EPIDEMIOLOGY AND POPULATION STRUCTURE OF *PHYTOPHTHORA* SPECIES CAUSING DISEASE OF COCONUT IN INDONESIA

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ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
BP	Black Pod
BR	Budrot
BALITKA	Balai Penelitian Tanaman Kelapa Dan Palma Lain
CPCRI	Central Plantation Crops Research Institute
DFID	Department for International Development
DFID CPP	DFID Crop Protection Programme
GO	Orange dwarf
ITS	Internal Transcribed Sequence
ITS-RFLP	ITS-Restriction Fragment Linked Polymorphism
NYD	Nias Yellow Dwarf
MLEE	Multi-locus enzyme electrophoresis
PNF	Premature nutfall
RAPD	Randomly Amplified Polymorphic DNA
RICP	Research Institute for Coconut and Palmae
SCDP	Smallholder Coconut Development Programme
WAT	West Africa Tall

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1. EXECUTIVE SUMMARY

Coconut is a major commodity of Indonesia and is predominately a smallholder concern. A varietal improvement programme aimed to introduce dwarf, high yielding coconut germplasms to Indonesia. These initiatives culminated in the breeding and promotion of variety PB121. In the early 1980's a new disease was observed affecting coconut palms causing budrot (BR) and premature nutfall (PNF) symptoms. PB121 and its parental lines were highly susceptible to BR and PNF. Spread of the disease(s) in Indonesia was rapid and losses caused were reported as high. Research identified the causal organism as *Phytophthora palmivora*, although other *Phytophthora* spp. have been associated with disease(s) of similar aetiology in other countries. This project aimed to investigate the epidemiology of the BR and PNF causal organisms under mixed coconut based cropping system of Indonesia and to identify cropping practices that affected the prevalence of the disease(s).

An socio-economic study was conducted to appraise the current status of BR and PNF and palm replanting strategies. This study concluded that with the current replacement programme for PB121 with purportedly BR and PNF resistant palm varieties, such as Khina, and existing PB121 palm stands having progressed above a height commonly affected by BR and PNF, the disease(s) would become insignificant in coming years. Accordingly, a research focus on rehabilitation programmes for coconut smallholder affected by BR and PNF that gave due attention to maintaining financial income lines in the period between replanting and palms reaching maturity was needed. The truths of this statement are unsubstantiated as 1) the parentage of the current recommended varieties, such as Khina, continue to include BR and PNF susceptible lines and BR and PNF resistance screening is not a component of varietal selection and 2) knowledge of the epidemiology of BR and PNF is largely anecdotal.

The study of over 100 *Phytophthora* isolates obtained from BR and PNF infected palms and crops associated with coconut, ostensibly black pod of cocoa (BP), confirmed *P. palmivora* as almost exclusively the casual organism. Molecular analysis by ITS-RFLP proved an effective method of species identification and also identified an anomalous multiple ITS sequence prevalent in Indonesian *P. palmivora* populations. AFLP analysis revealed *P. palmivora* populations to be broadly homogeneous but delineated between coconut and cocoa isolates. These results were substantiated by pathogenicity studies. An analogous study on *Phytophthora* spp. affecting coconut/cocoa/arecanut based cropping systems of India provided comparable data. The ITS-RFLP data from these studies added value to the *Phytophthora* information web site <PhytID.org> [DFID CPP R7337].

Plantation based studies aimed to determine the mode of BR and PNF infection. Spore trapping by mechanical means proved insufficiently sensitive to detect background levels of *P. palmivora* sporangia and biotic baiting approaches proved inconclusive. Visual observations on the incidence of BR and PNF recorded no positive correlation between these symptom types, and showed that PNF lesions were ostensibly positioned with an outward projection from the palm trunk and had a similar distribution on BR and non-BR infected palms. These data suggest distinct modes of infection for BR and PNF, and that for PNF an air-borne source of infection is probable. Studies on potential insect vectors suggested a possible role with *P. palmivora* isolated from *Orycetes* sp., *Rhyncophorus* sp., *Forficula* sp. and *Dio calandra* sp., but robust data was not obtained. Similarly, studies on spatial distribution of BR proved difficult to execute. The inability to capture infectious propagules of *P. palmivora* within the plantation reduced the scope for molecular marker assisted epidemiological studies.

2. BACKGROUND

Coconut (*Cocos nucifera*) is economically very important in Indonesia and figures prominently in social and cultural activities. It is grown mainly as a smallholding crop, providing coconut meat, copra (from which coconut oil is derived), wood shell and coconut milk. Coconut stands are frequently underplanted with cocoa (*Theobroma cacao*); pepper (*Piper nigrum*) in mixed cropping systems (see Plates 1a & b, respectively). The productive phase of a coconut is variety dependent, but for current varieties cropping can be expected at 4 - 6 years and can continue upto 40 - 60 years. Harvesting is normally undertaken every 3 months.

Indonesia has the largest percentage area of land devoted to coconut in the world and the crop has spread to almost all the islands of the archipelago: Sumatra 30.9%, Java 26%, Celebes 19%, Borneo 7.9% and other islands 15.4%. The total area of coconut plantations in Indonesia is about 3.7 million hectares of which over 95% is held by smallholders. In Indonesia an estimated 3.2 million people depend on coconut based cropping system as a source of livelihood. However, productivity is recognised as being low, notably lower than the Philippines which has a comparable area to cultivation (Persley, 1992; Muljoharjo, 1993): Average productivity of copra in 1991 was 1.03 ton/ha compared to an expected 2-6 ton/ha under optimal conditions (Mahmud and Novarianto, 1992).

The national importance of coconut has long been recognised and the introduction of new hybrids started as part of an Indonesian Government programme to increase coconut production. In 1977 coconut hybrids were imported from Ivory Coast via the Smallholder Coconut Development Project (SCDP) with funding from the World Bank. These hybrids aimed to combine the early cropping of dwarf coconut varieties with other desired attributes of tall varieties. The hybrid PB121, a cross between Nias Yellow Dwarf (NYD) and West Africa Tall (WAT), was the variety most widely planted by smalholders through this programme.

In the early 1980's two new diseases were reported on PB-121 and NYD in North Sulawesi (Bennett et al., 1986) causing a budrot (BR) and premature nutfall (PNF), respectively (see Plate 2 and 3). These diseases subsequently increased in incidence and in 1992 some 7,000 ha of PB-121 were reported as affected by BR and PNF diseases (Mardi, 1992). The extensive planting of PB-121 and the susceptibility of this variety to BR and PNF are considered to be key factors in the increased incidence of the diseases that now represent the main diseases affecting coconut in Indonesia. Levels of incidence (palms affected) have been estimated in certain areas up to 50% for budrot and 20 – 30% for premature nutfall (Renard and Darwis, 1992).

Diagnostic examination identified the causal agent(s) as *Phytophthora palmivora* for both diseases. This is a recognised pathogen affecting coconut in other countries, although other *Phytophthora* spp. have also been associated with a disease of similar symptomolgy with country-specific distributions. For example *P. palmivora* has been identified as the primary causal agent in Indonesia and the Philippines, whereas *P. katsurae* is the main causal agent in Cote-d'Ivoire (Hall & Warokka, 1994). A previous EU project (EEC STD 3) revealed that of 240 *Phytophthora* isolates obtained from coconut based cropping systems in Indonesia 95% were *P. palmivora*/*P. arecae* and 5% were *P. nicotianae* (Ortiz Garcia and Blaha, 1994). The designation of species status for *P. palmivora and P. arecae* was questionable from these and related data (Blaha et al., 1994) and in a study by Mchan and Coffey (1994) these species were described as cospecific. Mchan and Coffey also suggested that Southwest Asia might be the region of origin for *P. palmivora*, since coconut is indigenous to this area of the world.

Control measures centre on cultural control through cutting out diseased palms and removing fallen coconuts. Chemical control can be achieved, for example stem injections with phosphonates can act as a prophylactic against BR, however, it is not economically viable to do so in the majority of cases, particularly for smallhold farmers, and its effectiveness is questioned. Accordingly, more appropriate control strategies are required. Current research strategies include the development of resistant cultivars and improved cultural practices. In this context an improved understanding of the epidemiology and genetic diversity of *P. palmivora* is essential.

Some aspects of the epidemiology of BR and PNF diseases were addressed in the previous EEC/STD 3 project. Spore trapping at ground level showed that *P. palmivora* was splashed from the ground to a height of at least 75 cm. However, there was no evidence to link soil borne *P. palmivora* populations with *P. palmivora* isolated from organic matter collected in the axils of palm fronds of healthy and diseased palms (Thevenin, 1994). This observation may suggest the role of an insect vector as has been established in the spread of *P. palmivora* in cocoa in West Africa (Gregory & Maddison, 1981), and of *P. katsurae* on coconut in Côte d'Ivoire (Pohe, 1992), although this has not been specifically studied in Indonesia. A recurrent problem with these types of study has been the inability to track individual isolates due to the lack of suitable isolate-specific markers, such as a rare MLEE or an AFLP.

To date, characterisation of *P. palmivora* isolates from coconut of Indonesia and elsewhere has focused on morphological features and isozyme analysis. These studies have shown only limited diversity. For example, Mchau and Coffery (1994) described 8 electropheritic types (ETs) through MLEE of 14 enzymes amongst a collection of *P. palmivora* isolates of world-wide origins. Greater powers of resolution are possible through molecular fingerprinting methods such as RAPD and AFLP. Although no studies have been undertaken on *P. palmivora*, comparable studies on *P. megakarya* (Nyasse et al., 1999) and particularly *P. infestans* (Drenth et al., 1994). In both of these studies, although the populations comprised A1 and A2 mating types the population structures were consistent with a predominantly clonal nature of reproduction, rather than one where sexual exchange was a frequent event. The potential significance of non-clonal reproduction, even if at a minor frequency, is significant.

An array of molecular approaches are available for the characterisation of fungi, the selection of which depends on the objective. In the context of *Phytophthora* spp. the lack of stable diagnostic characters frequently makes species determination by molecular means desirable. This was recognised by Brasier in 1983 who suggested the need to relate morphological criteria to molecular markers. Sutton (1992) also indicated that no progress in the genetic relationship and identification of phytopathogenic fungi was likely if based on morphology alone. An appropriate method in this regard is restriction analysis of the internal transcribed spacer region (ITS-RFLP). Using this method ITS-RFLP profiles are generated that are specific at the species level (Lee and Taylor, 1992; Sherriff et al., 1994; Cook et al., 2000).

