENCES

ARMSTRONG, G. M. & ARMSTRONG, J. K., 1975. Reflections on the wilt Fusaria. *Annual Review of Phytopathology* 13: 95-103.

ARMSTRONG, G. M. & ARMSTRONG, J. K., 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. Pages 391-399. *In:* Fusarium, Diseases, Biology, and Taxonomy. P. E. Nelson, T. A. Toussoun & R. J. Cook, Eds. The Pennsylvania State University Press, University Park.

BAAYEN, R. P. & DE MAAT, A. L., 1987. Passive transport of microconidia of *Fusarium oxysporum* f. sp. *dianthi* in carnation after root inoculation. *Netherlands Journal of Plant Pathology* 93: 3-13.

BECKMANN, J. S. & SOLLER, M., 1986. Restriction fragment length polymorphisms in plant genetic improvement. Pages 197-250. *In:* Oxford surveys of plant molecular and cell biology. B. J. Miflin, Ed. Oxford Press, Oxford.

BOSLAND, P. W. & WILLIAMS, P. H., 1987. An evaluation of *Fusarium oxysporum* from crucifers based on pathogenicity, isozyme polymorphism, vegatative compatibility, and geographic origin. *Canadian Journal of Botany* 65: 2067-2073.

CORRELL, J. C., PUHALLA, J.E. & SCHNEIDER, R. W., 1986. Identification of *Fusarium oxysporum* f.sp. *apii* on the basis of colony size, virulence, and vegetative compatibility. *Phytopathology* 76: 396-400.

CORRELL, J. C., PUHALLA, J.E., SCHNEIDER, R. W. & KRAFT, J. M., 1985. Differentiating races of *Fusarium oxysporum* f.sp. *pisi* based on vegetative compatibility. *Phytopathology* 75: 1347 (abs.).

CORRELL, J. C., KLITTICH, C. J. R. & LESLIE, J. F., 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77: 1640-1646.

GARIBALDI, A., 1977. Race differentiation in *Fusarium oxysporum* f. sp. *dianthi* and varietal susceptibility. *Acta Horticulturae* 71: 97-101.

GARIBALDI, A., 1983. Resistenza di cultivar di garofano nei confronti di otto patotipi di *Fusarium oxysporum* f. sp. *dianthi* (Prill. et Del.) Snyd. et Hans. *Rivista della Ortoflorofrutticoltura Italiana* 67: 261-270.

HART, L. P. & ENDO, R. M., 1981. The effect of time of exposure to inoculum, plant age, root development, and root wounding on *Fusarium* yellows of celery. *Phytopathology* 71: 77-79.

IRELAND, K. F. & LACEY, M. L., 1986. Laboratory identification of pathogenic isolates of *Fusarium oxysporum* f. sp. *apii* race 2. *Phytopathology* 76: 956 (abs.).

KATAN, T. & KATAN, J., 1988. Vegatative-compatibility grouping of *Fusarium oxysporum* f. sp. *vasinfectum* from tissue and rhizosphere of cotton plants. *Phytopathology* 78: 852-855.

KISTLER, H. C., BOSLAND, P. W., BENNY, U., LEONG, S. & WILLIAMS, P. H., 1987. Relatedness of strains of *Fusarium oxysporum* from crucifers measured by examination of mitochondrial and ribosomal DNA. *Phytopathology* 77: 1289-1293.

KRAFT, J. M. & HAGLUND, W. A., 1978. A reappraisal of the race classification of *Fusarium oxysporum* f. sp. *pisi*. *Phytopathology* 68: 273-275.

MANICOM, B. Q., BAR-JOSEPH, M., ROSNER, A., VIGODSKY-HAAS, H. & KOTZE, J.M., 1987. Potential applications of random DNA probes and restriction fragment length polymorphisims in the taxonomy of the Fusaria. *Phytopathology* 77: 669-672.

MEINKOTH, J. & WAHL, G., 1984. Hybridization of nucleic acids immobilized on solid supports. *Analytical Biochemistry* 138: 267-284.

NELSON, P. E., TOUSSON, T. A. & MARASAS, W. F. O., 1983. *Fusarium* species. An illustrated manual for identification. The Pennsylvania State University Press, University Park.

PLOETZ, R. C. & CORRELL, J. C., 1988. Vegetative compatibility among races of *Fusarium oxysporum* f. sp. *cubense*. *Plant Disease* 72: 325-328.

POUND, G. S. & FOWLER, D. L., 1953. *Fusarium* wilt of radish in Wisconsin. *Phytopathology* 43: 277-280.

PUHALLA, J. E., 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatability. *Canadian Journal of Botany* 63: 179-183.

QUEEN, C. & KORN, L. J., 1984. A comprehensive sequence analysis program for the IBM personal computor. *Nucleic Acids Research* 12: 581-599.

RAEDER, U. & BRODA, P., 1985. Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* 1: 17-20.

CHAPTER 4

AN INVESTIGATION OF THE PROBE D4 WITH SPECIFICITY FOR *FUSARIUM OXYSPORUM* F. SP. *DIANTHI*

INTRODUCTION

The peculiar reaction and specificity of the random probe D4 and its possible linkage to vegetative compatibility groups (VCGs) and pathogenicity aroused interest in knowing its origin and any unusual features at the DNA level.

At the inception it was known that the probe was a random Hind III cut fragment, of approximately 3400 base pairs (bp), from a partial digest of a total DNA extract of *Fusarium oxysporum* f. sp. *dianthi*. This had been cloned into pBR322 and was found to give up to 14 RFLP bands with certain *F. oxysporum* isolates, making it useful in the differentiation of isolates of f. sp. *dianthi*, *lycopersici* and *gladioli*. It further did not react with other species of *Fusarium* or other f. spp. of *F. oxysporum* (Manicom *et al.* 1987).

Accordingly, its hybridisation to DNA fractions was investigated, a restriction map was constructed, and subclones were tested for their ability to differentiate among isolates of *F. oxysporum* f. sp. *dianthi*. Sequence data was also obtained and compared with data banks of known sequences.

MATERIALS AND METHODS

Probe origin

Total DNA was isolated by the method of Raeder & Broda (1985) from 3 gm of freeze dried mycelium of isolate SS1 of *Fusarium oxysporum* f. sp. *dianthi*, from which the probe was originally cloned (Manicom *et al.*, 1987). This was dissolved in 10 ml of TE buffer, (10 mM tris-HCl, 1 mM EDTA, pH 8,0), solid cesium chloride was added to attain a density of 1,685 gm/ml and 120 ug/ml bisbenzimide (Hoechst 33258) was included. The solution was split between two tubes and centrifuged at 150.000 g_{max} (Beckman Ti 50 rotor) for 48 hr. The mitochondrial (mtDNA) and nuclear DNA (nDNA) bands were removed separately by side puncture with a syringe from one tube. The second tube was bottom punctured and after discarding the first approx. 2 ml, 100 µL lots were collected. The dye was removed by extraction with isopropanol, fractions were diluted with 3 vol. of TE and precipitated with 2 vol. of ethanol and taken up in 20 µL of TE. The mtDNA and nDNA bands were taken up in 200 µL and 500 µL TE respectively.

Twenty µL portions of the fractions were digested with Hind III according to manufacturers instructions and run on 1% agarose gels, blotted, and probed with nick translated probe D4, as previously described (Manicom *et al.*, 1987).

Hybridisation to known probes

A few known probes were obtained as a gift from Dr. A. Breiman of Tel Aviv University. pTA71 comprised a 9kb EcoR1 fragment containing the wheat nuclear 18S and 26S nuclear ribosomal genes, cloned into pBR322. 2A6 was a 5,5 kb Sal I fragment containing the 18S wheat mitochondrial ribosome gene cloned in the cosmid pHC79, and Cox II contained the maize cytochrome oxidase gene subunit II cloned into the Sma site of pUC 9. These were nick translated and used to probe blots of Hind III digests of D4 in pBR322, according to standard procedures

(Meinkoth & Wahl, 1983).

Restriction analysis

The probe D4 in pBR322 was digested singly and with combinations of a number of restriction enzymes according to the enzyme manufacturers' protocols (See Fig. 2 for a representative example). The enzymes used were selected according to availability and with a view to subsequent cloning into the polylinker of phage M13. Size estimates of the digestion fragments were made graphically by plotting mobility against the log of base length using phage lambda/Hind III and pBR322/Hae III digests as markers. A restriction map was constructed using the methods of Fitch *et al.* (1983), (Fig. 3).

