APPLICATION OF THE TECHNIQUES OF MOLECULAR BIOLOGY TO COCOA PATHOLOGY

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INTRODUCTION

The overwhelming public perception of the recent advances in molecular genetics is that such techniques are primarily used in the production of transgenic crop plants or in animal cloning (e.g. FlavrSavr™ tomatoes, Dolly the sheep). However, the same technological advances have revolutionised many areas of biological research, where they do not give rise to similar ethical or ecological concerns. This article will illustrate the impact of this technology on cocoa pathology and give an indication of its future potential.

Plant pathology, like all aspects of microbiological research, faces the inherent problem that the organisms under study are microscopic and generally only visible to the naked eye when causing disease lesions on a host plant or producing macroscopic fruiting structures (e.g. mushrooms). Even during microscopic examination, few phenotypic characters are discernible and the reliable identification of microbial pathogens to the species level requires a significant degree of expertise. For the cocoa-grower, it can often be important to obtain a correct and precise identification of the causal agent of a disease (preferably at an early stage in the disease cycle), so that appropriate control measures can be implemented. It may also be important to achieve identification below the species level, for example where two races of a given pathogen differing in their pathogenicity to particular hosts may be present.

Where morphological identification is problematic or unreliable, pathologists have developed a range of biochemical, microscopic and genetical tools to aid the investigative process. The most powerful of these tools are those of molecular biology, which permit direct analysis of the genetic material (the DNA) of the pathogen. At the simplest level, genetic features characteristic of a particular pathogen can be elucidated, though the power of these techniques is such that it is also possible to gain an insight at the molecular level into the processes which underlie pathogenesis. Such a detailed understanding of the events which take place during the process of host infection can identify targets for disease control such that pesticides with specific activity against the target pathogen can be developed.

Cocoa trees are susceptible to a panoply of microbial pathogens, many of which are still poorly understood. Of these the three which are considered most important and have thus received the greatest attention are *Phytophthora* pod rot (PPR or Black Pod caused by Oomycete pseudofungi), witches' broom disease (caused by the basidiomycete fungus *Crinipellis perniciosa*) and swollen shoot disease (caused by the Cocoa Swollen Shoot Virus (CSSV), a non-enveloped bacilliform badnavirus). All three have been the subject of study by molecular plant pathologists to varying degrees. The advances permitted by the advent of molecular biology for each of these are described below *Phytophthora* Pod Rot (PPR), also widely known as black pod, is found in all cocoa-growing regions of the world, though its severity varies depending on climatic conditions, the variety of cocoa being grown, the

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relative importance of PPR compared to other diseases in a particular areas (e.g. South America) and also the precise nature of the causal agent itself. PPR disease of cocoa causes some 45% of the total global crop loss attributed to pest and diseases though locally, however, it can cause losses of up to 80% (Lass, 1985).

It is now recognised that PPR is caused by several species belonging to the genus *Phytophthora* rather than by a single pathogen (*P. palmivora*), as was originally believed (Waterhouse, 1963). Brasier and Griffin (1977) identified consistent morphological and karyotypic differences between isolates morphologically distinct forms of *P. palmivora*. This evidence resulted in the erection of a new species, *P. megakarya* (Sansome et. al., 1975), members of which possessed 5-6 larger chromosomes (L-type = large chromosomes, hence *mega-karya*) as opposed to the 9-12 smaller chromosomes (S-type = small chromosomes) found in isolates more typical of *P. palmivora*. The various morphological forms (denoted MF1 to MF4) of *Phytophthora* pathogens which are found on cocoa have now been identified as *P. palmivora* (MF1 and MF2), *P. megakarya* (MF3) and *P. capsici* (MF4) (Förster et. al., 1987; Griffin, 1977; Oudemans and Coffey, 1991). However, there remains some confusion about the extent to which MF4 and *P. capsici* are entirely synonymous (Ortiz Garcia, 1996). An additional complication is that PPR is occasionally caused by other *Phytophthora* species, such as *P. citrophthora* in Brazil and some parts of West Africa (Campêlo and Luz, 1981; Kellam and Zentmyer, 1981; Ortiz Garcia, 1996).