If greater resolution is required, intra-species variation, then a number of genomic fingerprinting methods may be applied. Those most frequently used include random amplified polymorphic DNA (RAPD) (Samuels et al., 1995; Welsh et al., 1990, Williams et al., 1991) and, more recently, amplified fragment length polymorphism (AFLP) (Vos et al., 1995). AFLP is recognised as particularly suitable, combining high levels of discrimination with reproducibility. The AFLP technique as described by Vos et al (1995) involves digesting of genomic DNA with two restriction endonucleases, followed by the ligation of the restriction products to specific double-stranded oligonucleotide adapters.

The adapter/restriction fragments are amplified by PCR with selective primers containing nucleotides homologous to the adapters with a 1 - 3bp extensions at the 3' end. DNA fragments complementary to the extensions are amplified selectively, separated on a polyacrylamide gel and visualised (Vos et al., 1995; Muller et al., 1996; O'Niel et al., 1997). A simplified procedure has subsequently been described by Muller et al (1996) that uses a single restriction enzyme (Pst1) and adapter. This method produces fewer DNA bands and thus allows separation of the fragments by standard agarose gel.

The main research themes of this project have been formed in line with the conclusions and recommendations of the Final Workshop of the previous EEC/STD 3 funded project held in Manado in January 1996 and prioritised at the national level by the Research Institute for Coconut and Palmae.

In addition to these main objectives a comparative study was undertaken on the diversity of *Phytophthora* populations affecting coconut/cocoa/arecanut based cropping system in India. This research was conducted by Dr. P. Chowdappa from the Central Plantation Crops Research Institute (CPCRI), through attachment to CABI Bioscience UK Centre Egham.

3. PROJECT PURPOSE

Budrot (BR) and premature nutfall (PNF) caused by *Phytophthora* spp. are the major plant disease problems affecting coconut in Indonesia, notably PB121: a variety promoted under various smallholder coconut rehabilitation programmes. This project aimed to increase our knowledge of BR and PNF in Indonesia through socio-economic review of smallholder coconut based cropping systems and analysis of the genetic structure, host specificity and distribution of *Phytophthora* spp. populations. Based on this enhanced knowledge the epidemiology of the disease(s) was to be studied in order to determine mechanisms of spore spread, the role of any vectors and alternative hosts and mechanisms of fungal survival, and ultimately to develop smallholder-appropriate recommendations targeted at the alleviation of the constraints. Throughout the project, a strong emphasis was placed on the training of counterpart Indonesia scientists in skills relevant to ensuring a capability in pathology and molecular biology beyond the project end date.

4. RESEARCH ACTIVITIES

4.1 ECONOMIC CONSEQUENCES OF *PHYTOPHTHORA* DISEASES IN COCONUT AND MECHANISMS FOR RESEARCH DISSEMINATION

The aim of this socio-economic component was to gauge the impact of the disease at the micro and macro level, then to suggest how the research might generate technologies of control and how well these may be taken up by farmers, and to determine linkages to be established with other institutes to develop and deliver effective extension messages resulting from the RICP research.

The format for the review entailed the visitation of Drs P. Oldham (NRI) and M. Holderness (CABI Bioscience UK Centre) to North Sulawesi, Manado for a 2 - 3 week period in November 1996 for discussion with RICP, other related projects, smallholder farmers and coconut processing factories. The findings of the visit were assimilated into a report.

4.2. COLLECTION OF *PHYTOPHTHORA* SPP. FROM COCONUT BASED CROPPING SYSTEMS OF INDONESIA

An extensive collection of *Phytophthora* spp. of coconut based cropping systems of Indonesia had been collected previously under the EEC/STD 3 funded project. This included representation of *P.palmivora* of BR, PNF, BP and environmental sources (soil mainly). Isolation from environmental sources had been achieved by baiting coconut nuts and isolating from PNF like infections. The viability of these cultures held at RICP was tested, and new stock cultures made and deposited in the CABI Bioscience genetic resource collection.

This collection was added to over the duration of the project by routinely isolating from new BR and PNF infections, and from suspected *Phytophthora* infections on crops associated with coconut cropping systems (particularly black pod of cocoa).

All isolates were maintained on V8 agar with antibiotics. For morphological characterisation, all cultures were transferred on V8 agar without antibiotics. For DNA extraction, isolates were grown on V8 broth as stationary cultures within 250ml conical flasks.

Species assignment had been previously been undertaken on the EEC/STD 3 collection. These identification and those of the additional isolates collected were confirmed/determined to species level using the keys of Stamp et al. (1990).

4.3. GENOMIC ANALYSIS OF *PHYTOPHTHORA* SPP. FROM COCONUT BASED CROPPING SYSTEMS OF INDONESIA

This research aimed to develop robust molecular tools for 1) species confirmation and 2) genomic fingerprinting *Phytophthora* populations. The outputs of this study were primarily to investigate host specialisation and provide baseline data to enable marker-assisted epidemiological analysis. The methods developed for the DNA analysis were selected with a view of transference to RICP in Manado.

4.3.1. DNA EXTRACTION

DNA was extracted using the method described by Cenis (1992). In brief, cultures were incubated as stationary cultures at 25°C for 3-4 days in 20% V8 broth. Harvested mycelium was pelleted by centrifugation at 13000 rpm for 5 minutes, washed in TE buffer and then pelleted again. The TE was decanted and replaced with 300ul of extraction buffer (200mMTris pH 8.5; 250mM NaCl; 25mM EDTA; 0.5% SDS). The mycelium was then macerated by use of a conical grinder at 200 rpm for 2-3 minutes. To this solution 150ul of 3M Na acetate (pH 5.2) was added and mixed, prior to placing at –20°C for 10 minutes. Cell debris was pelleted by centrifugation at 13000 rpm for 5 minutes and the supernatant retained. DNA was precipitated by adding an equal volume of ice-cold isopropanol, with a further wash of the pelleted DNA in 70% ethanol. The DNA was quantified visually in a 2 % LE agarose gel (Flowgen) by comparison to serial dilutions of lambda DNA of known concentration.

4.3.2. ITS-RFLP ANALYSIS FOR CONFIRMATION OF SPECIES

Studies initiated by the Scottish Crop Research Institute have shown that *Phytophthora* species can be reliably diagnosed by RFLP analysis of the ITS region. A database on RFLP profiles is used for this purpose.

The ITS region was amplified using the primers ITS1 and ITS4. Detail of the primers, PCR reaction components and thermal cycling are presented in Table 1. PCR products were resolved by electrophoresis for 2 hour in a 2% LE agarose gel (Flowgen), stained with ethidium bromide and observed under UV light.

Restriction enzyme digestion were performed using an array of enzymes to identify those that restricted the ITS fragment. The products of digestion were separated by electrophoresis for 3 hours in a 2% LE agarose gel (Flowgen), stained with ethidium bromide and observed under UV light.

4.3.3. GENOMIC FINGERPRINTING ANALYSIS

Screening of PCR primers to elucidate genetic diversity was carried out using a selection of RAPD, repetitive sequence and AFLP primers. Detail of the primers, PCR reaction components and thermal cycling are presented in Table 1. AFLP analysis was adapted from Muller et al. (1996): fungal DNA was restricted with the endonuclease Pst1 and ligated to double stranded Pst1 adapters, prior to a PCR with AFLP preamplification and selection primers. PCR products were separated by electrophoresis for 6 hours in a 2% LE agarose gel (Flowgen), stained with ethidium bromide and observed under UV light.

4.4. PATHOGENICITY ASSESSMENTS ON *PHYTOPHTHORA* SPP. FROM COCONUT BASED CROPPING SYSTEMS OF INDONESIA

Research described under this section aimed to determine the pathogenicity of *Phytophthora* isolates obtained from coconut and crops associated with the coconut from coconut based cropping system of Indonesia. The selection of isolates to be tested was based on their source of origin (geographic and substrate) and genomic fingerprint type.

4.4.1. VALIDATION OF INOCULULATION METHOD

Experimental outline:

Phytophthora isolate	–	99P83
Coconut variety	–	NYD
Treatment	–	Coconut nuts inoculated with *Phytophthora* zoospore or, 7 or 21 day old mycelium + sporangia culture (grown on V8 medium at 30ºC); each coconut nut inoculated at 2 positions; controls were non inoculated treatments
Inoculation method	–	4 mm wide and 2-3 mm deep cores removed from the equatorial area of coconut nuts; zoospore (25ul) suspensions or agar based mycelial plug inserted in cavity and secured with clear sticky tape
Coconut nut maturity	–	Immature (<2 months), maturing (3 – 6 months) and mature (>9 months)
Experimental design	–	Randomised complete block design; with 3 coconut nuts per replicate and 4 replicates
Data recorded	–	Diameter of PNF lesion at 2, 4 and 6 days post inoculation: Scored as 0 = 0 mm; 1 = <1 cm, 2 = <3 cm, 3 = <8 cm 4 = >8 cm
Statistical analysis	–	Hypothesis tested: affect of inoculum types and coconut nut stage of maturity on pathogenicity by generalised linear regression (Genstat)

4.4.2. ASSESSMENT ON DETACHED COCONUT NUTS

Experimental outline:

Phytophthora isolate	–	A total of 60 isolates were assessed (see Appendix A-i)
Coconut variety	–	NYD
Treatment	–	Coconut nut inoculated with 10 day old *Phytophthora* mycelium + sporangia culture (grown on V8 medium at 30ºC); each coconut nut inoculated at 2 positions; control was a non inoculated treatment
Inoculation method	–	4 mm wide by 2-3 mm deep cores removed from the equatorial area of coconut nuts; agar based mycelial plug inserted into cavity and secured with clear sticky tape
Experimental design	–	Randomised complete block design; with 2 coconut nuts per replicate and 5 replicates
Data recorded	–	Diameter of PNF lesion at 2, 4 and 6 days post inoculation: Scored as 0 = 0 mm; 1 = <1 cm, 2 = <3 cm, 3 = <8 cm 4 = >8 cm
Statistical analysis	–	Hypothesis tested: pathogenicity of *Phytophthora* isolates associated with coconut cropping system of Indonesia on coconut nuts by generalised linear regression (Genstat)

4.4.3. ASSESSMENT ON DETACHED COCOA PODS

Experimental outline:

Phytophthora isolate	–	A total of 60 isolates were assessed (see Appendix A-i)
Cocoa variety	–	local variety
Treatment	–	Cocoa pod inoculated with 10 day old *Phytophthora* mycelium + sporangia culture (grown on V8 medium at 30ºC); each cocoa pod inoculated at 2 positions; control was a non inoculated treatment
Inoculation method	–	4 mm wide by 2-3 mm deep cores removed from the equatorial area of cocoa pods; agar based mycelial plug inserted into cavity and secured with clear sticky tape
Experimental design	–	Randomised complete block design; with 1 cocoa pod per replicate and 5 replicates
Data recorded	–	Diameter of PNF lesion at 2, 4 and 6 days post inoculation: Scored as 0 = 0 mm; 1 = <1 cm, 2 = <3 cm, 3 = <8 cm 4 = >8 cm
Statistical analysis	–	Hypothesis tested: pathogenicity of *Phytophthora* isolates associated with coconut cropping systems of Indonesia on cocoa pods by generalised linear regression (Genstat)