Subcloning

Five fragments were initially chosen (Fig. 3). Early confusion in the probe map caused by incomplete Bam H1 digests resulted in SB5 being taken in the expectation that this covered the area of the Sal I - Bam H1 280 bp region. The fragments obtained by digestion with the appropriate enzymes were separated on 0,7% Seaplaque agarose as it was found that other low melting temperature agaroses inhibited further reactions. The methods of Crouse *et al.* (1983) were then followed for the cloning of the fragments directly from the gel into M13mp8 and M13mp9. All enzymes and reagents were sourced from Boehringer Mannheim. The subclones were then tested by hybridisation for their specific banding patterns against four Hind III digested isolates of *Fusarium oxysporum* f. sp. *dianthi* each representative of a particular RFLP pattern (See Chap. 3).

Hybridization

The methods of Meinkoth and Wahl (1983) were used for blotting and hybridisation. Either BA85 nitrocellulose or Hybond N nylon membranes were

used for Southern blotting. DNA was fixed to membranes by exposure to U.V. light (312 nm) for a predetermined time. Probes were nick translated using the Amersham kit N. 5000 and according to the protocol supplied. Autoradiography was performed on DuPont Cronex film with DuPont "HiPlus" screens at -70 C overnight.

Sequencing

Based on the patterns obtained it was decided to sequence subclones HB3, BS4 and BS5. The Amersham kit N. 4502 and recommended procedures for the standard Sanger di-deoxy chain termination sequencing method were used. Either $dCTP^{32}$ or [a-S^{35}]dCTP was used as the radiolabel, depending on availability. Acrylamide gels and buffers were according to standard procedures suggested for the Amersham kit except that wedge gels were used starting at 0,4 mm and extending to 1,2 mm in thickness. Glass plates were silanased ("Bind-Silane" and "Repel-Silane", LKB) and the gels dried thereon for autoradiography. The gels were covered with cling film and exposed to DuPont "Cronex" film at room temperature overnight. Sequencing was performed in both directions, i.e. using the clone in M13mp8 and M13mp9.

Computer search and analysis

The sequence of SB5 (which contains HB3) was matched against the EMBL sequence library in a homology search pattern using the program implemented on a PDP-11 at the Weitzman Institute, Israel and also the GENBANK library (Oct. 1987 version) using the MicroGenie program on a microcomputer at the University of Pretoria (Queen & Korn, 1984). The sequence was further examined for internal homologies, repeats, inverted repeats etc. using the suite of programs developed by Pustell & Kafatos (1982).

RESULTS

Probe origin

Bisbenzimide binds preferentially to AT sequences. This reduces the buoyant density of DNA fractions containing multiple copies of AT-rich sequences and serves to separate (in descending density) the mitochondrial (mtDNA), ribosomal RNA coding nuclear DNA (rDNA) and nuclear (nDNA) fractions (Garber & Yoder, 1983). The rDNA band is not always seen, and its appearance depends more on the isolate than age of the mycelium or preparation method.

Two bands were obtained on CsCl gradients with isolate SS1. The hybridisation of probe D4 to representative fractions as shown in Fig. 1 indicates that the probe is associated with the nuclear DNA fraction. The weak patterns with the mitochondrial fractions where the entire gradient was fractionated are presumed due to contamination, as bottom puncture was used for collection. Where the bands were individually removed the contamination is much reduced. It will be noted that the autoradiograph pattern does not coincide with the bands generated by digestion of the mtDNA fractions. None of the ribosomal and mitochondrial probes used reacted with D4 further confirming its nuclear origin, and excluding the 18S and 26S nuclear ribosomal genes.

Restriction map

The restriction map, as also the position of the subclones is shown in Fig. 3. There were no restriction sites for Cla I, Eco R1, Pst I or Sma I.

Subclone specificity

The patterns obtained with the subclones are shown in Fig. 4. HH3 did not react with the isolates. SB5 which comprised HB3 and 147 bp of HH3 gave the

same pattern as HB3 and has been excluded from the figure. It will be noted that the complete probe (D4) pattern is the summation of the patterns given by HB3 and BH18. The BS4 pattern is almost identical to that of HB3 except for a missing band at approximately 6.5 kbp in the D4-12 isolate.

The representative single and double Hind III, Bam HI and Sal I digests shown in Fig. 2 were also blotted and probed with subclone HB3. The fragments containing the Sal I - Bam HI 280 bp fragment which was not cloned did not hybridise to HB3, indicating that this fragment did not contribute to the band patterns obtained (data not shown).

Sequencing

Subclones HB3 and SB5 gave relatively few problems and these could be elucidated either by the sequence from the alternate direction or by raising the reaction temperature to 37 C. The sequence of SB5 confirmed and extended the sequence of HB3.

Clone BS4 however, proved totally refractory to sequencing, the autoradiographs having numerous shadow bands and bands at the same position extending over more than one channel, which made reading the sequence impossible. Various modifications of the reaction were tried, without success. Among them: short growth times to avoid defective bacteriophage production; reducing the ionic strength of the annealing and Klenow buffer; raising reaction temperature to 60 C; and using reverse transcriptase in place of Klenow enzyme (Ornstein & Kashdan, 1985; Williams *et al.*, 1986). This is not easily understandable as the RFLP patterns given by this subclone are almost identical to that of subclone HB3 and one would expect a very similar sequence. Where the sequence of BS4 could be read with confidence (approx. 50% of the total 400 presumed bases), it was characterised by a high proportion of adenosine and guanosine bases in doublets, triplets and runs of up to five, but these portions could not be matched in any logical

manner to clone HB3. The sequence of the clone HB3 is given in Fig. 5.

Computer search and analysis

The sequenced segment does not match any currently known sequence. There is a short homology with part of the *Aspergillus niger* aldA aldehyde dehydrogenase gene (22 of 27 bp matching), another with part of the *Saccharomyces cerevisiae* RP031 gene encoding RNA polymerase III (22 of 27 bp matching), and a third with the *Dictyostelium discoideum* prestalk D11 gene (17 of 19 bases matching), but none of these is regarded as being of any significance.

No significant features in terms of structure were found.

DISCUSSION

It was disappointing, but not unexpected, to be unable to assign the probe DNA to a known part of the genome. The multiband pattern generated from such a short probe has the immediate implication that the probe is associated with some form of repeated or multicopy DNA. The logical candidates are the mitochondrial and ribosomal DNA. Mitochondrial DNA has been excluded, as have the 18S and 23S RNA coding DNA and their flanking regions as these can be expected to be conserved, and thus plant derived probes should still hybridise with fungal rDNA. When one comes to look at the hybridisation of the probe with other *Fusarium* spp., it has a very limited range, being specific to *F. oxysporum dianthi, lycopersici and gladioli* of the *F. oxysporum* tested and also not reacting with other Fusaria (Manicom *et al.*, 1987).

Further, as seen here, the 376 bp portion of the probe responsible for most of the multiband pattern does not react with *F. oxysporum* f. sp. *dianthi* identical in all respects except for the fact of belonging to a different vegetative compatibility

group. This could be explained if the cloned fragment happened to be part of, or close to, one of the VCG genes. There are several nuclear genes controlling vegetative compatibility and only strains with the same alleles at all loci will form heterokaryons (Puhalla & Speith, 1983). It does not, however, explain the multiple banding pattern seen or the fact that the probe also recognises some other species, unless the probe comprises a controlling or flanking sequence to a number of genes.

We are here entering the realms of pure speculation. All that can currently be said is that this random probe is useful in the differentiation and identification of two VCGs of *Fusarium oxysporum* f. sp. *dianthi*. The implications for a simple dot blot test have not been overlooked.

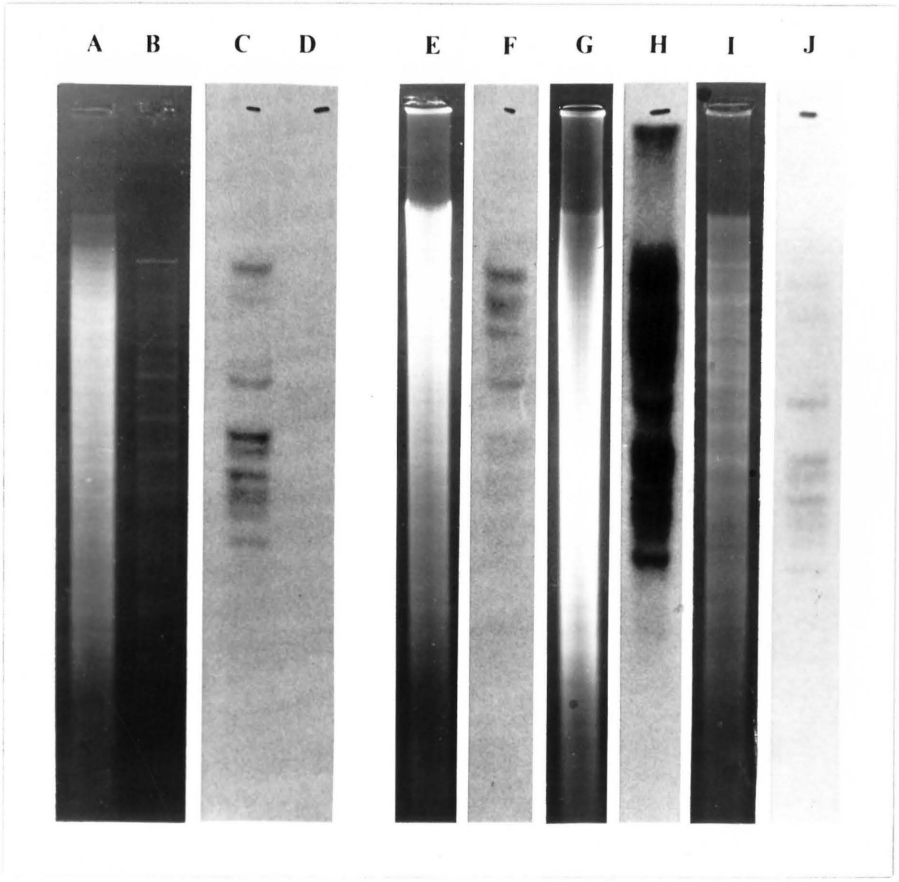


Fig. 1. Hybridisation of probe D4 to blots of Hind III digested nDNA and mtDNA fractions.