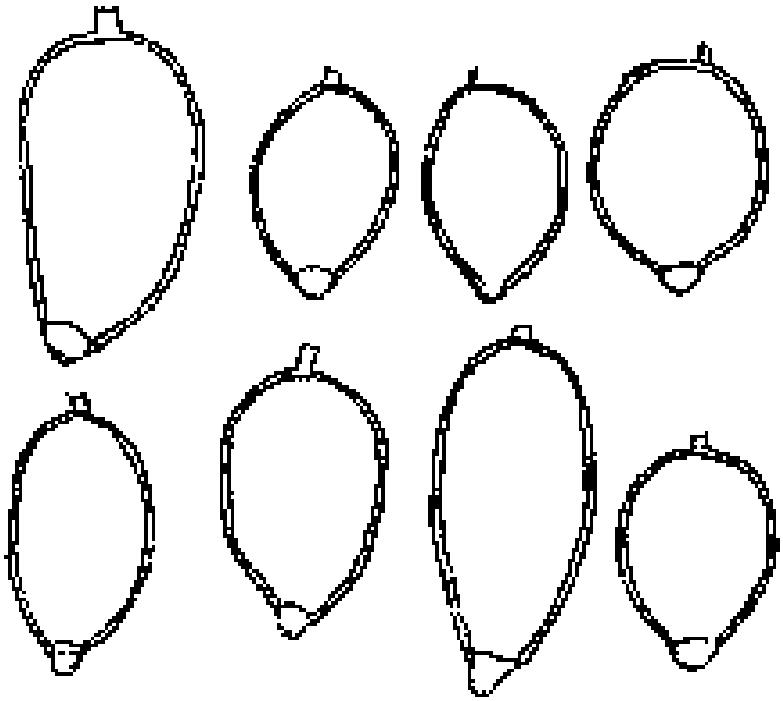
Of the three main species of *Phytophthora* associated with PPR, *P. palmivora* is world-wide in its distribution and *P. capsici* is locally important in several areas, notably parts of Brazil, Mexico, the Caribbean and India (Chowdappa and Mohanan, 1994; Lass, 1985; Ortiz Garcia, 1996). Of greatest concern however (particularly in the context of cocoa supplied to Europe), is *P. megakarya*, which causes the most severe symptoms and over recent decades has spread progressively through the cocoa-growing areas of West Africa. At present, *P. megakarya* has not been reported from the more westerly parts of West Africa (e.g. Ivory Coast), nor has it spread to cocoa-growing regions outside Africa (Luterbacher and Akrofi, 1994).

It is possible to distinguish *P. palmivora* and *P. megakarya* on the basis of sporangial morphology (*P. palmivora* isolates have smaller protrusions [papillae and pedicels] on their sporangia) (Figure 1), as well as macroscopic features, such as lesion morphology (*P. megakarya* lesions have a more powdery appearance) and colony appearance on agar (*P. palmivora* generally has a stellate appearance in culture). However, reliable differentiation of the two species based on these features is a specialist skill, usually requiring the time-consuming isolation of the pathogen into pure culture on selective media. Characterisation of the two species using biochemical markers was first reported by Idosu and Zentmyer (1978) and Ersulius and Shaw (1982), who found differences in isoenzyme and total protein profiles respectively between the two species. A more detailed study of isoenzyme variability by Oudemans and Coffey (1991) found *P. palmivora* isolates from a wide variety of hosts throughout the world to be genetically very homogeneous, while isolates of *P. megakarya* were polymorphic (variable) at several loci. They defined two groupings within *P. megakarya*, with group MK1 being found in Nigeria and Equatorial Guinea and group MK2 being found in Cameroon.