4.4.4. ASSESSMENT ON COCONUT SEEDLINGS

Experimental outline:

Phytophthora isolate	–	A total of 60 isolates were assessed (see Appendix A-i)
Coconut variety	–	PB121 seedlings (3 months old)
Location	–	Mapanget, Manado
Treatment	–	Coconut seedlings inoculated with 10 day old *Phytophthora* mycelium + sporangia culture (grown on V8 medium at 30ºC); control was a non inoculated treatment
Inoculation method	–	Lower leaf pulled back, inner palm stem 3-5 cm from soil level slashed with a sharp knife to make a V shaped incision, cut surface inoculated with 4 mm agar based mycelial plug, lower leaf pulled back
Experimental design	–	Randomised complete block design; with 5 coconut seedlings per replicate and 5 replicates
Data recorded	–	Presence or absence of disease symptoms, 0 and 1, respectively recorded at 2, 3 and 5 weeks post inoculation
Statistical analysis	–	Hypothesis tested: pathogenicity of *Phytophthora* isolates obtained from coconut cropping systems of Indonesia on coconut seedlings by generalised regression analysis modelling binomial proportions e.g. by logits (Genstat)

4.4.5. ASSESSMENT ON CROPS ASSOCIATED WITH COCONUT BASED CROPPING SYSTEMS

Experimental outline:

Phytophthora isolate	–	A total of 24 isolates were assessed (see Appendix A-i)
Host	–	Coconut (*Cocos nucifera*), arecanut (*Areca catechu*), papaya (*Carica papaya*), cocoa (*Theobroma cacao*); pepper (*Piper nigrum*) and vanilla (*Vanilla planifolia*). These plants were all planted in 10l pots and were about 3 months old
Treatment	–	Test plant inoculated with 10 day old *Phytophthora* mycelium + sporangia culture (grown on V8 medium at 30ºC); control was a non inoculated treatment
Inoculation method	–	Coconut and arecanut (as described for coconut seedling assessment); papaya – V shaped incision at base of stem inoculated with 4 mm agar based mycelial plug; pepper, cocoa and vanilla – agar based mycelial plug positioned on leaf surface, secured by sticky tape with pin-prick wound driven through mycelium/leaf interface; leaf inoculations comprised 3 inoculation points (leaves) per replicate; control treatment was a non inoculated agar plug
Experimental design	–	Complete randomised block design; with 2 replicates

Data recorded – Disease observations recorded as either present or absent, 0 and 1, respectively, at 2, 4 and 6 days post inoculation for leaf inoculation and 1, 2 and 3 weeks post inoculation for stem inoculation treatments

Statistical analysis – This assessment aimed to give qualitative data and hence no statistical analysis was performed

4.5. EPIDEMIOLOGICAL STUDIES ON PNF AND BR

Research described under this section aimed to investigate spatial aspects of the incidence of BR and PNF and potential involvement of vectors (wind/rain/insect) in the spread of the diseases.

4.5.1. PNF INFECTION WITH AND WITHOUT WOUNDING

Experimental outline:

Phytophthora isolate – 99P83

Coconut variety – NYD

Treatment – Coconut nuts inoculated with 10 day old *Phytophthora* mycelium and sporangia culture (grown on V8 medium at 30ºC); control was a non inoculated treatment

Inoculum method – 4 mm agar based mycelial plug positioned equatorially on coconut nut surface, secured by clear sticky tape; wound treatment by pin-prick through mycelial/leaf interface; each coconut nut inoculated at 2 positions; control treatment was a non inoculated agar plug

Experimental design – Complete randomised block design comprising wound and non-wound treatments, with 3 coconut nuts per replicate and 4 replicates

Data recorded – Diameter of PNF lesion at 2, 4 and 6 days post inoculation: Scored as 0 = 0 mm; 1 = <1 cm, 2 = <3 cm, 3 = <8 cm 4 = >8 cm

Statistical analysis – Hypothesis tested: incidence of PNF infection with and without wounding by generalised linear regression (Genstat)

4.5.2. DISTRIBUTION OF PNF ON COCONUTS IN THE PLANTATION

This research aimed to determine the distribution of early PNF infection sites in the plantation in relation to their orientation to the main palm stem. These data would give an indication as to whether PNF involved physical damage, an insect vector, wind or rain as a dispersal mechanism.

Experimental outline:

Location – Paniki and Mapanget

Coconut varieties – 25 cross hybrid trial (Paniki experimental plots); NYD and GO (Mapanget)

Experimental design – Observations taken on PNF location within plantation stands

Data recorded	–	Infection location were assigned a 2 character code (see Table 2)
Statistical analysis	–	Hypothesis tested: distribution of PNF infection sites in relation to aspect of palm by chi square analysis

4.5.3. CORRELATION BETWEEN INCIDENCE OF PNF ON BR AND NON-BUDROT INFECTED COCONUT PALMS

This research aimed to establish if BR infected palms had a higher incidence of PNF than non-BR infected palms. These data would provide insight to potential mechanisms of spread of BR and PNF infections.

Experimental outline:

Loaction	–	Various single variety plantations about Paniki and Mapanget
Coconut varieties	–	PB121, GO and NYD
Experimental design	–	Pairwise comparison on PNF taken on neighbouring palms with and without BR; non-BR infected palm identified randomly from 1 of 4 palms neighbouring the BR infected palm
Data recorded	–	Incidence of PNF recorded from the main coconut branches and expressed as number of PNF infected coconut nuts per total number of coconuts on each branch
Statistical analysis	–	Hypothesis tested: proportion of PNF infected to healthy coconut nuts/branch between BR and non-BR infected palm by split plot analysis where palm = block, branch number = main plot and number of infected coconut nuts/branch = split factor (Gensat)

4.5.4. MAPPING ANALYSIS ON THE DISTRIBUTION OF BR INFECTED PALMS IN 3 COCONUT PLANTATIONS

This research aimed to investigate the spatial patterns of BR infections within coconut plantations. Three plantations were selected, Mapanget, Tetey and Karegesan. Tetey and Karegesan plantations were pure stands of coconut variety PB121; Mapanget was a mixed plantation of PB121 and Khina. Keregesan was underplanted with cocoa; Mapanget and Tetey were managed grassland.

The analysis of spatial mapping was to be supported by molecular analysis of the *P. palmivora* isolate responsible for the BR. This required the felling of newly diseased palms and isolation of the *P. palmivora* from the meristem.

4.5.5. INVESTIGATION ON THE POTENTIAL OF INSECTS AS VECTORS OF PNF AND BR

This research aimed to determine the prevalence of insect species within the crown of coconut palms and whether these species were potential vectors of BR and PNF.

Experimental outline:

Location	–	Mapanget
Coconut varieties	–	PB121, NYD and GO

Experimental design	–	Insects of healthy, BR and PNF palms collected, identified to genus level; macerated and baited in 4 mm wide x 2-3 mm deep cavity of susceptible coconut nuts (NYD); PNF like symptoms confirmed by fungal isolation on V8 agar and morphological examination
Data recorded	–	Incidence of PNF symptoms associated with baited insects from healthy, BR and PNF diseased palms
Statistical analysis	–	Hypothesis tested: incidence of *P. palmivora* isolated from insects from healthy and BR and/or PNF infected palms and association with insect groups by generalised linear regression (Genstat)

4.5.6. MONITORING SOURCES OF INOCULUM IN THE PLATATION

This research aimed to identify the primary sources of infection of BR and PNF within coconut cropping systems of Indonesia.

Analysis of trapping system for airborne spores

This research aimed to identify the most suitable method for capturing air-born *P. palmivora* infectious propagules, and to implement this methodology in coconut plantations of Indonesia so as to identify the primary sources of PNF and BR inoculum and the conditions that favoured dispersal.

Mr. Lodrik of RICP undertook a 3 month attachment at IACR-Rothamsted under the supervision of Dr. Alastair McCartney for familiarise with spore trapping techniques. Comparisons were made between Burkard spore trap, miniature suction trap and Rota Rod trap systems using various inoculum sources and dispersal mechanisms. The methods used are reported on fully under the report *'A study of different spore trap systems and Phytophthora spore dispersal by splash and wind'*. Subsequent research aimed to validate the recommendations of this research under laboratory and field conditions in Indonesia.

Analysis of trapping system for water dispersed spores

This research explored the role of rain displacement and dispersal of *P. palmivara* infectious propagules from BR, PNF infected and healthy palms.

Experimental outline:

Location	–	Mapanget and Paniki
Coconut variety	–	NYD and GO (Mapanget) 25 hybrid cross (Paniki)
Experimental design	–	Beakers were positioned using string lines at 50 cm interval radiating vertically and horizontally from BR, PNF and healthy palms; in each instance the horizontal test radiated towards a healthy palm; water collected was baited in coconut husk cubes (CHC) (3 x 3 cm) containing a 4 mm wide x 2-3 mm deep cavity; 20 CHC tested per treatment combination; CHC were incubated at room temperature for 4 days when incidence of PNF-like symptoms was recorded; confirmation of PNF infection was performed by fungal isolation on V8

agar and morphological analysis. control treatments comprised an inoculation treatment of rain water collected from outside the coconut plantation; each treatment (BR, PNF and healthy palm) was replicated 5 times and repeated over the course of 1 year

Data recorded – Positive PNF infections per ml of rainwater collected

Statistical analysis – Hypothesis tested: number and distribution of *P. palmivora* infectious propagules associated with BR, PNF and healthy palms by generalised linear regression (Genstat)

4.6. COMPARATIVE STUDY ON GENETIC DIVERSITY OF *PHYTOPHTHORA* SPP. AFFECTING COCONUT/COCOA/ARECANUT CROPPING BASED SYSTEMS IN INDIA

During the course of the project, add-on funding was secured to undertake a parallel study on the species and genetic make-up of *Phytophthora* populations affecting coconut/cocoa/arecanut based cropping systems in India.

This research was conducted by Dr. P. Chowdappa from the Central Plantation Crops Research Institute (CPCRI) in India through a 3 month attachment to CABI Bioscience UK Centre [Egham] (February- May 1999). CPCRI already maintained an extensive collection of *Phytophthora* isolates associated with coconut/cocoa/arecanut plantations of India and this population was made available to study. The molecular methods used in this study are as described previously. The collection of *Phytophthora* cultures have been deposited with the reserve collection at CABI Bioscience UK Centre [Egham].

5. RESEARCH OUTPUTS

5.1 ECONOMIC CONSEQUENCES OF *PHYTOPHTHORA* DISEASES IN COCONUT AND MECHANISMS FOR RESEARCH DISSEMINATION

The summary from this reported is abstracted below. The full report is presented in Appendix B-i.