Lanes A & B; ethidium stained gel of syringe separated nDNA and mtDNA, respectively, Lanes C & D; corresponding hybridisation patterns.

Lane E; fraction 2, beginning of nDNA band, Lane G; fraction 8, major part of nDNA band, Lane I; fraction 13, central fraction of mt DNA band.

Lanes F, H, J; corresponding hybridisation patterns.

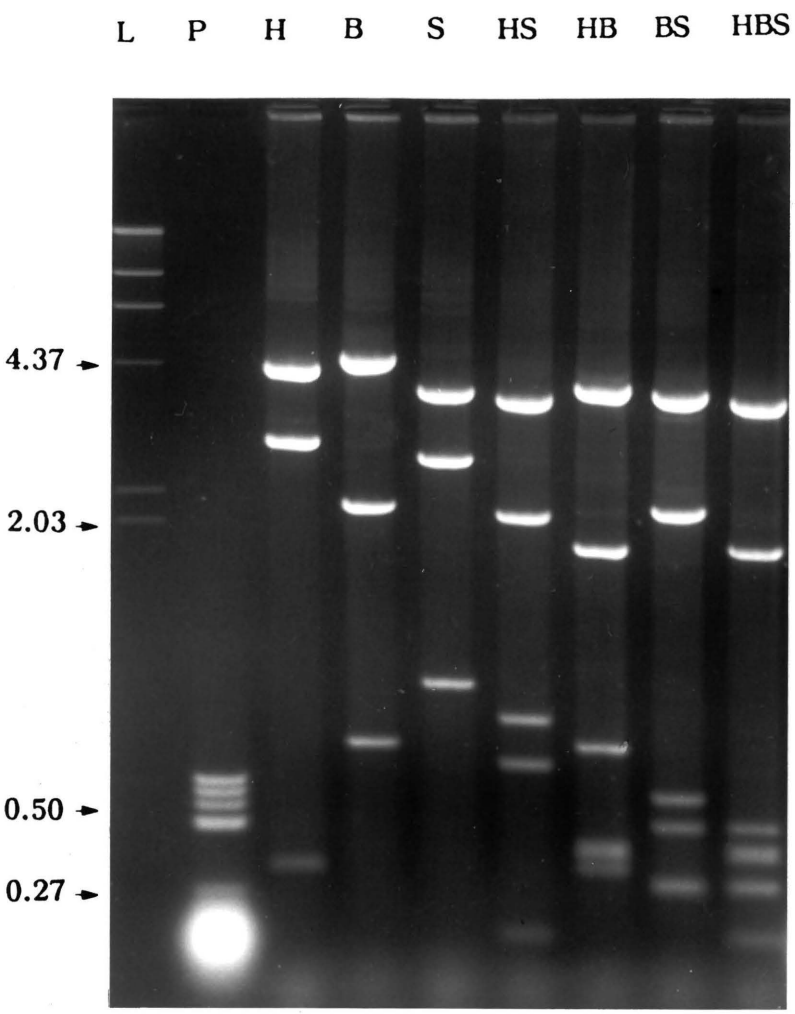


Fig. 2. Representative single and double digests of probe D4 in pBR322. H = Hind III, S = Sal I, B = Bam HI. L = phage Lambda digested with Hind III as markers, P = pBR322 digested with Hae III as markers. Representative base pair size markers have been indicated (X1000).

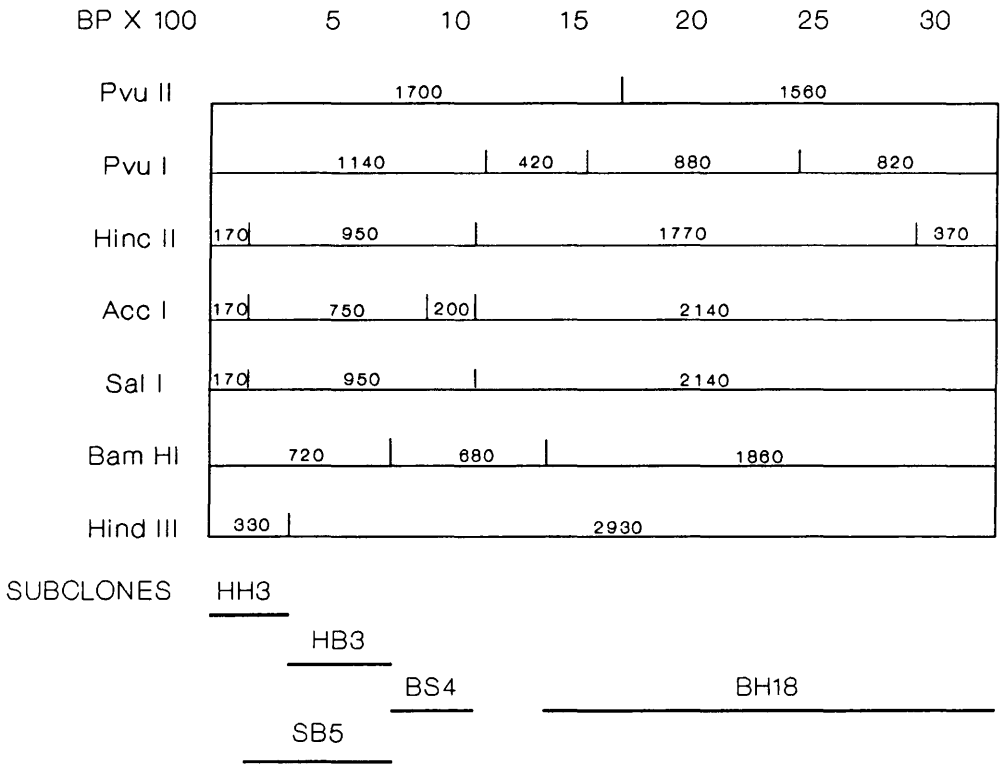


Fig. 3. Restriction map of probe D4 indicating sites and approximate fragment lengths when restricted with various enzymes. Subclones used as probes and for sequencing are also indicated. The total length is approximately 3250 bp.