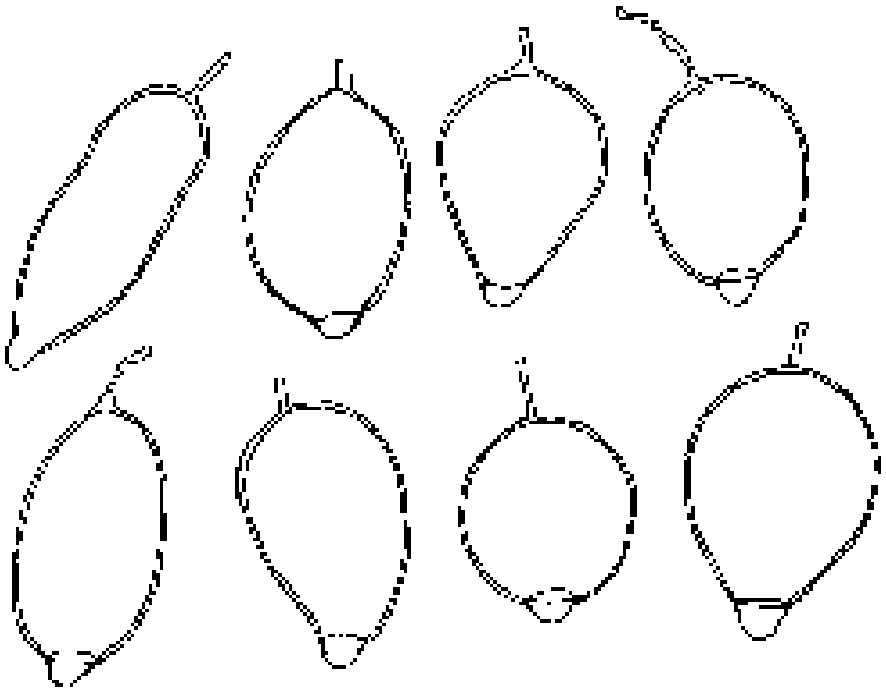
The advent of molecular biology has provided the technology for more direct and detailed analysis of genetic variability at the DNA level within and between PPR pathogens. A number of more general investigations have been conducted to examine genetic variation within and between various *Phytophthora* species (many of which are important pathogens of crops other than cocoa).

Figure 1. Variability in sporangium morphology of (A) *Phytophthora palmivora* and (B) *P. megakarya*

(A)



(B)



Source: Redrawn from Brasier and Griffin (1977). Note the variability both in sporangium size and shape. Bar equals 50 μm.

Förster et. al. (1990) purified mitochondrial DNA from a large number of *Phytophthora* isolates and the restriction maps (obtained by locating the cutting sites for several restriction enzymes) for these broadly confirmed those of previous isoenzyme studies (Oudemans and Coffey, 1991), in that *P. palmivora* was found to be genetically very homogeneous and that *P. megakarya* isolates were split into two groups. They suggested that *P. megakarya* was indigenous to West Africa on an unknown host and that present populations found causing black pod in different areas might have independent origins. A more detailed study of isoenzyme polymorphisms alone (Blaha, 1994) and together with RAPD (see below) polymorphisms Nyassé (1997; in press) confirmed the existence of two distinct populations in West and Central Africa, with the greatest genetic diversity being found in Cameroon. These data further suggested that though most populations were clonal in structure, there was evidence of sexual reproduction in field populations from Cameroon.

Earlier studies on PPR pathogens were originally prompted by a desire to establish the relatedness of the various pathogens to each other and to other *Phytophthora* species,. The emphasis has now shifted towards the development of rapid methods of distinguishing the various pathogens. In Ghana (where *P. megakarya* is locally present) and Ivory Coast (where currently only *P. palmivora* is reported to occur), such methods will permit accurate monitoring of the distribution of *P. megakarya*. One such diagnostic assay has been published by Sackey and Luterbacher (1994), in which DNA probes specific to *P. megakarya* and *P. palmivora* were isolated and cloned. By hybridisation of labelled probes against DNA from PPR isolates collected from the field in a dot blot assay, it was possible to distinguish the two species from as little as 0.1 ng of DNA from a pure culture (it should also serve to identify pathogens from disease lesions).