Summary of findings

Phytophthora bud rot is primarily associated with the PB 121 variety of coconut planted under a number of coconut rehabilitation programmes in Indonesia. The total number of PB121 palms at risk in Indonesia is around 80,000 ha (total coconut area in Indonesia is 3.5 million ha), of which about 10 to 12% have already been affected. The risk of the lethal disease spreading further in the future is probably reducing as no PB 121 has been planted in affected areas since 1989, and it is thought that palms are not at risk of bud rot infection once they achieve a height of 10 metres (around 15 years of age).

The immediate solution to bud rot is to replace palms that die with a variety that is not at present susceptible - cv. ‘Khina’ is used and has been resistant to date. However, this carries a penalty of lost production until the new palm comes into bearing. For those farms still susceptible to attack, research should endeavour to make prediction of the likely spread of the disease easier and to target dissemination mechanisms, so that the farmer can take appropriate action to replant or limit spread at an earlier stage. Future rehabilitation programmes will need to take full account of the risks posed to monocultures by diseases of this kind and the possible impact of mixed systems on disease spread and economic returns. The economic impact of nutfall has not yet been

fully gauged and survey data is required to determine the link between this syndrome and bud rot and the economic losses caused.

How will the results of research be transmitted to and taken up by farmers? The results of the chemical treatment of trees at risk using phosphonic acid suggest that farmers are reluctant to spend cash on disease prevention, especially when the results claimed are not guaranteed and without clearer knowledge of the risks of disease spread. To improve the dissemination of research information to farmers, close involvement of the project with the agencies concerned in coconut rehabilitation programmes, notably TCSDP and Dinas Perkebunan, should ensure the progressive transfer of information and be of considerable value in selection of field trial sites and in obtaining pertinent information from farmers. The Research Assessment Institutes (BPTP) also offer a useful site for managed field trials, particularly on the impact of intercrops.

5.2. COLLECTION OF *PHYTOPHTHORA* SPP. FROM COCONUT BASED CROPPING SYSTEMS OF INDONESIA

The existing collection of *Phytophthora* isolates held at RICP had not been maintained satisfactorily since the termination of the previous EEC/STD 3 funded project, and consequently isolate viability was at about 50%. This population was added to under the current project with isolations made from the North Sulawesi region and isolates held in the CABI Bioscience genetic resource. Details on the cultures studied in this project are presented in Appendix A-i.

During the course of the project it proved difficult to maintain all cultures in a viable state. This problem was exacerbated by recurrent mite infestations experienced at RICP that at its worst required the replacement of all cultures held at RICP from the reserved collection held at CABI Bioscience. At the close of project activities, duplicate viable cultures were held of 120 *Phytophthora* spp. of coconut cropping systems of Indonesia at RICP and CABI Bioscience UK Centre [Egham] as indicated in Appendix A-i. No contingency allows for these cultures to be maintained at CABI Bioscience beyond the project end date.

Summary: The morphological analysis of these cultures identified *P. palmivora* as the only *Phytophthora* sp. isolated from BR and PNF infections of coconut, and the predominant *Phytophthora* sp. isolated from black pod of cocoa. One *P. citrophthora* isolates and 1 *P. capsici* isolate were also recorded from cocoa and pepper, respectively. No morphological evidence was found to support the presence of *P. arecae* as was reported under the EEC/STD 3 funded project.

5.3. GENOMIC ANALYSIS OF *PHYTOPHTHORA* SPP. FROM COCONUT CROPPING BASED SYSTEMS OF INDONESIA

A focus of this aspect of the project was to familiarise RICP counterpart scientist Mr Lolong in methods of molecular biology appropriate to delineating *Phytophthora* populations, and to transfer this capability to RICP so as to enable molecular activities to be progressed in-house. A primary output of this transference was the production of a user guide to molecular biology. This is presented in Appendix B-ii. Accordingly, Mr Lolong spent year one of the project based at CABI Bioscience UK Centre [Egham] initiating the molecular research aspects of the project that were subsequently successfully transferred to RICP.

5.3.1. DNA EXTRACTION

DNA was successfully extracted from 130 *Phytophthora* isolates and quantified in accordance with the procedure outlined in Appendix B-ii.

5.3.2. ITS-RFLP analysis for confirmation of species

Amplification with the ITS1 and ITS4 primers produced a single fragment of approx. 900bp. Of the restriction enzymes analysed Msp1, Hinf1 and Alu1 gave rise to RFLPs (see Table 3; Plate 4a, b & c). Using these enzymes and comparison to the ITS-RFLP data base under development (DFID RNRKS CPP R7337) the morphological assignment of species was confirmed as stipulated in 5.3.1. No evidence was found to support the species status of *P. arecae*. However, within the *P. palmivora* population an aberrant ITS-RFLP feature was observed in 40% of isolates under digestion with Hinf1 and Msp1. The atypical isolates appeared to comprise the typical ITS-RFLP profile and 2 additional bands close to the 310bp fragment (see Plate 5a). Thus, the product of the observed band sizes exceeded that of the undigested ITS fragment. Single tip hyphal sub-culturing confirmed that the atypical ITS-RFLP was not due to a mixed culture and it was proposed that the atypical isolates of *P. palmivora* contained more than 1 ITS sequence. To substantiate this observation it was necessary to clone single copies of the ITS sequence prior to sequencing. From a study of 28 clones 2 distinct ITS-RFLPs were obtained at a near equal frequency (Plate 5b) and shown to differ by a 22/23 base deletion (Plate 5c). This accounted for 2 of the bands observed in the parent isolate only. The 3rd band was shown to be an artefact of chimaric (hybrid) ITS DNA fragments having an distinct mobility under electrophoresis (Plate 5d)

An overview on the distribution of the 2 *P. palmivora* ITS types observed indicated no strong geographic localisation suggesting that both populations had been present during the early phases of the spread of the disease in Indonesia. The assignment of ITS-RFLP type to each isolate is presented in Appendix A-i.

5.3.3. GENOMIC FINGERPRINTING ANALYSIS

Of the 1 RAPD, 7 repetitive sequence and 20 AFLP primers screened, primers #71 and AFLP primers E, H and Q gave rise to the most useful banding patterns, producing between 15-20 fragment within the size range 100 – 2000 bps. The remaining primers yielded profiles with either too many or too few bands. The profiles generated by #71 were shown to have only moderate reproducibility, requiring caution in the analysis of the data; whereas the AFLP primers were highly reproducible. Based on these data AFLP analysis with primers E, H, and Q was selected as the method of choice, combining robust reproducibility with high resolution between isolates.

AFLP data was generated on 120 isolates and analysed as described in Appendix B-ii. This analysis identified amongst the *P. palmivora* isolates a very high degree of homology. Ostensibly only 2 robust AFLP profile types were identified amongst the *P. palmivora* isolates with AFLP primer E by the prsence of a characteristic triple band (see Plate 6). It was observed that isolate membership to AFLP groupings 1 and 2 delineated between *P. palmivora* isolates of coconut and cocoa, respectively, in all but one case, 93P105, which was isolated from soil baited by coconut. The assignment of AFLP type to each isolate is presented in Appendix A-i. No evidence in support of *P. arecae* isolates was obtained. *P. megakaryia*, *P. capsici*, *P. katsurae* and *P. citrophthora* all gave distinct AFLP profiles.

SUMMARY

Effective molecular methods to support species identification and to determine genetic diversity amongst field populations of *Phytophthora* spp. affecting coconut based cropping systems in Indonesia were developed and transferred to RICP, Manado (see Plate 7). Extensive training was given to Mr Lolong and fellow RICP researchers to ensure the molecular research could be maintained in Manado. Mr Lolong successfully typed by ITS-RFLP and AFLP over 20 isolates at RICP. A manual was produced in support of this part of the project (Appendix B-ii).

The main findings of the genetic analysis were the confirmation of *P. palmivora* as the main causal organism for BR, PNF and BP, and that this population was highly homogenous despite both A1 and A2 mating types being present. This low level of diversity suggests that asexual reproduction is the main method of survival and that the sexual stage either occurs rarely or the progeny of sexual recombination do not contribute to the pathogenic gene pool. Nevertheless, sufficient genetic diversity was evident for marker assisted epidemiological studies.

Two AFLP groupings of isolates were identified that appeared to be associated with BR and PNF, and BP. The characteristic triple band of the BP type isolates (AFLP 2) is shown in Plate 6. This was potentially an important finding if the delineation was supported by pathogenicity assessments. Distinct *P. palmivora* types specialising on coconut and cocoa have been described previously under the EEC/STD 3 project by MLEE. Unfortunately, the lack of overlap between the *P. palmivora* populations studied under the respective projects prevented the corroboration of these data sets. In the few cases where comparisons could be made no correlation was apparent. No evidence was apparent from ITS-RFLP and AFLP analysis to support the assignment of *P. arecae*.

An anomalous ITS-RFLP profile was observed that was shown to be due to multiple ITS sequences (22/23bp deletion) held within the genome of a single isolate. No epidemiological significance could be attributed to the occurrence of the 2 ITS sequence types that was present across Indonesia, although it was not recorded amongst the AFLP 2 isolate types. The widespread distribution of the multiple ITS isolate type suggests that it was present in the early stages of the diseases' spread. Multiple ITS sequences have been described previously in a few fungi only (Fatehi et al., 1998).

This research represented the first extensive assessment on the genetic diversity of *P. palmivora* isolates in Indonesia.

5.4. PATHOGENICITY ASSESSMENTS ON *PHYTOPHTHORA* SPP. FROM COCONUT BASED CROPPING SYSTEMS OF INDONESIA

5.4.1. VALIDATION OF INOCULATION METHOD

These data are presented in Graph 1, with a summary of the data in Appendix A-ii and statistical analysis in Appendix C.

Overview of the data suggested Observation date 2 gave the most meaningful data. Statistical analysis at Observation date 2 indicated that PNF expression was affected by coconut nut age ($P<0.001$) and inoculum type ($P<0.001$) and that these factors interacted ($P<0.001$). The interaction between these factors was shown to be mainly associated with zoospore and old mycelium inoculum types and coconut age, with no

interaction apparent between new mycelium and coconut age. By observation date 3 no differences were apparent between zoospore and mycelium inoculum types.

It was recommended that this assessment should be extended to include additional isolates and to study the effect of inoculum concentration, however, this was not undertaken. Based on these preliminary data it was decided to undertake pathogenicity screening with new mycelium (mycelium + sporangia cultures grown for between 7-10 days on V8 agar medium) on maturing (3 - 6 months) coconut nuts.