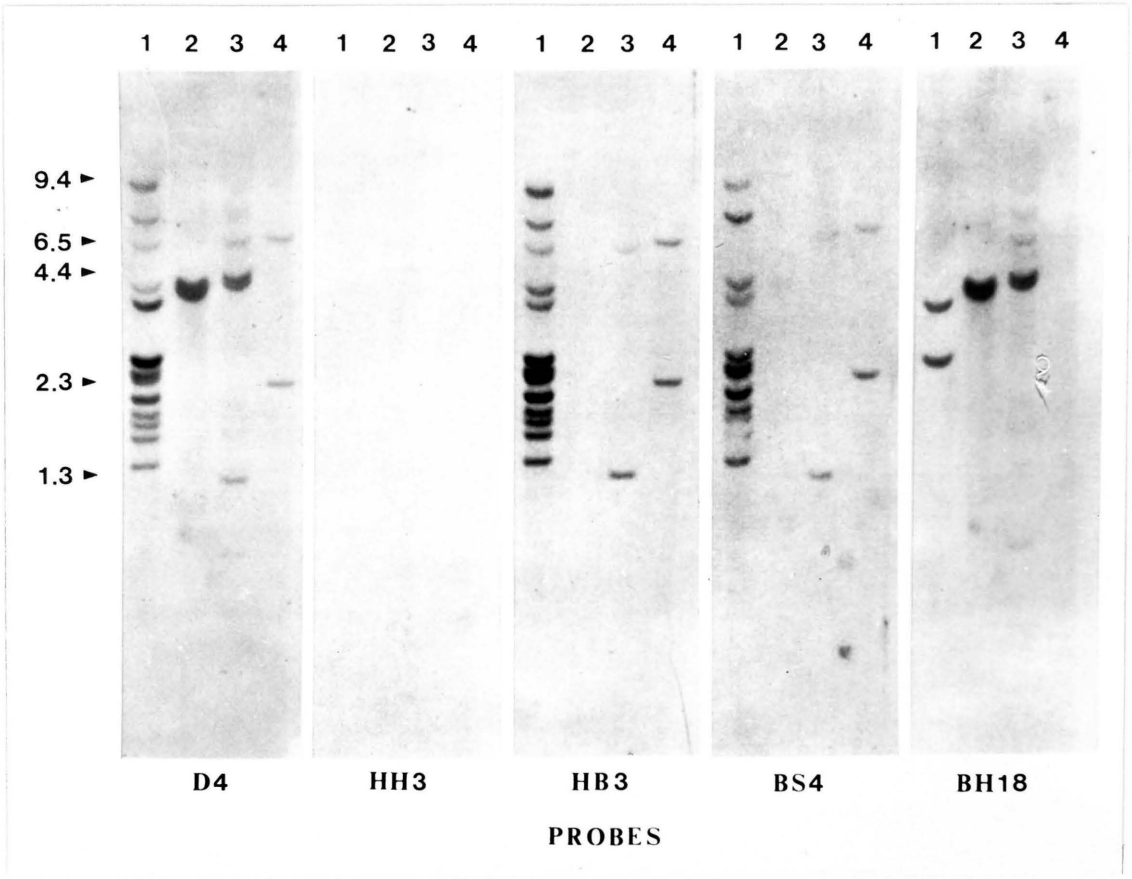


Fig. 4. Banding patterns obtained with the subclones on the four types of fungal groups of *Fusarium oxysporum* f. sp. *dianthi*. 1 = D4-12 pattern, VCG 0021, isolate 30; 2 = D4-01 pattern, VCG 0022, isolate 42; 3 = isolate 32 and 4 = isolate 23.

```
  1  AGCTTCTTTC ACTCCGGAGA ACATCGCCAG TGGGTGGAGA
 41  CGGATGGGTC TCAAGCCTTT CGATCCCGAC GTCGTCCTAT
 81  CTCAGGTTTC GAAGAATACG GACGATGACT CAGATGTGGA
121  AAGTGGCTTG GATGATTCCA TTGCTTTACA AGAACCTACT
161  GCACGAGAAT TACGGCGACT TGTTGATCAT GTCGTGAAGC
201  AATCCTCAGT CAGCTCTGAC ACAGGCGCCC GAAAGCTAAA
241  GAGAACCCTT GAAAGCCTTC AGGCCGAGGT TGAACTACTC
281  AGGCATGAAA ACCAAGGCCT CCGTGAGACA ATTATCCGTA
321  AAGAACAGCG CAGGCAGCGT GGTAAAGCCC TGAAAGACTA
361  TATCTTTGAT CGTGTG
```

Fig. 5. Sequence of clone HB3.

REFERENCES

CROUSE, G. F., FRISCHHAUF, A. & LEHRACH, H., 1983. An integrated and simplified approach to cloning into plasmids and single-stranded phages. *Methods in Enzymology* 101: 78-89.

FITCH, W. M., SMITH, T. F. & RALPH, W. W., 1983. Mapping the order of DNA restriction fragments. *Gene* 22: 19-29.

GARBER, R. C. & YODER, R. C., 1983. Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal, and plasmid components. *Analytical Biochemistry* 135: 416-422.

MANICOM, B. Q., BAR-JOSEPH, M., ROSNER, A., VIGODSKY-HAAS, H. & KOTZE, J.M., 1987. Potential applications of random DNA probes and restriction fragment length polymorphisims in the taxonomy of the Fusaria. *Phytopathology* 77: 669-672.

MEINKOTH, J. & WAHL, G., 1983. Hybridisation of nucleic acids immobilised on solid supports. *Analytical Biochemistry* 138: 267-284.

ORNSTEIN, D. L. & KASHDAN, M. A., 1985. Sequencing DNA using ^{35}S-labeling: A troubleshooting guide. *BioTechniques* 3: 476-483.

PUHALLA, J. E. & SPIETH, P. T., 1983. Heterokaryosis in *Fusarium moniliforme*. *Experimental Mycology* 7: 328-335.

PUSTELL, J. & KAFATOS, F. C., 1982. A high speed, high capacity homology matrix: zooming through SV40 and polyoma. *Nucleic Acids Research* 10: 4765-4781.

QUEEN, C. & KORN, L. J., 1984. A comprehensive sequence analysis program for the IBM personal computer. *Nucleic Acids Research* 12: 581-599.

RAEDER, U. & BRODA, P., 1985. Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* 1: 17-20.

WILLIAMS, S. A., SLATKO, L. S., MORAN, L. S. & DeSIMONE, S. M., 1986. Sequencing in the fast lane: A rapid protocol for [a-^{35}S] dATP dideoxy DNA sequencing. *BioTechniques* 4: 138-147.

CHAPTER 5

A NON-RADIOACTIVE DOT BLOT METHOD FOR THE IDENTIFICATION OF TWO VCGS OF *FUSARIUM OXYSPORUM* F. SP. *DIANTHI*

INTRODUCTION

Non-radioactive detection methods for DNA hybridised to target sequences offer important advantages in avoiding the dangers of radio-active materials and the inconvenience and expense of products with a short half-life. Consequently, considerable efforts have been expended over the past few years in the development of alternative probe labelling systems, most of these based on the biotin-avidin system (Langer *et al.*, 1981; Leary *et al.*, 1983) and new labelling methods have continued to be developed (reviewed by Matthews & Kricka, 1988). The sensitivity of these tests has been extended into the femtogram range (Chan *et al.*, 1985).

Progress has been such that several non-isotopic detection kits are now commercially available. Those from ONCOR, BRL and ENZO are based on nick translation with biotinylated deoxy-nucleotides and that from Vector links biotin to the probe by photoactivation (Forster *et al.*, 1985). Chemiprobe from FMC BioProducts chemically links a sulphone hapten to the DNA probe and the kit from Boehringer uses the oligolabelling procedure of Fienberg & Vogelstein (1983) to incorporate a digoxigenin hapten labelled deoxy-uridine triphosphate into the probe. The various reporter molecules are then detected by enzyme-linked antibodies or avidin. The latter two hapten based kits were tested in this study.

Given a suitable probe, dot blots are far quicker and easier than Southern blotting where a simple yes/no answer is required. Further investigation of a random DNA probe previously described (Manicom *et al.*, 1987; Manicom *et al.*, submitted), suggested that subclones of one of these probes would provide a differential test for the two major vegetative compatibility groups (VCGs) of *Fusarium oxysporum* f. sp. *dianthi* found in Israel, and also the non-pathogenic Fusaria and thus give a rapid pathogenicity test.

A fast hybridisation/detection system is not necessarily an advantage if sample preparation is difficult and thus attention was also paid to the rapid preparation of fungal DNA.

METHODS

Fungal isolates and probes

The isolates used have been previously described and are repeated here in Table 1 for convenience (Manicom *et al.*, submitted). Briefly, 47 isolates of *Fusarium* from diseased carnations submitted to a diagnostic clinic were analysed for restriction fragment length polymorphisms (RFLPs) with the probe D4 (Manicom *et al.*, 1987), VCGs and pathogenicity. The isolates fell into three major groups; RFLP pattern D4-14 = VCG 0021, RFLP pattern D4-01 = VCG 0022, and those isolates which did not react with the probe. The first two groups were pathogenic and thus identified as *F. oxysporum* f. sp. *dianthi*, the others were non pathogenic *F. oxysporum* or in two cases, *F. moniliforme*.

Four isolates from each of RFLP patterns D4-14 and D4-01, and three isolates which did not react with the probe were chosen for this study. Two isolates were also included which gave different RFLP patterns (Manicom *et al.*, submitted). Isolate 23 was known to be non-pathogenic and the status of isolate 32

was unknown (Table 1).

The full D4 probe was an approx. 3400 base pair (bp) random nuclear fragment originally cloned from an isolate of *F. oxysporum* f. sp. *dianthi*. This was further restricted and fragments sub-cloned into M13mp8 by the methods of Crouse *et al.* (1983), and the subclones tested against Southern blots representative of the RFLP patterns, according to standard procedures (Meinkoth & Wahl, 1984). A Hind III - Bam H1, 376 bp subclone (HB3) was found to react only with VCG 0021, thus permitting a differential dot blot for the identification of the two major VCGs.