The PCR technique (see Appendix I – attached to this article) has revolutionised diagnostic assays in plant pathology. It is both sensitive enough to use on small quantities of biological material yet specific enough to be highly discriminating. The first use of PCR amplification to differentiate PPR pathogens involved the RAPD technique (see below) though this can only be used with DNA from pure cultures (John Spence, *pers. comm.*; Denis Despréaux, *pers. comm.*).

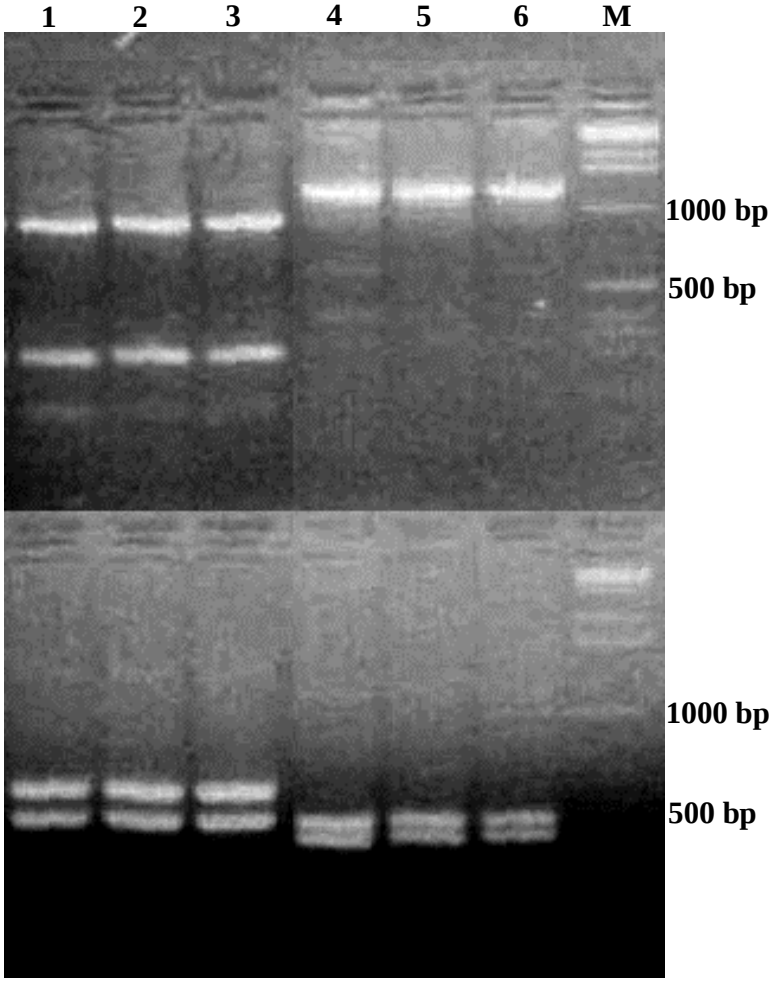
A feature of the all methods described above is that not only must the pathogen be isolated into pure culture, a process which may take several weeks to complete but also in some protocols, large quantities of mycelium are required for analysis. For instance, purification of mitochondrial DNA (mtDNA) is a protracted process which initially requires that several grams of mycelium be grown up in liquid culture (e.g. Förster et. al., 1990). Protocols such as isoenzyme analysis also involve the use of hazardous stain compounds to test for enzyme activity. Since the aim of a diagnostic assay is to provide an identification both rapidly and economically, an ideal method would permit pathogen identification directly from disease lesions within a few days of sampling.

Based on the observations of Förster et. al. (1990) that polymorphisms exist between the mitochondrial DNA (mtDNA) of *P. palmivora* and *P. megakarya*, a recent study funded by the BCCCA (Griffith and Jones, 1997) has developed a number of PCR probes to amplify polymorphic regions of mtDNA and which permit differentiation of the two pathogens. Primers were designed based on sequence information available for the mtDNA genome of *Phytophthora infestans* (which causes late-blight of potato). When cut with restriction enzymes the amplified fragments reveal polymorphisms between the two species in the form of distinctive banding patterns on an agarose gel. The specificity of this assay (due to the fact

that *Phytophthora* spp. are only very distantly related to both other fungi and plants) is such that DNA extracted directly from disease lesions can be analysed directly.