5.4.2. ASSESSMENT ON DETACHED COCONUT NUTS

A total of 62 *Phytophthora* isolates were tested for pathogenicity (see Plate 8a & b). These data are presented in Graph 2, with a summary of the data in Appendix A-iii and statistical analysis in Appendix C (non *P. palmivora* isolates excluded).

An overview of this data suggested that a small number of the isolates had lost pathogenicity through storage and/or sub-culturing. It was, however, evident by grouping isolates according to source of origin that coconut BR, PNF and soil isolates were significantly ($P<0.001$) more aggressive than BP derived isolates on coconut nuts (Graph 2). Isolates of soil appeared as aggressive as BR and PNF. *P. capsici* and *P citrophthora* isolates were not pathogenic on coconut nuts.

5.4.3. ASSESSMENT ON DETACHED COCOA PODS

The same 62 *Phytophthora* isolates were tested for pathogenicity as used in 5.4.2 (see Plate 9a & b). These data are presented in Graph 3, with a summary of the data in Appendix A-iv and statistical analysis in Appendix C (non *P. palmivora* isolates excluded).

As was observed with the detached coconut nut assessment, a small number of isolates gave no BP symptoms and may have lost pathogenicity, and more meaningful information was obtained by grouping isolates according to source of origin. Analysis of these data identified BP isolates as significantly more ($P<0.001$) aggressive on cocoa pods than BR, PNF or soil isolates, the reciprocal of the assessment on coconut nuts (Graph 2). *P. capsici* and *P citrophthora* isolates were both highly pathogenic on cocoa pods.

5.4.4. ASSESSMENT ON COCONUT SEEDLINGS

The field trial was successfully established with the same 62 *Phytophthora* isolates as tested for pathogenicity on detached coconut nuts and cocoa pods, and maintained for 2 months after which time no new BR was observed (see Plate 10a & b). Mirroring the data obtained on detached coconut nuts differential levels of aggressiveness (host preference) were recorded between coconut (BR and PNF) and cocoa pod isolates ($P<0.001$). Similarly isolates of soil origin were aggressive to coconut seedlings. These data are presented in Graph 4, with a summary of the data in Appendix A-v and statistical analysis in Appendix C (non *P. palmivora* isolates excluded).

5.4.5. ASSESSMENT ON CROPS ASSOCIATED WITH COCONUT CROPPING SYSTEMS

This assessment aimed to determine the potential host range of *P. palmivora* amongst crops associated with coconut cropping systems of Indonesia. It was not intended to quantify pathogenicity/aggressiveness in a statistical sense. Accordingly, the data from this assessment is presented qualitatively in Appendix A-vi. From this assessment it

was apparent that *P. palmivora* isolates were potentially capable of causing disease on pepper, vanilla and papaya. However, it should be noted that disease expression on pepper and vanilla was more rapid with the *P. capsici* isolate suggesting that under natural conditions infection of these crops by *P. palmivora* may not happen. The disease symptoms observed on papaya were quite striking and shown to be reproducible (see Plate 11). Whether such infection occurs in the field was not substantiated and no genomic basis to explain the pathogenicity of isolate 99P106 over other BR and PNF isolates was evident.

SUMMARY

This research represented the first extensive assessment of pathogenicity of *P. palmivora* isolates of coconut and cocoa. An overview of the data does not support the hypothesis that BR and PNF isolates possess specialisation to their respective disease types: in both assessments on coconut nuts and coconut seedlings similar pathogenicty profiles were evident. Accordingly these data support that both BR and PNF can act as sources of inoculum for both disease types within a plantation.

Comparison of *P. palmivora* isolates from BP, PNF and BR on cocoa and coconut clearly identified host preference towards the respective source of origin. This was particularly strong with regards to BP isolates that showed very low aggressiveness on coconut (nuts and seedlings), whereas *P. palmivora* isolates of BR and PNF caused moderate levels of BP infection. This distinction between *P. palmivora* populations based on pathogenicity correlated with AFLP groupings (see Graph 5). Indeed isolate 99P105 that showed unexpected levels of pathogenicty on coconut was found to be a natural example of cross-pathogenicity between the AFLP groups thus enforcing the robustness of the AFLP type and pathogenicity association. Thus it is concluded that the potential detrimental interaction between mixed cropping of coconut and cocoa is less than expected, although an example of cross-pathogenicty occurring in the plantation was recorded. It could be speculated based on these data that the risk posed by *P. palmivora* of coconut (AFLP 1) to cocoa is more than that of *P. palmivora* of cocoa (AFLP 2) to coconut. Clearly rain spread would be an exacerbating factor here.

It is evident from these data that *P. palmivora* of soil is highly pathogenic to coconut and therefore a source of inoculum. The observed dominance of the AFLP 1 type of isolates obtained from soil probably reflects the method of isolation that relied on baiting coconut nuts, followed by isolation onto selective medium. Thus no inference on the proportions of AFLP 1 and 2 in soils can be made.

Analysis of ITS and pathogenicity was not conclusive with contradicting levels of association recorded with detached nut and seedling assessments on coconut. (see Appendix C). In both cases isolates with a single ITS copy were the more aggressive.

Assessment on the potential of *P. palmivora* to infect other crops provided some evidence that pepper, vanilla and papaya could be potential hosts. Isolate 99P106 was particularly aggressive to papaya.

5.5. EPIDEMIOLOGICAL STUDIES ON PNF AND BR

5.5.1. PNF INFECTION WITH AND WITHOUT WOUNDING

The data from this assessment is presented in Appendix A-vii with statistical analysis in Appendix C. Th percentage of coconuts that developed PNF disease symptoms with and without wounding was 100 and 62.5%, respectively. This difference was shown to

be significant (P<0.001), however, of those coconut nuts that developed disease the disease index was not significantly different, 3.2 and 3.6, respectively after 6 days.

From an epidemiological standpoint the significance of these data is that wounding is not a prerequisite to infection.

5.5.2. Distribution of PNF on coconuts in the plantation

The position of 315 PNF lesion were recorded from 120 palms from 2 plantations based on the 2 character spatial description code described in Table 2. The frequency of occurrence of these positional codes are presented in Graph 6, with a summary of the data in Appendix A-viii and statistical analysis in Appendix C.

The hypothesis tested in this analysis was that the positional codes of PNF infection would conform to a 1:1 distribution. Chi square analysis showed a significant deviation from this ratio (P<0.001). From Graph 6 it is evident that the region of a coconut nut most frequently infected corresponded to the equatorial region and particularly the regions that were outward facing from the palm trunk (see Plate 3). These findings suggest strongly that infectious *P. palmivora* propagules alight on the surface of coconut nuts passively on air currents and under conducive environmental conditions are able to infect.

5.5.3. Correlation between incidence of PNF on BR and non-budrot infected coconut palms

This research tested the hypothesis that the incidence of PNF is positively associated with BR infection. A total of 40 pairwise comparison were made on the incidence of PNF on BR and a non-BR neighbouring palms. These comparisons were made with pure stands of coconut, including PB121, NYD and GO. Of these varieties incidence of PNF was frequently observed on GO only. The findings of these data are presented in Graph 7 and 8, with a summary of the data in Appendix A-ix and statistical analysis in Appendix C.

Given the low incidence of PNF on varieties PB121 and NYD, it was only valid to analyse the 10 pariwise combinations of variety GO. This analyses showed that the incidence of PNF on BR and non-BR infected palm was not statistically different, despite the apparent higher incidence on the BR infected palms. This is a surprising result as the pathogenicity data showed these diseases to cross-infect and it would be logical to expect *P. palmivora* infectious propagules to be higher on BR infected than non-BR infected palm, and that this would manifest as a higher incidence of PNF. It may be that the non-significant analysis was due to the small sample size and the variability in the data set. However, if a positive correlation had been observed then it may also of been expected that the distribution of PNF on a BR and non-BR infected palm would differ reflecting the sources of inoculum. There was no evidence to support that this was the case (Graph 8 and Budrot.Branch-number interaction = P>0.05). Hence, it may be speculated that separate mechanism for the spread of infectious propagules of *P. palmivora* causing BR and PNF exist.

From these data it was also possible to analyse the number of coconut nuts on palms affected and not affected with BR or PNF. No significant difference was apparent on the number of coconut nuts on BR and non-BR infected palms, whereas for variety GO incidence of PNF resulted in a 10% reduction in the total number of coconut nut per palm.

5.5.4. MAPPING ANALYSIS ON THE DISTRIBUTION OF BR INFECTED PALMS IN 3 COCONUT PLANTATIONS

Distribution maps on BR infections at 3 sites are presented in Appendix A-x.

The implementation of this research theme was difficult, as the plantations were physically hard to map accurately, especially where BR had led to the removal of a number of palms. In addition, the objective to link this activity with genomic analysis of new BR infected palms was not undertaken as this required destructive sampling of the palm which would remove the natural source of infection and thus nullified the objective of observing natural BR progression within a plantation.

Statistical analysis on the mapping data has not been completed.

5.5.5. INVESTIGATION ON THE POTENTIAL OF INSECTS AS VECTORS OF PNF AND BR

This assessment was not undertaken as advised as only insects from BR infected palms were collected. From these assessments *P. palmivora* was isolated from *Orycetes* sp., *Rhyncophorus* sp., *Forficula* sp. and *Dio calandra* sp.. No positive isolations were obtained from ant sp., however, the method of testing did not allow for analysing the plantation debris that the ants were seen to transport very extensively within and between palms and that accumulated in the axils of the palm fronds. No quantitative assessment were possible with the data collected.

5.5.6. MONITORING SOURCES OF INOCULUM IN THE PLANTATION

Analysis of trapping system for airborne spores

A comparison of spore trapping using Burkard spore trap, miniature suction trap and Rota Rod trap was undertaken. In these comparisons consideration was given to the need to sample at multiple sites within the coconut plantation.

1) Burkard spore samplers were shown to be effective at spore and pollen capture under UK field conditions, but were considered too expensive and bulky to be used within a coconut plantation.

2) Direct comparison between miniature suction traps and Rota Rods were undertaken. In these assessment *Lycopodium* and puff ball spores provided the source of inoculum placed in the IARC-Rothamsted wind-tunnel ran at 1, 2.5 and $4 ms^{-1}$. These assessments established higher rates of spore recovery with the Rota Rods (Graph 9). The effect of Rota Rod velocity was also assessed by use of 10, 12 and 14V batteries. This assessment indicated that spore recovery was not markedly affected by the speed of the Rota Rods within these parameters (data not shown).

Additional tests to replicate these finding with Rota Rods using *P. palmivora* sporangia seeded into soils and splash dispersed into an air current were largely unsuccessful. Splash dispersal of sporangia was recorded by microscopic examination of water droplets at distances of 45 cm vertical and 90 cm horizontal from the source of inoculum.