Rapid mini-preps of fungal DNA

Three methods were tested: a scaled down version of the Murray & Thompson (1980) cetyltrimethylammonium bromide (CTAB) procedure as proposed by Rogers & Bendich (1985); the method of Miller *et al.* (1988) which uses no toxic chemicals, and that of Raeder & Broda (1985). The following slight variant of the latter was adopted.

Isolates were grown in potato dextrose broth in petri plates, including 250 mg/L of chloramphenicol to ensure the absence of bacterial contamination, for 2-3 days. Mycelium was harvested by filtration through Miracloth and freeze dried or blotted dry.

Fifty mg of freeze dried mycelium was ground in a mortar with a little sand. This was transferred to an Eppendorf tube, and 500 µL extraction buffer was added (200 mM Tris-HCl, pH 8,5, 250 mM NaCl, 25 mM EDTA, 0,5% SDS). The powder was suspended with a toothpick and briefly vortexed. To this was added 350 µL water saturated phenol and 150 µL chloroform/isoamyl alcohol (24:1), the mixture was vortexed, then centrifuged for 10 min. 400 µL of the supernatant was transferred to a new tube, 10 µL of 10mg/ml RNAse A was added, (cloudiness can be ignored) and the mixture was incubated at 37 C for 20 min. A

second chloroform/isoamyl alcohol extraction and 5 min centrifugation was per formed and 350 uL of the upper phase was transferred to a new tube. To this, . µL iso-propanol was added, the mixture shaken briefly and centrifuged for 2 min The supernatant was discarded, the pellet rinsed with 500 µL 70% ethanol, vacuu dried, and taken up in 100 µL TE (10 mM Tris - HCl, pH 8,0, 1 mM EDTA).

Alternatively, mycelium from liquid culture, or scraped from a solid culture, was pressed dry with blotting paper and 200 mg ground in 1 mL extraction buffer. This produced a thick slurry to which phenol and chloroform were added and the procedure continued as above except that all volumes were doubled.

Preparation of probes

The RF forms of the plasmids were extracted by the alkali lysis procedure (Maniatis *et al.*, 1983). Crude preparations were restricted with the relevant enzymes, separated electrophoretically on an agarose gel and the fragments to be used as probes (D4 and HB3) were eluted by isotachoelectrophoresis (see appendix for method).

Probes were prepared according to the protocols supplied except that labelled probe was not purified from the reaction mixture. For the Boehringer kit, 1 µg of probe was used in the labelling reaction which was conducted for 1 - 2hr, stopped with EDTA, and diluted to 50 µL. The Chemiprobe reaction was conducted with 50 µL of probe at a concentration of 500 ng/µL and the reaction scaled down accordingly.

Dot blotting

DNA was quantitated spectrophotometrically at 260 nm. Pilot runs established that 0,5 µg per dot was adequate for detection and 1,0 µg per dot was taken as the standard. Nylon membranes required extra blocking to reduce background and thus nitrocellulose was used routinely (Schleicher & Schuell, BA85). A com-

mercial dot blot vacuum apparatus was used to apply DNA to the membranes. Membranes were briefly wet in distilled water, soaked for a few minutes in 20X SSPE (3,6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7,4) and placed in the apparatus on two sheets of filter paper also prewet with 20X SSPE.

DNA samples were diluted in distilled water to provide a loading of 1 µg per dot, boiled for 10 min and rapidly chilled, diluted with an equal volume of 20X SSPE and applied to the membrane. Dot chambers were washed through with 200 µL of 10X SSPE and the membrane baked for 5 - 10 min at 80 C until completely dry or exposed to 300 nm UV irradiation for a predetermined time.

Hybridisation

Membranes were prehybridised for 10 min at 68 C in the hybridisation mixture provided with the Boehringer kit at approx. 80 µL/cm sq. The heat denatured probe was then added at a ratio of 1% of the hybridisation mixture (i.e. 200 ng of starting material per ml, equivalent to approx. 50 ng labelled probe) and the blots were hybridised for 2 hr at 68 C.

A similar procedure was followed for the Chemiprobe except that the hybridisation mixture comprised 5X SSPE, 5X Denhardt's solution, 0,1% SDS and the prehybridisation mixture included 100 µg/ml denatured heterologous DNA. The probe was used at a concentration of 1 µg/ml.

Blots were washed for 3 X 1 min in 2X SSPE plus 0,1% SDS, followed by the high stringency wash of 2 X 5 min at 68 C in 0,1X SSPE, 0,1% SDS.

Detection

Blots according to the Boehringer system were soaked for 2 min in hybridisation mixture, rinsed in TBST (100 mM Tris-HCl, pH 7,4, 150 mM NaCl, 0,05% Tween 20), and further blocked in TBST for 2 min. They were then shaken gently at room temperature for 30 min in the digoxigenin antibody supplied,

diluted 1:5000 with TBST at approx. 800 µL/cm sq. Excess antibody was washed out for 3 X 2 min in TBST, and blots were then rinsed in substrate buffer (100 mM Tris-HCl, pH 9,5, 100 mM NaCL, 50 mM $MgCl_2$. They were then placed in substrate (337 µg/ml nitroblue tetrazolium, 175 µg/ml 5-bromo-4-chloro-3-indolyl phosphate in substrate buffer) and developed for 1-3 hr although this could be extended overnight.

Chemiprobe blots were blocked in TBST for 2 X 5 min with shaking, and then shaken at room temperature for 30 min in the first antibody diluted in blocking solution according to the protocol supplied, washed 4 X 5 min. in TBST and shaken in the second antibody similarly diluted for 30 min. Blots were again washed for 4 X 5 min. in TBST and developed as above.

RESULTS

Rapid mini-preps of fungal DNA

Typical examples of preparations obtained by the various methods are given in Fig. 1. The Raeder/Broda method as described here yielded 20 - 25 µg DNA, of a size a little in excess of 23 kbp, starting from either wet or freeze dried mycelium. Note that there was some degradation of the DNA, although this is unimportant for a dot blot. The centrifugation steps were considerably shortened from the original protocol and reliance was placed on incomplete transfer of the supernates for the elimination of polysaccharides.

The CTAB method yielded similarly from freeze dried material, but had a low yield from fresh mycelium. The recommended short centrifugation times led to massive polysaccharide contamination. Where these were increased the method lost its time advantage and elimination of CTAB from the DNA often required an additional precipitation.

The method of Miller *et al.* (1988) was inappropriate for this use as chloroform and RNAse steps had to be added to eliminate fats and RNA respectively, and co-precipitation of polysaccharides inhibited restriction enzymes. Yield was in the order of 10 μg from freeze dried material but was mostly degraded.

Preparation of probe

A weak point in both kits is the matter of probe size. The highest signal to noise ratio is obtained with probes in the range of 500-1500 bp (Meinkoth & Wahl, 1984; Dykes, 1988). Sizing gels performed with the labelled probes indicated that the Boehringer kit reduced the size to 70% of the starting fragment where the Chemiprobe kit preserved the original length. As the probes were excised from the vector this gave a suboptimal size of approx. 300 bp for probe HB3, which was nevertheless effective, and in the case of probe D4 the fragment was cut with restriction enzymes to give fragments of 400-1100 bp before labelling.

The precipitation clean-up steps after labelling were generally found to be unnecessary except for one batch of HB3 in which some slight non-specificity was eliminated by the clean-up procedure. Some of the probe is lost during the procedure.

Hybridisation and Detection

The approach adopted here was to trade ultimate sensitivity for rapidity. However, even using the standard protocol and controls supplied by the manufacturer the ultimate detection sensitivity attained was only 10 pg of homologous DNA using the Boehringer kit and 100 pg with the Chemiprobe kit, and this could be duplicated using the shortened protocol described here.

Surprisingly a 2 hr hybridisation gave a better signal to noise ratio than an overnight hybridisation. UV fixation of DNA to nitrocellulose gave a slightly higher signal than baking, but the parameters of membrane dampness and time of

exposure were critical and thus baking was generally used for its consistency.

The Boehringer detection system is relatively fast (60 min to staining) and under these conditions gave a low background. Staining was routinely terminated after 3 hr although results were normally obvious after an hour. Tween 20 was added to the antibody incubation and washing buffer as this was found to further reduce background staining. The Chemiprobe kit is a two step process and the full detection protocol requires 7-9 hr to the beginning of staining. This could be reduced along the lines described to a little over an hour without sacrificing sensitivity, but by either procedure background staining was high, confirming the experience of Dykes (1988). Far more important was the lack of specificity of probe HB3 when used with the Chemiprobe procedure and for these reasons this detection system was discarded.

Detection of *F. oxysporum* f. sp. *dianthi* VCGs

Results of dot blot tests are given in Fig. 2. As can be seen the complete probe D4 reacts with both VCG 0021 and 0022 but not with other isolates of *Fusarium oxysporum* from carnations. Probe HB3 on the other hand reacts only with VCG 0021, thus enabling one to distinguish between the two VCGs.