Small pieces of tissue cut from black pod lesions (and which may be preserved by air-drying) are used as the starting material. DNA is extracted from this tissue using a simplified extraction method and a small amount of the purified DNA is used in the PCR reaction. After the PCR amplification, the resultant PCR products are cut with a restriction enzyme and subjected to gel electrophoresis to separate fragments of different length (Figure 2). Samples are generally analysed in sets of 24 or 48 and a single operator can obtain results from the samples within 24 hours. The assay is economic (consumable cost of ca. £1-2 per sample) and although specialised equipment is required, such equipment is now commonplace in many biological research laboratories.

Figure 2. PCR-RFLP Digest of *Phytophthora megakarya* and *P. palmivora*



A 1300 bp fragment of mitochondrial DNA was amplified by PCR (with the primers F3A and R3A) and digested with the restriction enzymes *HaeIII*. (above) and *NdeII* (below). Lanes 1-3 *P. palmivora* (isolates TR1 [from Trinidad], CIV01, CIV04 [both from Ivory Coast]); lanes 4-6 *P. megakarya* (isolates GHBA6 [from Ghana], TG3, TG7 [both from Togo]); Lane M denotes a molecular weight marker (1 kb ladder; Gibco BRL Ltd.). Following agarose gel electrophoresis, isolates belonging to the two species clearly show distinct banding patterns.

WITCHES' BROOM DISEASE (WBD)

Of all the diseases of cocoa, WBD is currently the source of greatest concern. Its recent spread to Bahia State has decimated the Brazilian cocoa industry, as it did earlier this century in other Latin American countries, notably Ecuador. The possibility, however remote that it might spread beyond South America is viewed with considerable trepidation by the world's cocoa growers.

Unlike PPR, there is only one causal agent of WBD, namely the basidiomycete *Crinipellis perniciosa* though there is evidence that the fungus varies in pathogenicity in different parts of South America (Wheeler and Mepsted, 1984). Genetic differences between pathogen populations have previously been examined indirectly by investigation of basidiocarp morphology (Hedger et. al., 1987), isoenzyme polymorphisms (Griffith, 1989) and the identification of somatic incompatibility groupings (Griffith and Hedger, 1994a,b; McGeary and Wheeler, 1988; Wheeler and Mepsted, 1982; Wheeler and Mepsted, 1984, 1988).

The application of molecular biology techniques to the study of WBD is in its infancy. By Southern hybridisation with a mitochondrial DNA probe from *Coprinus cinereus*, Wilson (1995) (also detailed in Griffith et. al., 1994) identified RFLPs between isolates of *Crinipellis perniciosa* belonging to the three different biotypes (Cocoa (C-), Liana (L-) and *Solanum* (S-) biotypes; Griffith and Hedger, 1994a) though this method did not differentiate between isolates belonging to the pathogenic C-biotype. Andebrhan and Furtek (1994a,b) conducted RAPD analysis (see below) to assess levels of genetic variability between Brazilian isolates *C. perniciosa* from different host plants. They found an isolate of the S-biotype to be distinct from isolates of the C-biotype (including isolates found on relatives of *T.cacao* and the shrub *Bixa orellana*) but that within the C-biotype isolates genetic relatedness was more dependent on geographical proximity than occurrence on a particular host.

Future work of this nature making use of recent advances in molecular technology (for example using the Amplified Fragment Length Polymorphism (AFLP) technique or DNA sequencing) will provide more detailed information about how the spread of the pathogen between different areas, the relationship between the various biotypes and the rate at which current populations are evolving in the field. This last aspect is of particular importance for the development of durable control strategies in the future. Analysis of the process of pathogenicity by more detailed study of the process of meristem infection and pathogen development *in planta* (for example, which host or pathogen genes are turned on and off during pathogenesis) may also elucidate new targets for disease control. Research into this last aspect is currently being funded by Cocoa Research UK Ltd. at University of Wales, Aberystwyth.