From these assessments it was concluded that Rota Rods were potentially the more effective method of monitoring sporangial dispersal, however, the findings were not highly encouraging. Attempts to transfer these methodologies to Indonesia, both in laboratory and field (see Plate 12a & b), failed to recover *P. palmivora* sporangia.

Analysis of trapping systems for water dispersed spores

In view of the failed spore trapping approaches alternative methods were sought. Laboratory assessments on baiting coconut husk cubes with environmental samples were shown to have promise (see Plate 12c and d). Accordingly, assessments were initiated to determine the quantitative nature of the method by serial dilution of a *P. palmivora* sporangial/mycelial suspension. The initial assessment on this proved inconclusive and was to be repeated. However, with repeated experimentation in the laboratory a contaminating saprophytic fungi of the husks became prevalent and masked PNF disease symptoms.

These assessments culminated in the collection of rainwater about BR, PNF and healthy palms with a view to quantifying inoculum potentials of infected palms in the plantation (see Plate 12e). A single assessment was undertaken, but did not result in meaningful data. Modifications on these themes were proposed but were not implemented.

Summary

This research demonstrated that wounding was not a prerequisite to PNF infection as evidenced by artificial inoculation assessments and observation on the position of PNF infection sites on coconut nuts when still attached to the palm. These data suggest an air-borne infection mechanism for PNF. Whether this is also the mechanism of spread for BR seems doubtful, as no strong correlation could be found between the incidence of PNF on BR and non-BR infected palm and the distribution of PNF on BR and non-BR infected palms was highly similar: it was hypothesised that PNF would be more prevalent on BR than non-BR infected palms and that the distribution of PNF amongst the coconut nuts of the bunches would be different reflecting a proximate high and disparate low inoculum source, respectively. The probable role of insects as vectors was poorly addressed and although positive isolations of *P. palmivora* infectious propagules were obtained from *Orycetes* sp., *Rhyncophorus* sp., *Forficula* sp. and *Dio calandra* sp. these observations were not quantified or investigated further.

Initial approaches to monitor *P. palmivora* infectious propagules in the plantation proved unsuccessful. The Rota Rods appeared to lack the sampling capacity to detect *P. palmivora* infectious propagules. However, the baiting method using coconut husk cubes was innovative and showed promise despite a problem with a saprophytic contaminant that masked PNF symptoms. It was thought that the problem with the saprophytic contaminant could have been managed with improved hygiene and laboratory practices and if this were achieved the coconut husk cube method would have provided the data sought on inoculum potential sources within the plantation.

5.6. COMPARATIVE STUDY ON GENETIC DIVERSITY OF *PHYTOPHTHORA* SPP. AFFECTING COCONUT/COCOA/ARECANUT CROPPING BASED SYSTEMS IN INDIA

Overview of plantation systems of India

Plantation crops are perennial crops that occupy about 4 million hectares in India, amounting to 2.3% of the total land area under cultivation. They contribute about Rs 2,98,500 million (£4500 million) to the gross national product and about Rs 30,295 million (£445 million) through export earnings, approximately 27% of the total export revenue for India (George, 1997). Coconut, cocoa, arecanut, rubber, black pepper, cardamom and vanilla are the main plantation crops and production is mainly centred in the south of India (see Table 4). The prevailing climate of this region during the monsoon season is highly conducive to disease and numerous *Phytophthora* species have been associated with plant disorders. The main *Phytophthora* diseases are listed

in Table 4 along with associated *Phytophthora* sp. and estimates of yield loss. The mixed, multi-stratified nature of plantation cropping allows for complex interrelationships between *Phytophthora* sp. and with different hosts (cross-pathogenicity). Knowledge of the host range of *Phytophthora* species affecting plantation based systems and the genetic diversity within these populations is fundamental to the development of effective disease control strategies.

Research summary

A population of 77*Phytophthora* isolates were obtained from a range of crops associated with coconut plantations of India. These cultures are listed in Appendix A-xi. Morphological (Plates 13a, b, c & d) and ITS-RFLP (Plate 16) analysis with reference to type culture held in CABI Bioscience Genetic Resource identified the primary *Phytophthora*/host associations as presented in Table 3 and Appendix A-xi.

A group of isolates of coconut identified as *P. palmivora* on morphological analysis and yielding a characteristic 900bp ITS fragment did not conform to the type *P. palmivora* ITS-RFLP profile, nor of another known ITS-RFLP profile. Subsequent, more detailed morphological analysis revealed these isolates to belong to *Pythium vexans*. No multiple ITS sequences were evident..

AFLP analysis on isolates of *P. palmivora* of coconut and cocoa revealed minor variation and were highly homologous to *P. palmivora* isolates of Indonesia (Plate 17). Interestingly, amongst the Indian *P. palmivora* isolates the triple band feature that was characteristic of AFLP 2 *P.palmivora* isolates of cocoa from Indonesia was again evident in the cocoa isolates and absent in the coconut isolates (Plate 17): the robustness of this differentiation is uncertain as only 3 *P. palmivora* of coconut were studied.

AFLP analysis of *P. meadii* isolates of arecanut formed a homogenous group; whereas the 4 *P. nicotianae* isolates gave distinct AFLPs and amongst the 7 *P. capsici* isolates a further 4 AFLP types were observed (Plate 18).

Summary

This study on *Phytophthora* populations affecting coconut plantations in India aimed to complement the analogous study undertaken in Indonesian. From a cropping practice perspective the farmer systems in India appeared more complex, with substantial mix cropping of coconut, arecanut and cocoa as primary crops, with black pepper also a notable component. This diversity was mirrored in the *Phytophthora* spp. present that exhibited cross-host pathogenicity traits. For example, *P. meadii* was pathogenic to arecanut and rubber, *P. palmivora* was pathogenic cocoa, jack fruit and coconut and *P. capsici*. was pathogenic to cocoa, black pepper and capsicum. AFLP analysis revealed varying levels of genetic diversity within these *Pytophthpora*/host associations: for *P. palmivora* and *P. meadii* minor genetic diversity was evident , whereas for *P. capsici* and *P. nicotianae* greater genetic diversity was observed. Diversity within *P. capsici* has been documented before with CapA and CapB type strains proposed and associated with distinct hosts (Mchau and Coffey, 1995). The AFLP profiles and host associations recorded here support this view, although additional isolates are needed to draw robust conclusions. Collectively, these data on species specific ITS-RFLPs and homogeneous/diverse AFLP profiles amongst *Phytophthora* sp. give weight to the robustness of an ITS-RFLP based approach to species identification and an AFLP based approach to population studies. These data added value to the *Phytophthora* information web site <PhytID.org> [DFID CPP R7337].

Comparison of the Indonesian and Indian *P. palmivora* AFLP types showed these populations to be broadly homogeneous and that the AFLP1 and 2 groups identified in Indonesia were also present in India and again aligned to coconut and cocoa hosts, respectively.

The significance of the high proportion of *Pythium* vexans isolates obtained from coconut exhibiting BR is unknown.

6. CONTRIBUTION OF OUTPUTS TO PURPOSE

Contribution of outputs towards DFID's developmental goal

The research activities of this project:

Identified the need to address farmer-accepted mechanism(s) of diseased palm replacement with new purportedly resistant palm types;

Confirmed the robustness and value of ITS-RFLP analysis for the identification of *Phytophthora* spp..

Identified a low level of genetic diversity amongst *P. palmivora* isolates of coconut/cocoa in Indonesia. Sufficient genetic diversity was recorded for marker assisted epidemiological studies.

Recorded no systematic genetic or pathogenic distinction between *P. palmivora* isolates of BR and PNF that correlated to their source of isolation

Identified through molecular (AFLP) and pathogenicity studies host preference of *P. palmivora* isolates towards coconut (BR and PNF) and cocoa (BP).

Developed and validated a rapid artificial method for inoculating coconut seedlings with *P. palmivora*.

Failed to deliver in the time available effective protocols that would enable the monitoring of *P. palmivora* infectious propagules in a plantation. An approach to measure infectious propagules obtained from run-off plantation water using coconut nut husk cubes was developed by project end and showed promise in resolving this difficult aspect of the research..

Built national capacity in plant pathology (Indonesia) and molecular biology (Indonesia and India).

Showed coconut/cocoa/arecanut plantations of India to be affected by a more complex mix of *Phytophthora* spp. than in Indonesia, although coconut was affected by *P. palmivora* (and *Pythium vexans*) only. Genetic diversity and host associations amongst *P. palmivora* isolates of India were consistent with that observed amongst Indonesian populations.

Implementation of these finding will result in:

A focus on rehabilitation programmes for coconut smallholder affected by BR and PNF that gives due attention to maintaining financial income lines in the interim period between replanting diseased palms and palms reaching maturity (coconut production);

Expeditious and focused breeding programmes for BR and PNF resistance, embracing scientifically sound pathogenicity protocols and a robust knowledge of the genetic basis of the BR and PNF pathogenic population, with spin-off value to breeding programmes of cocoa against BP;

Reduced intercropping of cocoa with coconut, with a concomitant increase in alternative understorey crops or livestock within coconut plantations;

Enhanced national research in plant pathology, particularly the molecular biology aspects of plant pathology.

Identified promotion pathways to target institutes and beneficiaries

The outputs of this project have not resulted in the development of a device, material or process, but an enhanced knowledge on *P. palmivora* populations affecting coconut-based farming systems of Indonesia that will facilitate future research initiatives targeting the alleviation of these constraints to production. In this context, the target institute for uptake of project outputs remains the RICP, holder of the national mandate for coconut and palmae research. The inclusion of research findings within breeding programmes presents the strongest uptake route for the main project outputs. Supporting linkages to improve the dissemination of research information to farmers should be fostered with organisations involved in coconut rehabilitation programmes, notably Tree Crop Smallholder Development Project and Dinas Perkebunan.

What further follow-up action/research is necessary to promote the findings of the work to achieve their development benefit?

Current breeding programmes for new coconut varieties do not have a specific focus on screening for BR and PNF resistance. This represents the same positioning of research priorities that led to the initial and poorly advised introduction of PB121 in the early 1980s. Accordingly, the current recommended coconut varieties, such as Khina, have not been rigorously tested for BR and PNF resistance, and purported resistance qualities are poorly documented. Considering that one of the parental lines of Khina, NYD, is known to be highly susceptible to BR and PNF the continued overlooking of this key facet of breeding is surprising: Khina palms were badly affected by BR in the Mapanget plantation!