Isolate 23 reacts weakly with the complete probe and is below the detection limit for probe HB3 at this loading. Another sub-clone of the full probe does not recognise isolate 23 (data not given). Isolate 32 responds in the fashion of VCG 0022, but its pathogenicity is unknown. The other *F. oxysporum* isolates used did not react with the probes.

An example of the test set probed with Chemiprobe labelled HB3 is also included showing that this method is unsuitable for this use. The contrast of positive dots over background staining can be enhanced by wetting with toluene to make the nitrocellulose transparent.

DISCUSSION

The aim of developing a rapid method for the screening of fungal isolates for pathogenicity to carnations in this situation has been achieved. The entire process from fungal sample to developed dot blot is easily completed in a day. Freeze dried mycelium is easier to work with and gives a higher DNA recovery, but is not essential. It should be possible to lyse fungal colonies directly on the membrane along the lines suggested by Crowley & Oliver (1987) but this method has not as yet been tested with this system. With the additional use of a sub-probe the isolate can be further classified as to VCG.

There are limits to the technique at present. The probe is known not to react with the *Fusarium* species outside of *F. oxysporum* in the Snyder & Hansen classification, as also some *F. oxysporum* f. spp. but does react with f. spp. *lycopersici* and *gladioli* (Manicom *et al.*, 1987). Further, within the original 47 isolates there were two which gave RFLPs unlike the D4-14 or D4-01 patterns and reacted with the probes. One can be excluded on the basis of its weak reaction or by the use of an additional sub-clone of the probe; the pathogenicity of the other is currently unknown. Within the limits of only subjecting isolates from diseased carnations to testing, accuracy is high, and given defined probes the technique is faster than matching isolates against tester mutants for VCG determination.

Where specific identification is needed at various sub-specific levels, be it for ecological studies or diagnostics, suitable probes can be found by screening a cloned library of the target fungus with total DNA of undesired isolates to determine those that only react at the level sought.

TABLE 1. Isolates of *Fusarium* used in dot blot testing, their RFLP and VCG groups, and pathogenicity.

Isolate code	RFLP group	VCG	[1]Species	Pathogenicity
SS1	D4-14	0021	Fox d	+
3	D4-14	0021	Fox d	+
16	D4-14	0021	Fox d	+
46	D4-14	0021	Fox d	+
21	D4-01	0022	Fox d	+
40	D4-01	0022	Fox d	+
42	D4-01	0022	Fox d	+
44	D4-01	0022	Fox d	+
24	NIL		Fox	-
48	NIL		Fox	-
50	NIL		Fox	-
23	TWO		Fox	-
32	1+1		Fox	NA
TM			Fox c	
MRC 2325			Fox	
MRC 2676			Fox m	
MRC 1873			Fox s	

[1] Fox d = *F. oxysporum* f. sp. *dianthi*; Fox = *F. oxysporum*; Fox c = *F. oxysporum* f. sp. *cubense*; Fox m = *F. moniliforme*; Fox s = *F. solani*

NA = Not available.

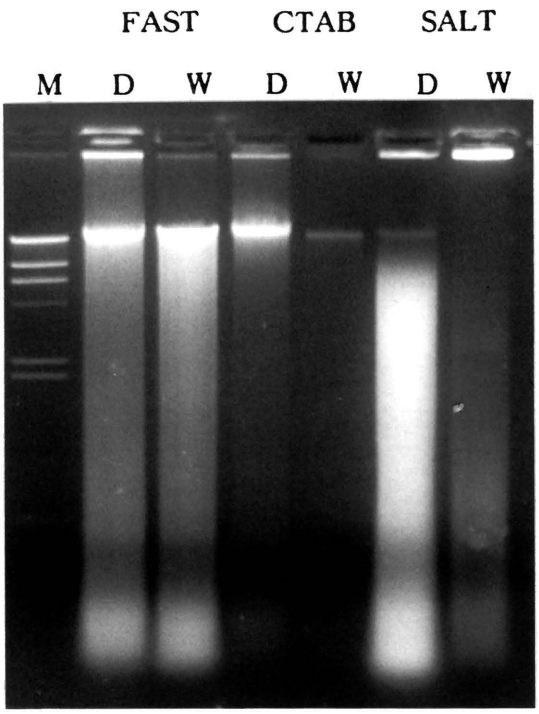
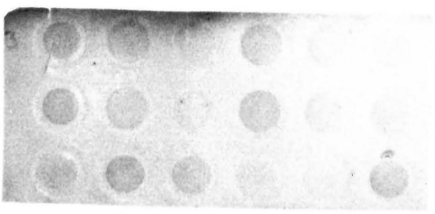


Fig. 1. Agarose gel electrophoretic analysis of DNA quality of three extraction methods on freeze dried or press dried mycelium. M = Phage Lambda/Hind III size markers. D = freeze dried. W = press dried mycelium. Fast = Raeder/Broda method, CTAB = CTAB method, SALT = Miller salt method. Ten µL of a standard preparation were loaded per lane.

Boehringer system
Probe D4.

Boehringer system
Probe HB3.

Chemiprobe system
Probe HB3.



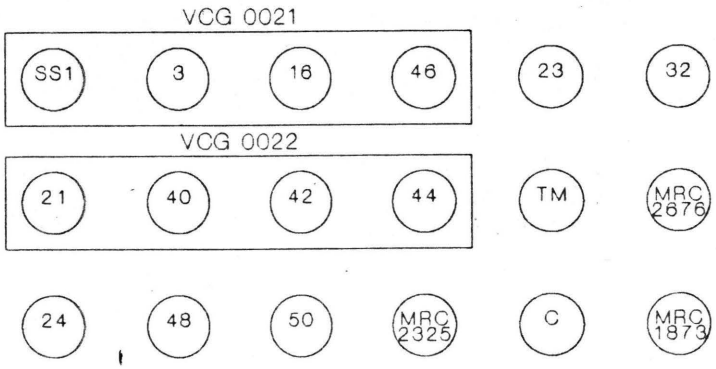


Fig. 2. Results of dot blots of the test isolates. The diagram gives the position of the various isolates on the membranes and the VCG groups. Codes are as per Table 1. C = Control dot, no DNA.

REFERENCES

CHAN, V. T. W., FLEMING, K. A. & McGEE, J. O'D., 1985. Detection of sub-picogram quantities of specific DNA sequences on blot hybridization with biotinylated probes. *Nucleic Acids Research* 13: 8083-8090.

CROUSE, G. F., FRISCHHAUF, A. & LEHRACH, H., 1983. An integrated and simplified approach to cloning into plasmids and single-stranded phages. *Methods in Enzymology* 101: 78-89.

CROWLEY, P. & OLIVER, S. G., 1987. A microculture hybridization technique for the detection of specific DNA sequences in filamentous fungi. *Experimental Mycology* 11: 70-73.

DYKES, D. D., 1988. The use of biotinylated DNA probes in parentage testing: Non-isotopic labeling and non-toxic extraction. *Electrophoresis* 9: 359-368.

FIENBERG, A. P. & VOGELSTEIN, B., 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* 132: 6-13.

FORSTER, A. C., McINNES, J. L., SKINGLE, D. C. & SYMONS, R. H., 1985. Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. *Nucleic Acids Research* 13: 745-761.

LANGER, P. R., WALDROP, A. A. & WARD, D. C., 1981. Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. *Proceedings of the National Academy of Science of the USA* 78: 6633-6637.

LEARY, J. J., BRIGATI, D. J. & WARD, D. C., 1983. Rapid and sensitive colorimetric method for visualising biotin-labeled DNA probes hybridized to DNA and RNA immobilized on nitrocellulose: Bio-blots. *Proceedings of the National Academy of Science of the USA* 80: 4045-4049.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J., 1982. Molecular cloning, a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

MANICOM, B. Q., BAR-JOSEPH, M., ROSNER, A., VIGODSKY-HAAS, H. & KOTZE, J.M., 1987. Potential applications of random DNA probes and restriction fragment length polymorphisims in the taxonomy of the Fusaria. *Phytopathology* 77: 669-672.

MANICOM, B. Q., BAR-JOSEPH, M., KOTZE, J. M. & BECKER, M. M. An RFLP probe relating vegetative compatibility groups and pathogenicity in *Fusarium oxysporum* f. sp. *dianthi*. *Phytopathology* (Submitted).

MEINKOTH, J. & WAHL, G., 1984. Hybridization of nucleic acids immobilized on solid supports. *Analytical Biochemistry* 138: 267-284.

MILLER, S. A., DYKES, D. D. & POLESKY, H., 1988. A salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* 16: 1215.

MURRAY, M. G. & THOMPSON, W. F., 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* 8: 4321- 4325.

RAEDER, U. & BRODA, P., 1985. Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* 1: 17-20.