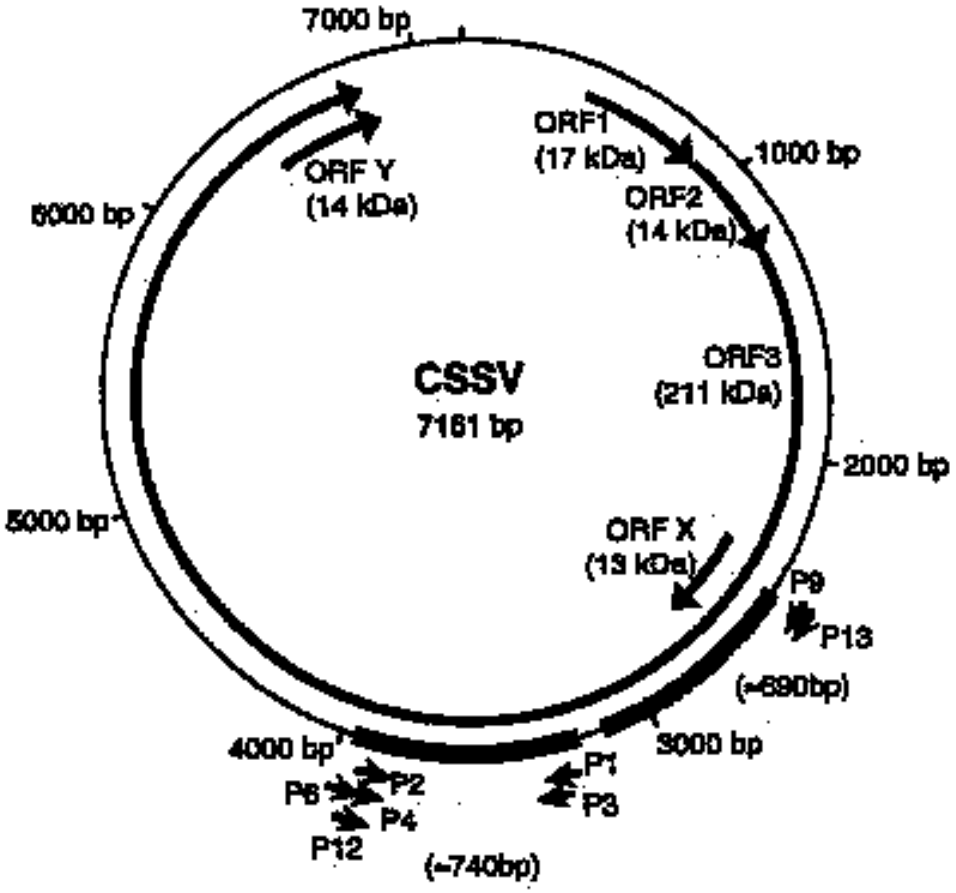
COCOA SWOLLEN SHOOT DISEASE

Cocoa swollen shoot disease was first reported on cocoa in West Africa in 1936, and although it was fairly quickly established that control could be achieved by total removal of infected trees, the difficulty of achieving this and the neglect of sanitation programmes during the Second World War resulted in a significant exacerbation of the disease problem for many years thereafter. The removal of almost 200 million trees has been necessary to bring the situation under control in the intervening period. The pathogen was identified as a virus by Posnette in 1940 and its mechanism of spread via mealybugs has also been long appreciated (Posnette, 1940; Posnette, 1947). Since viral infections cannot be cured (i.e. the pathogen cannot be killed, since viruses are not 'alive' in the normal sense of the word), preventative

strategies (as opposed to the control strategy of removing infected trees) rely on breeding resistant varieties, control of the vector or early identification of disease. The latent infection of trees is known to be a problem and it has been established that latently infected trees (i.e. not yet showing symptoms) can act as a source for further transmission (Legg, 1982).