A primary finding of this research was the identification of subspecies (AFLP 1 and 2) populations of *P. palmivora* in Indonesia and India with host preference to coconut and cocoa. This suggests that natural cross-infection between coconut and cocoa may occur less frequently than expected. Nevertheless, in the absence of field validated data it would be premature to conclude that *P. palmivora* of coconut and cocoa were not potential sources of cross-infection under plantation conditions. Given the obvious downward movement of inoculum for palm to cocoa the potential for loss in cocoa yield is potentially high. In recommending that cocoa is not an understorey crop to coconut in areas affected by BR and PN alternative crop or livestock practices needs to be identified and promoted. Vanilla or pepper may be options, as may banana and livestock, although market garden type crops should also be considered when in proximity to urban markets.

Additional research is also required to revisit the shortfalls experienced in identifying/monitoring the sources of BR and PNF infection in a plantation. It was generally viewed that the coconut husk cube method potentially could have proven

effective given additional investment in time and improved assessment hygiene protocols in the laboratory. The value of persisting with this study would be in identifying specific cultural practices that led to the reduction of *P. palmivora* inoculum reservoirs in coconut based farming systems with associated reductions in BR and PNF.

7. TABLES, GRAPHS AND PLATES

Primer name and sequence 5'-3'		Programme in ℃		
		Phase 1	Phase 2	Phase 3
ITS primers				
ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	94 for 4mins	94 for 1min 55 for 1min 72 for 1.5mins 30 cycles	72 for 5mins
RAPD and repetitive sequence primers				
# 71 MR RY GF GACA	CGGCTTGGGT GAGGGTGGCGGTTCT (CAG)5 (TCC)5 (GACA)4	94 for 4mins	94 for 1min 45 for 1min 72 for 1min 30 cycles	72 for 5mins
ERIC 1R ERIC 2 Box	ATGTAAGCTTCCTGGGGATTCAC AAGTAAGTGACTTGGGGTGAGCG CTACGGCAAGGCGACGCTGACG	94 for 4mins	94 for 1min 52 for 1min 65 for 8mins 30 cycles	72 for 5mins
AFLP primers				
AFLP-A AFLP-B AFLP-C AFLP-D AFLP-E AFLP-F AFLP-H AFLP-I AFLP-J AFLP-K AFLP-L AFLP-M AFLP-N AFLP-O AFLP-P AFLP-Q AFLP-EC AFLP-EA AFLP-AA AFLP-FA	GACTGCGTACATGCAGGT GACTGCGTACATGCAGGA GACTGCGTACATGCAGGC GACTGCGTACATGCAGAC GACTGCGTACATGCAGAG GACTGCGTACATGCAGCG GACTGCGTACATGCAGAA GACTGCGTACATGCAGAT GACTGCGTACATGCAGTA GACTGCGTACATGCAGTT GACTGCGTACATGCAGTG GACTGCGTACATGCAGTC GACTGCGTACATGCAGGG GACTGCGTACATGCAGCA GACTGCGTACATGCAGCT GACTGCGTACATGCAGCC GACTGCGTACATGCAGAGC GACTGCGTACATGCAGAGA GACTGCGTACATGCAGGTA GACTGCGTACATGCAGCGA	94 for 4mins	94 for 1min 60 for 1min 72 for 1.5mins 30 cycles	72 for 5mins

Table 1: List of primers and PCR thermal cycling parameters. In all cases reaction components were 30pmol of each primer, 2.5 units of Super Tth DNA polymerase (HT Biotechnology Ltd.) 0.2mM dNTP, 1X PCR buffer and 10-100ng of DNA template with a total reaction volume of 25ul.

Category 1	Category 2
A = Outward facing from the palm	D = Equitorial position
B = Inward facing the palm	E = Downward facing the soil
C = Between coconut nuts	F = Upward facing the sky

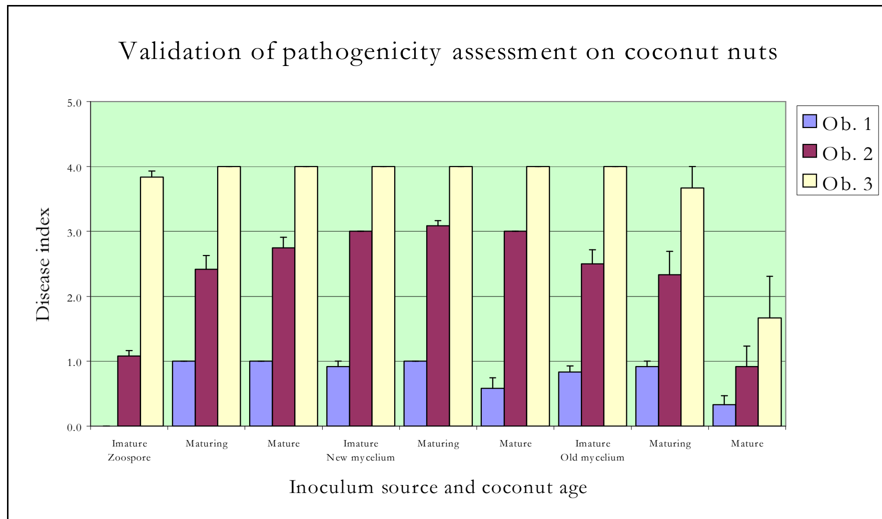
Table 2: Two character code assignment for PNF infection site on coconut nuts within coconut bunches

Phytophthora sp.	Country/source	Host	Hinf 1	Msp 1	Alu1
P .palmivora	Indonesia	Coconut, cocoa	320 (310 & 300*), 260, 180, 160	520, 380 (370)	500, 160, 130
	India	Coconut, cocoa, jack fruit	320, 250, 180, 160	520, 380	500, 160, 130
P. capsicii	Indonesia	Black pepper	300, 180, 150	350, 300, 200	540, 200, 150
	India	Cocoa, Black pepper	300, 180, 150	350, 300, 200	540, 200, 150
		Capsicum,	290, 200, 150	300, 220	535, 170, 150
P. meadii	India	Arecanut, rubber	300, 200, 150	350, 300, 220	500, 180, 170
P. nicotianae	India	Vinca, geranium, carnation, crassandra	400, 270, 200	400, 100	750, 120
P. citrophora	Indonesia	Cocoa	300, 180, 150, 100	380, 290, 220	550, 180, 160
P. megakarya	CABI Bioscience GRC		320, 260, 180, 140	500, 380	370, 220, 180, 110
P. katsurae	CABI Bioscience GRC		300, 180, 150, 100	370, 290, 220	570, 180

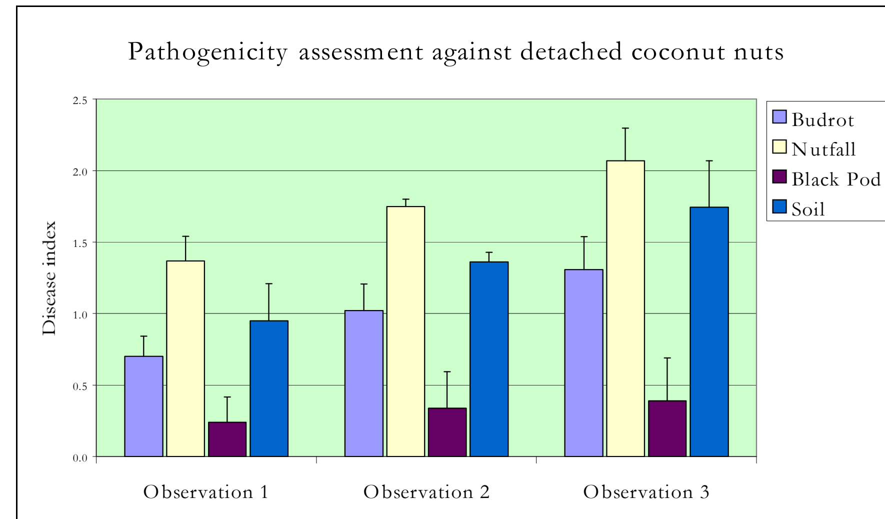
Table 3: Observed ITS-RFLP fragments of *Phytophthora* spp. of coconut based cropping systems of Indonesia and India, and from the CABI Bioscience Genetic Resource Collection
* figures in parenthesis indicate fragment size of multiple ITS sequence.

Crop	*Phytophthora* sp.	Disease	% loss
Coconut (*Cocus nucifera*)	*P. palmivora*	Budrot Premature nutfall	0.1 – 6.5 3.5 – 4.0*
Cocoa (*Theobroma cacao*)	*P. palmivora*	Black pod, Stem canker Seedling dieback	12 – 30
	P. capsici	Black pod Twig blight	
	P. citophthora	Black pod Twig blight Root rot	
Arecanut (*Areca catechu*)	*P. palmivora* *P. meadii*	Fruit rot	10 – 90
		Budrot	10 – 15
Black pepper (*Piper nigrum*)	*P. capsic* *P. parasitica var piperina*	Foot rot	25 – 40
Rubber (*Hevea brassiliensis*)	*P. palmivora* *P. meadii* *P. nicotianae var. parasitica* *P. botryosa*	Abnormal leaf fall, bark rot, patch canker	35 – 56
Cardamom (*Eletteria cardamomum*)	*P. meadii,* *P. palmivora* *P. nicotianae var nicottianae*	Capsule rot	30 – 40

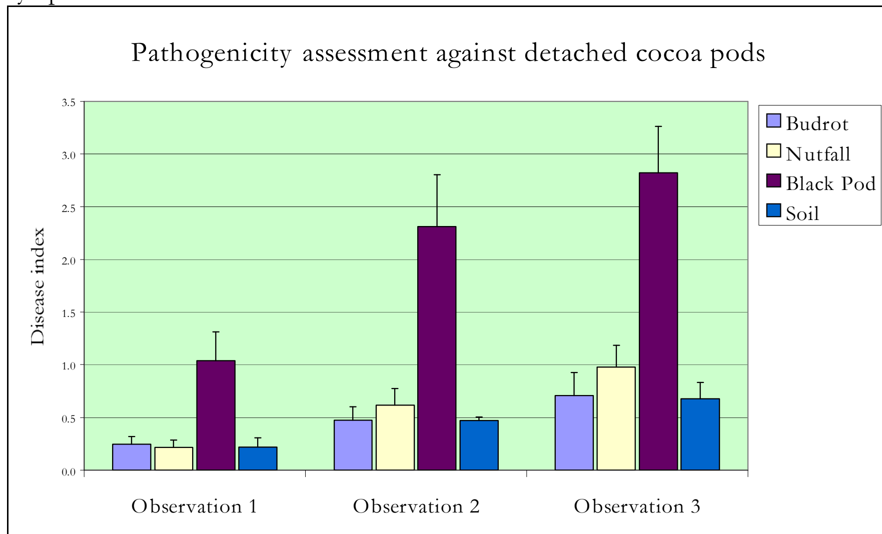
Table 4: *Phytophthora* spp. identified in plantation based cropping systems of India and hosts affected.