ROGERS, S. O. & BENDICH, A. J., 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology* 5: 69-76.

GENERAL DISCUSSION

The status of *Fusarium* taxonomy at the species level has reached a state of relative stasis not seen for many years. Whether one ascribes to the nine species concept of Snyder and Hansen, the multiple species varieties of Wollenweber, or somewhere in between (Nelson *et al.*, 1983), one can identify most isolates commonly encountered as to species.

At the subspecific level, particularly with *Fusarium oxysporum*, confusion reigns, with Armstrong & Armstrong (1981) listing 122 forma speciales and races. Two papers which have recently come to hand illustrate the problems: Bosland & Williams (1988) tested the pathogenicity of 103 isolates of *F. oxysporum* from crucifers on six differential hosts under strictly controlled conditions. On the results they grouped the isolates into five pathotypes comprising three f. spp., two with two races each. But within the data there were differential reactions, and the addition of two other hosts further increased the virulence profiles. The authors had to make a subjective judgment on the classification and others might see the data differently.

The second paper by the same group (Bosland *et al.*, 1988) discusses the effect of soil temperature on pathogenicity of these fungi. The important point is that below certain temperatures, some races are avirulent on the differential hosts. Not all pathogenicity studies are done in soil temperature tanks, and this has undoubtedly led to confusion over race classification in the past and will do so in the future.

Another problem is the extensive infrastructure required for pathogenicity tests and the lengthy incubation period. This raises difficulties where rapid diag-

nosis is needed before initiating control measures. There are also legal problems relating to the administration of phytosanitary regulations where precise identification is a prerequisite.

These and other considerations have driven the search for alternate, preferably laboratory based, host independent methods of identification. These are reviewed in chapter 1 of this work.

The most interesting advance in recent years has been the application of the technique of vegetative compatibility grouping (VCG) to *Fusarium*. The methodology is simple, and once tester strains are identified these can be maintained indefinitely, and new isolates easily matched against these. However, with 10 VCG loci, as Puhalla & Spieth (1985) found, both alleles of all of which need be identical for compatibility, Fusaria passing through a sexual stage have the potential for generating 1024 VCGs in a generation. In *F. oxysporum*, dependent on asexual processes for variation, the number will be much lower and natural selection will eliminate more. I believe that this technique will become a standard in the diagnostic laboratory for the identification of isolates in the near future. As knowledge builds up, certain VCGs will be associated with races and through this level with forma speciales, but for the reasons above it will always remain a low level taxonomic grouping.

Various molecular markers have been sought to aid in the grouping and identification of fungal isolates. Some, such as protein banding patterns have proved ineffective, others, such as monoclonal antibodies, will never see general use, because of the time and expense of producing the antibodies. These will only be of use in very specific situations.

The rapid development and dissemination of molecular biological techniques has opened a new vista on marker techniques. The nucleic acid component of an organism defines the organism, and it should be possible to select a marker which provides a distinction at any desired taxonomic level.

As regards the methodology, a prime necessity is rapid and easy isolation of DNA, and part of this study has been concerned with the evaluation of some protocols. I find variants of the Raeder & Broda (1985) method to be most suitable for *Fusarium*. This can be adapted to a micro scale, and sufficient DNA of an isolate for a number of analyses can be obtained in an hour.

In Chapter 1, I noted that it was surprising that the simple technique of ethidium stained restriction fragment length polymorphisms (RFLPs) of total DNA was not more used. I know of only the two papers mentioned using the method for fungi, although it appears to be becoming popular with nematologists (Kalinski & Huettel, 1988). My own experience has been that only two or three bands can be clearly seen against the DNA background and the multiple banding sometimes described is barely discernible in the accompanying photographs. In pilot studies, I found that Sma I is a suitable enzyme to use, confirming the results of Coddington *et al.* (1987). Using known probes I also established that the bright bands are due to nuclear ribosomal RNA coding DNA (rDNA) (unpublished). Using some of the *F. oxysporum* spp. described in this work, tentative results were that it was possible to separate species, but not make finer distinctions. This level of identification can more easily be done on the basis of morphology.

Also investigated was the use of RFLPs of mitochondrial DNA. The purification of the latter is tedious in the extreme, but because of the small, repetitive genome, clear, multiple band patterns are obtained. Fungal clones could be readily identified and these related to VCGs. However, attempts at a synthesis at race or higher level, using numerical taxonomy techniques, were highly dependent on the restriction enzyme used, and I believe that Taylor (1986) is correct where he states that mapping of the mitochondrial genome will be necessary to determine evolutionary and taxonomic clusters.

RFLPs can, by suitable choice of the DNA probe, theoretically be set at any desired taxonomic level. The rub lies in probe selection, but various strategies

can be adopted. For example rDNA is relatively conserved throughout the eukaryotes. However, regions between the genes are not essential to functioning and thus mutations build up in these regions over time and thus differing RFLPs are obtained. In another pilot study, using a wheat rDNA probe, I was able to identify races of *F. oxysporum* f. sp. *cubense* and show that race 4 from Taiwan, the Canary islands and South Africa appeared to be identical (unpublished).

The current work has been at a lower taxonomic level, with the aim of finding a pathogenicity test. This was achieved with a random probe. When one considers that only eight probes were initially screened, and only four used for detailed evaluation, where a specific aim is in view, the selection of a suitable probe is not difficult. Interesting, too, is the fact that the probe recognises f. sp. *gladioli* and *lycopersici*. This implies that these and f. sp. *dianthi* are closely related and may even be a group with multi-host pathogenicity.

The recent commercialisation of non-radioactive probes brings these techniques into the realms of everyday use. Minimal infrastructure and hazards are involved. Radioactive methods will continue to be used for developmental work, but once probes are obtained and defined they can be maintained indefinitely and distributed *ad lib*. Sensitivity is as yet lower than desired but it easy enough to use greater DNA loadings on the membranes.

The techniques are available, the taxonomic and identification problems are there. All that is needed is some hard work.

REFERENCES

ARMSTRONG, G. M. & ARMSTRONG, J. K., 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. Pages 391-399. *In:* Fusarium, Diseases, Biology, and Taxonomy. P. E. Nelson, T. A. Toussoun & R. J. Cook, Eds. The Pennsylvania State University Press, University Park.

BOSLAND, P. W. & WILLIAMS, P. H., 1988. Pathogenicity of geographic isolates of *Fusarium oxysporum* from crucifers on a differential set of crucifer seedlings. *Journal of Phytopathology* 123: 63-68.

BOSLAND, P. W. & WILLIAMS, P. H., 1988. Influence of soil temperature on the expression of yellows and wilt of crucifers by *Fusarium oxysporum. Plant Disease* 72: 777-780.

CODDINGTON, A., MATTHEWS, P. M., CULLIS, C. & SMITH, K. H., 1987. Restriction digest patterns of total DNA from different races of *Fusarium oxysporum* f. sp. *pisi* - an improved method of race classification. *Journal of Phytopathology* 118: 9-20.

KALINSKI, A. & HUETTEL, R. N., 1988. DNA restriction fragment length polymorphism in races of the soybean cyst nematode, *Heterodera glycines. Journal of Nematology* 20: 532-538.

NELSON, P. E., TOUSSON, T. A. & MARASAS, W. F. O., 1983. *Fusarium* species. An illustrated manual for identification. The Pennsylvania State University Press, University Park.

PUHALLA, J. E. & SPIETH, P. T., 1985. A comparison of heterokaryosis and vegetative incompatibility among varieties of *Gibberella fujikuroi (Fusarium moniliforme). Experimental Mycology* 9: 39-47.

RAEDER, U. & BRODA, P., 1985. Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* 1: 17-20.

TAYLOR, J. W., 1986. Fungal evolutionary biology and mitochondrial DNA. *Experimental Mycology* 10: 259-269.

APPENDIX

TECHNIQUES EVOLVED DURING THIS STUDY

A RAPID SPIN COLUMN TECHNIQUE FOR THE SEPARATION OF NICK TRANSLATION PRODUCTS USING A MICRO-CENTRIFUGE

A desirable step in the labeling of probes used in DNA hybridisation is the separation of unincorporated radionucleotides from the reaction products. Two methods are commonly used; repeated precipitation and column chromatography (Maniatis *et al.*, 1982; Perbal, 1984). The latter can be again divided into standard column chromatography and the spun column procedure using a clinical centrifuge. This note describes a rapid variant of the spun column procedure using a micro-centrifuge.

A miniature column is made by cutting off the collar of a 1 ml micropipette tip and inserting the body of the tip through the collar. This is then plugged with silanased glass wool and loaded with Bio-Gel P6, (50-150 mesh, molecular weight exclusion limit 4600) which has been preswollen in TE buffer (10 mM tris-HCl, 1 mM EDTA, pH 7,5). The column is drained and gel loaded to an approximate bed volume of 1 ml. This is then securely push fitted into a 1,5 ml Eppendorf tube. Alternative gels can be used but should be tested to establish that the beads are not crushed by the centrifugal forces involved.