The emphasis of recent CSSV research has been on detection of infection in the latent stage before any symptoms are manifest. Various immunological tests have been devised both to detect latent infection and also to differentiate the various strains of CSSV. An Enzyme-Linked Immunosorbent-Assay (ELISA) test devised by (Sagemann et. al., 1985; Sagemann et. al., 1983) was able to distinguish five CSSV serotypes in Ghana which correlated with differences in symptom production. However, this test is rather insensitive and only identifies the presence of CSSV in trees already showing symptoms. A more sensitive assay, the Virobacterial Agglutination (VBA) test has recently been developed (Hughes and Ollennu, 1993). This test differentiates 8 serotypes, as well as four subtypes of the well-characterised CSSV-1A group.

Figure 3. Map of the Genome of the Cocoa Swollen Shoot Virus



Genome organisation of the CSSV Agou1 based on the published nucleotide sequence (Hagen et. al., 1993; modified). The open reading frames (ORF) are indicated by long arrows. The predicted molecular weight (in kDa) of the proteins encoded by the individual ORF's is stated in parentheses under the ORF designation. The location of the primers (P1 to P4, P6, P9, P12 and P13) used for PCR amplification are shown by arrowheads outside the DNA molecule. The black and grey regions within the outer circle denote the location of the viral DNA of pCSSV9 (Maiss, unpublished) and that of the genome part sequenced after PCR respectively. Reproduced from (Hoffmann et. al., 1997).

CSSV (strain CSSV-1A) has the distinction of being the first cocoa pathogen to have its genome completely sequenced (Figure 3) (7161 bp - ca. 0.0001% of the size of the human genome!). This was achieved by Hagen et. al. (1993) and the complete sequence can be viewed at http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html (type accession number:- L14546). Five putative protein coding regions (open reading frames or ORFs) were identified within the genome, including a reverse transcriptase (for replication of the viral genome via RNA) and a coat protein.

By combining immunological purification of viral particles with PCR amplification (the IC-PCR method; immunocapture-PCR) based on information from the complete genome sequence, Hoffmann et. al. (1997) have devised an detection assay for identifying latent infection. However, this method is effective only for the CSSV-1A serotype and it remains to be established whether this method is more sensitive than the VBA test of Hughes and Ollennu (1993). Nevertheless, a significant degree of sequence polymorphism was detected within the ORF3 gene of CSSV-1A strains indicating a high level of genetic diversity within CSSV populations.

It has been proposed by (Hughes and Ollennu, 1994) and others that, in the light of the incomplete effectivenss of other control strategies it may be feasible to 'immunise' trees against infection by virulent strains of CSSV by inoculation with attenuated ('mild') strains of the virus. Some symptoms would be caused by these 'mild' strains but it is anticipated that overall crop losses would be reduced (Ollennu et. al., 1994a; Ollennu et. al., 1994b). However, such a strategy must be adopted with great care, since it must first be established that the cross-protection offered by 'mild'strains will be effective in controlling all the more virulent CSSV strains and also that 'mild' strains once inoculated will to be able to evolve higher levels of aggressiveness. Further, strain characterisation or even the construction of transgenic attenuated strains suggests a central role for molecular biology in these developments.

FUTURE DEVELOPMENTS

The progress outlined above has hitherto be made predominantly in the areas of discrimination and detection. Future investigation may use similar techniques to analyse in detail the process of pathogenesis (particularly in the case of WBD and CSSV, as described above) such that through a better understanding of the pathogens physiology, specific control strategies can be devised.

It is also important to consider the progress being made by plant geneticists and breeders. The advent of marker-assisted selection will ultimately speed up the process of plant breeding by identifying quantitative traits associated with degrees of resistance to certain pathogens. Again, the knowledge currently being obtained with regard to pathogen variability will play an important role in these breeding programme by permitting pathogen populations to be closely monitored. Although antibody-based ELISA assays (as used in pregnancy testing kits etc.) would provide a useful tool for cocoa farmers in accurate on-site disease diagnosis, the requirement for animal experimentation in the production of such antibodies can render such approaches ethically unacceptable. However, the combination of molecular genetics with immunology is likely to bypass such issues, as has recently been shown by He and Taussig (1997) who have developed an *in vitro* method for antibody selection.

The final area of advance is likely to stem from the interaction between molecular biology and information technology. The release of DNA sequence data is now a prerequisite for the

publication of results and as a result substantial databases of both sequence and other data (for example a website for the International Cocoa Germplasm Database [ICGD] will shortly be active) are now in the public domain and readily available via the Internet. Comparison of data between labs in different continents and the use of standardised methods for monitoring pathogen populations will thus be greatly facilitated.

ACKNOWLEDGEMENTS

The author would first like to thank the BCCCA for their financial support for our work on black pod diagnostics, and also to Drs. Michelle End and Martin Gilmour for their advice and encouragement. We are also grateful to Dr. Ann Parker, Mr. Wilberth Phillips, Dr, David Iwaro, Professor John Spence, Dr. Tony Lass, Dr. Denis Despréaux, Dr. Michel Ducamp, Dr. G. Blaha, Dr. Jeanne N'Goran, Dr. Carmen Suarez, Dr. Mark Luterbacher, Professor Brian Wheeler and Professor Paul Hadley.

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Appendix I. Description of PCR Methodologies

The polymerase chain reaction (PCR) is by now extensively used as a tool for identification of microbial pathogens. Only nanogram (1 ng = 10^{-9} gram) quantities (or less) of DNA as a template for amplification (which can be isolated from milligram or microgram quantities of biological material) are needed and the process can be made sufficiently specific that the DNA used need not necessarily be derived from a pure culture. It is thus possible to design methods of detection that are both highly sensitive (in theory able to detect as little as a single copy of the target DNA sequence), yet highly specific.

A technique that has been used extensively to date by plant pathologists is RAPD (Random Amplified Polymorphic DNA). In contrast to most applications of PCR, this technique does not require any *a priori* DNA sequence information about the organism being studied. It also only requires a single, short (usually 10 bp) random primer (a primer is a short single-stranded DNA sequence manufactured synthetically and which sticks to matching sequences on the target DNA to 'prime' DNA amplification) is used in the PCR reaction. By using a low primer annealing temperature, multiple-sized bands are amplified and these can be used as a 'bar-code' to type isolates. Similarity indices can be generated for comparison by scoring the frequency of shared bands between isolates. However, the technique has been bedevilled by difficulties in standardisation and there are many examples in the literature of different banding patterns being obtained from identical biological samples by different laboratories or when minor alterations are made to the DNA isolation or amplification methodology (e.g. McEwan et. al., 1998).

Most applications of PCR rely on the use of two short synthetic pieces of DNA (called 'primers', usually ca. 20 basepairs long and costing under £10 each to purchase) which match the two ends of the target region of DNA. The specificity of the PCR amplification reaction relies on designing suitable primers to avoid non-specific amplification of any DNA except the desired sequence. The design of these primers requires at least some DNA sequence information about the organisms under study. With the exception of a few 'model' organisms (e.g. humans, yeast, *E. coli* etc.), the amount of DNA sequence information for most organisms is limited or absent. However, primers can be designed using sequence information from related organisms, provided that reasonably conserved regions of DNA are chosen for study.

The most widely used target sequence for PCR-based studies in plant pathology is the rDNA (ribosomal DNA) locus. The rDNA locus is present in all living organisms (since all contain ribosomes) and certain regions of the locus are highly conserved between distantly related organisms. For instance, primers pairs have been designed which amplify parts of the rDNA locus from any fungi (White et. al., 1990). Once the PCR product is produced, genetic differences can be revealed either by restriction digestion of the amplified product or by sequencing all or part of the product. Cooke and Duncan (1997) have produced a phylogenetic tree from the genus *Phytophthora* based on rDNA sequences. Although PCR amplification of DNA from pure cultures works well with such primers, they are often non-specific and are thus of limited use for amplification of DNA from complex substrates. In disease lesions, for instance, it is possible that rDNA from other organisms (e.g. the host plant or contaminating fungi) may be also amplified in addition to that of the pathogen.

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