Shortly before use, the assembly is placed in a micro-centrifuge and spun for 5 seconds thus removing all the fluid from the void volume. This is removed from the Eppendorf tube and the unit reassembled. The completed nick translation reaction is loaded onto the damp column and the reaction tube rinsed with 100 ul

of 2% Dextran Blue in TE, which is also loaded onto the column and serves to monitor elution. The column is again centrifuged for 5 seconds, unincorporated nucleotides are retained in the gel and the labeled probe passes into the Eppendorf tube for further use. Six probes at a time can be cleanly separated in a matter of minutes and I have yet to record contamination of the centrifuge with radioactive material.

REFERENCES

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J., 1982. Molecular cloning, a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

PERBAL, B., 1984. A practical guide to molecular cloning. John Wiley & Sons, New York.

A SIMPLIFIED ISOTACHOELECTROPHORESIS METHOD FOR ELUTING DNA FROM AGAROSE GELS

Numerous methods have appeared in the literature for the elution of DNA from agarose and acrylamide gels. Representative of these are variants of electroelution (Ho, 1983; Stroop, 1988), centrifugal filtration (Zhu *et al.*, 1985), affinity columns (Vacek *et al.*, 1982), binding to glass (Vogelstein & Gillespie, 1979) and freeze-squeeze (Tautz & Renz, 1983). In such a case, where a plethora of methods exist, no one method is particularly good or applicable in all situations and generally a researcher will use that with which he is most familiar.

While on sabbatical I was introduced to the method of isotachoelectrophoresis, as used at the Division for Viroid Research of the Max Plank Institute in Martinsreid, for the recovery of DNA and RNA from agarose gels. Although recovery was excellent the apparatus was needlessly complex and required constant vigilance. The following variant of the method was developed.

The general requirements of the apparatus are most simply described by Ho (1983), (see Fig. 1). An alternative is to replace the nylon mesh with an agarose plug. I manufacture the columns from Eppendorf tips: 10-15 mm of the tip is cut off, inserted into the main portion and any protruding part cut off. A plug of silanased glass wool rests on the ledge thus formed to create the elution chamber.

The lower end of the Eppendorf tube is sealed with dialysis membrane which is held in place with a rubber O-ring and also secured with Parafilm. The whole is inverted, a few drops of buffer A (40 mM Tris-Cl, pH 8,3) are added to the column to help avoid air bubbles, a prewet plug of glass wool is set onto the ledge and the agarose slices containing the DNA to be eluted are inserted into the

tube. Sephadex G50 in buffer A is added to cover the slices and the surplus buffer removed with a pasteur pipette. The Sephadex aids in holding the gel slices in position and in layering on the next buffer.

A drop of buffer A containing a trace of phenol red is added to monitor the elution, and buffer B (100 mM amino-caproic acid) is layered onto the Sephadex surface. The membrane-sealed end of the tube is submerged in a reservoir of buffer A. Electrical connections are made to the reservoir and buffer B and electrophoresis is carried out at 1 - 2 mA per tube until the phenol red has moved into the lower chamber. The current is then reversed for 10 secs to detach DNA from the membrane. The tube is removed from the reservoir, the dialysis membrane punctured and the fluid from the lower chamber containing the DNA is recovered and precipitated with 0,25 vol. 7,5% ammonium acetate and 2 vol. ethanol.

In order to assess the recovery efficiency, an experiment was performed using a Hind III digest of phage lambda DNA. The fragments were electrophoretically separated on a 1% agarose gel, the loading being 12 µg per lane as I wished to avoid using radioactive tracers. The gel was stained with ethidium bromide and slices containing the fragments were recovered by observation under U.V. light.

The DNA was eluted as described above and the experiment was repeated using buffer A throughout to compare isotacho-electroelution with conventional electroelution. Precipitated DNA was taken up in 500 µL of water and the concentration measured spectrophotometrically at 260nm. The recovery of total fungal DNA was also compared by the two methods (Fig. 2).

It is clear from Fig. 2 that the sweeping effect of isotachoelectroelution enhances recovery at the higher molecular weights. Below 9 kbp there is no obvious advantage although electroelution required 2,5 hrs versus 40 min for isotacho-electroelution. A number of tubes can be run simultaneously unattended, as the membrane will retain DNA.

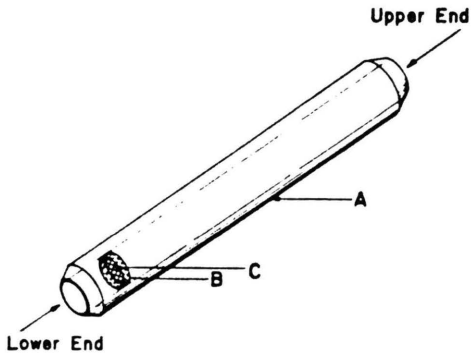


Fig. 1. The elution tube. A = Plexiglas tubing; B = polyethylene O-ring; C = nylon mesh. (Taken from Ho, N. W. Y., 1983. *Electrophoresis* 4: 168-170).

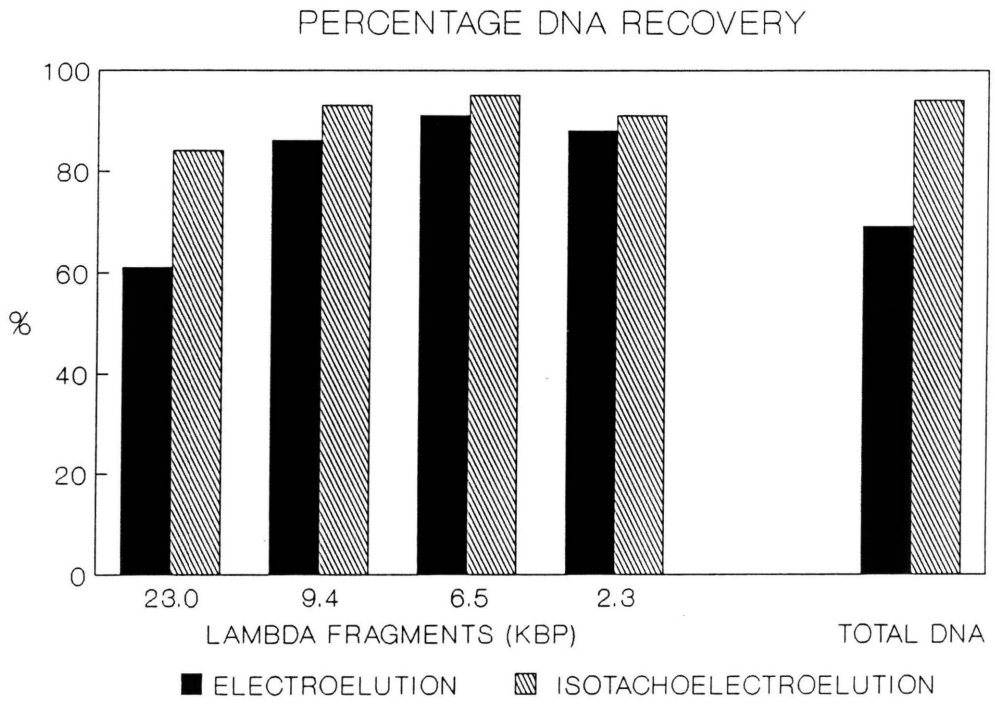


Fig. 2. Percentage DNA recovery of phage Lambda fragments and total fungal DNA by two methods.

REFERENCES

HO, N. W. Y., 1983. An improved electroelution method for the recovery of DNA fragments from agarose and acrylamide gels. *Electrophoresis* 4: 168-170.

STROOP, W. G., 1988. Simultaneous electroelution and concentration of DNA fragments from agarose gels. *Analytical Biochemistry* 169: 194-196.

TAUTZ, D. & RENZ, M., 1983. An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. *Analytical Biochemistry* 132: 14-19.

VACEK, A. T., BOURQUE, D. P. & HEWLETT, N. G., 1982. An ethidium-acrylamide affinity medium for the recovery of nucleic acids from free solution and from polyacrylamide and agarose gels. *Analytical Boichemistry* 124: 414-420.

VOGELSTEIN, B. & GILLESPIE, D., 1979. Purification of DNA fragments from agarose gels using glass powder. *Proceeedings of the National Academy of Sciences of the U.S.A.* 76: 615.

ZHU, J., KEMPENAERS, W., VAN DER STRATEN, D., CONTRERAS, R. & FIERS, W., 1985. A method for fast and pure DNA elution from agarose gels by centrifugal filtration. *Bio/Technology* 3: 1014-1016.