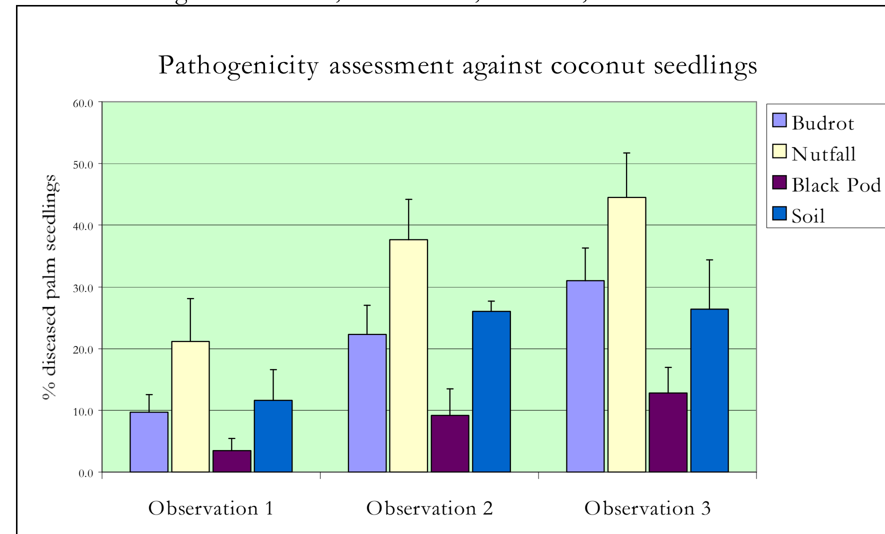
Graph 1: Effect of inoculum type and age of coconut on expression of PNF symptoms



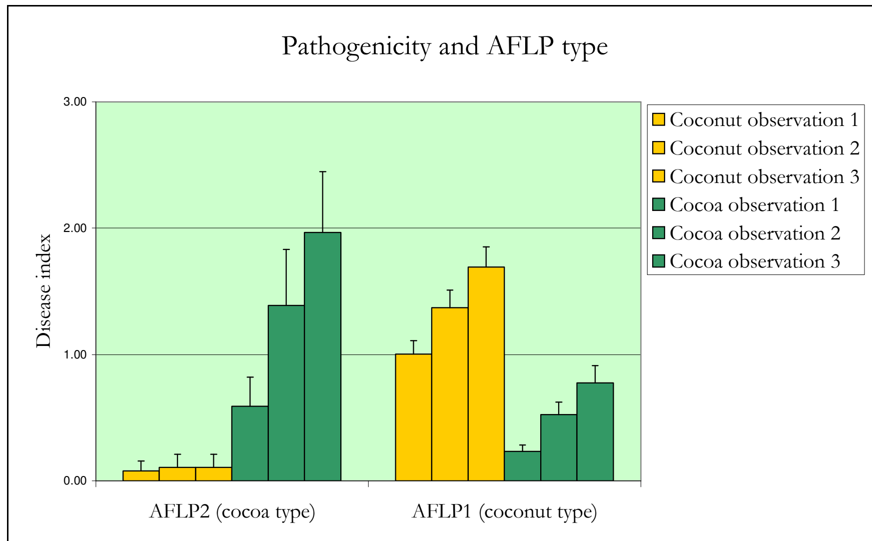
Graph 2: Pathogenicity of *P. palmivora* isolates on coconut nuts grouped according to source of origin: BR n = 20, PNF n = 16, BP n = 9, Soil n = 9



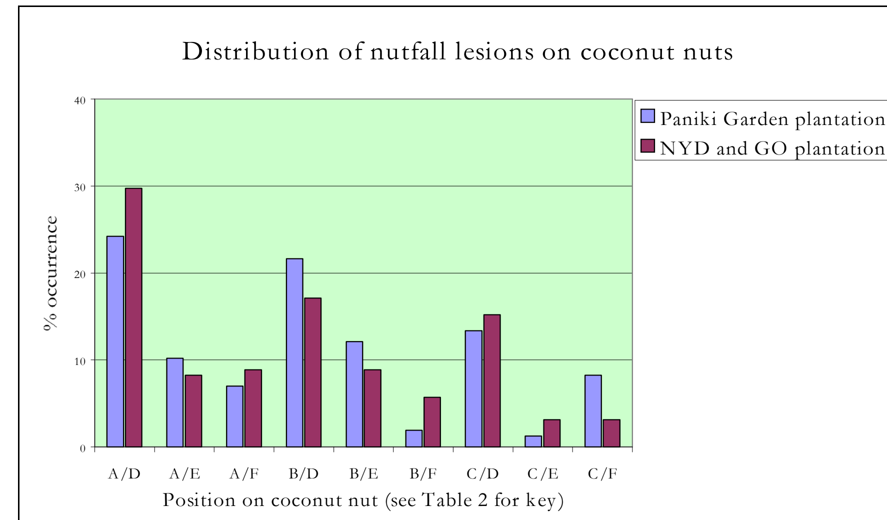
Graph 3: Pathogenicity of *P. palmivora* isolates on cocoa pods grouped according to source of origin: BR n = 20, PNF n = 16, BP n = 9, Soil n = 9



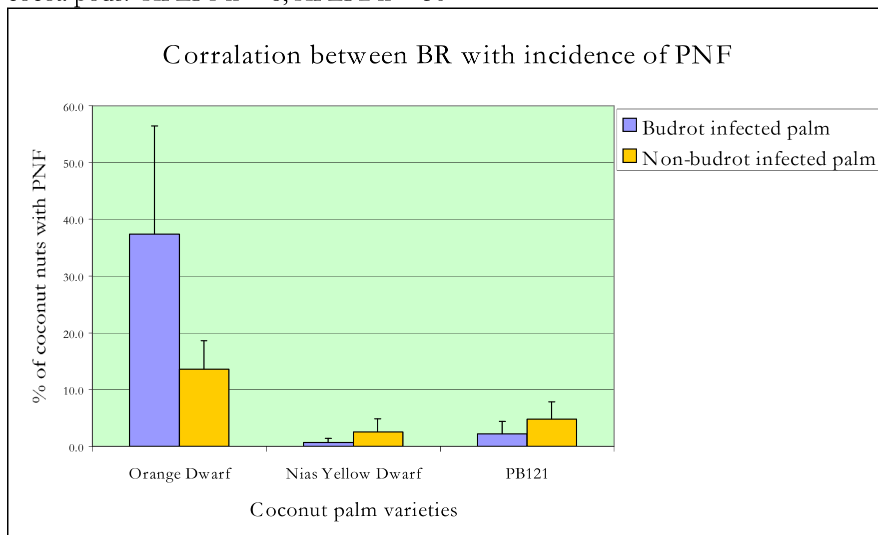
Graph 4: Pathogenicity of *P. palmivora* isolates on coconut seedlings grouped according to source of origin: BR n = 20, PNF n = 16, BP n = 9, Soil n = 9



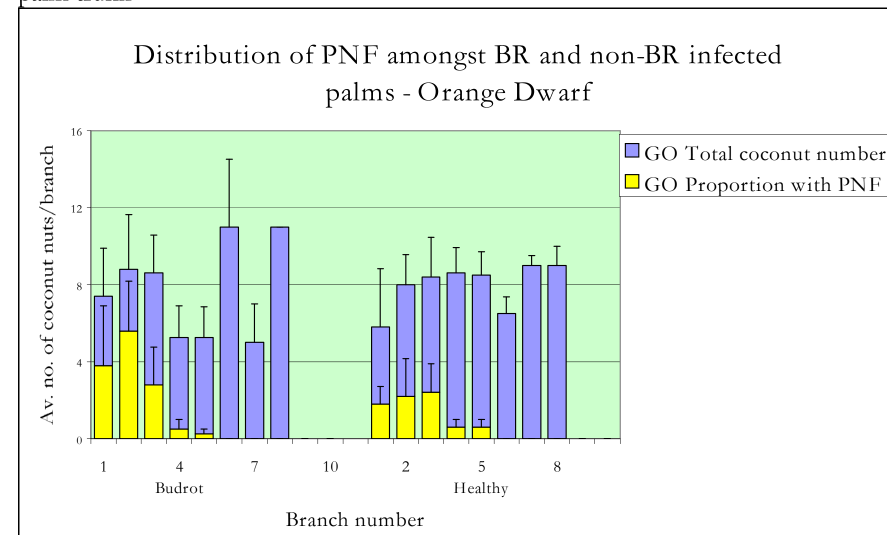
Graph 5: Correlation between AFLP type and pathogenicity on coconut nuts and cocoa pods: AFLP1 n = 8, AFLP2 n = 50



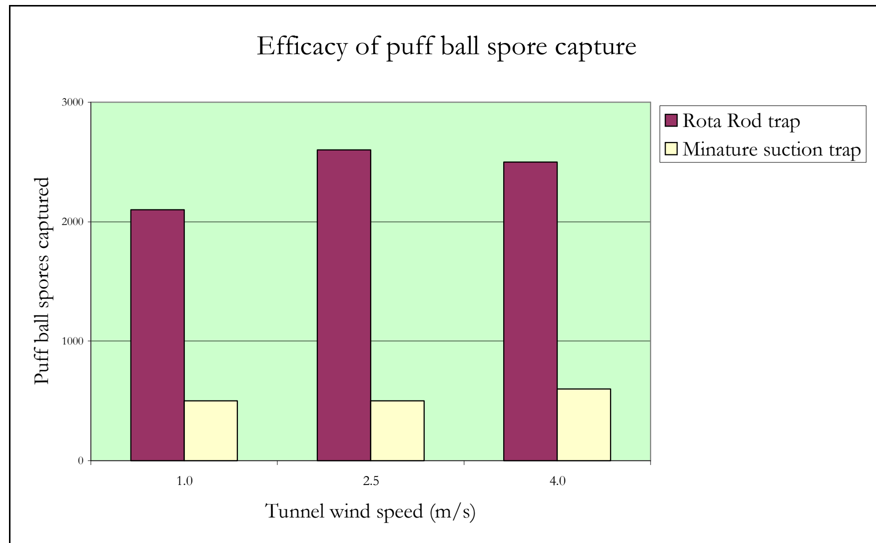
Graph 6: Positional frequency of PNF lesions on coconut nuts as orientated to the palm trunk



Graph 7: Epidemiological association between the incidence of PNF on BR and non-BR infected coconut palms



Graph 8: Distribution of PNF on BR and non-BR infected palms



Graph 9: Comparison of puff ball spore capture by Rota rod and miniature suction spore traps at varying wind speeds

Plate 1: Mixed coconut based cropping systems in Indonesia

Plate 2a: Coconut plantation with budrot diseased palm (centre)

Plate 2b: Early symptoms of budrot

Plate 2c: Budrot symptoms in meristem of palm

Plate 3: Symptoms of premature nutfall on coconut

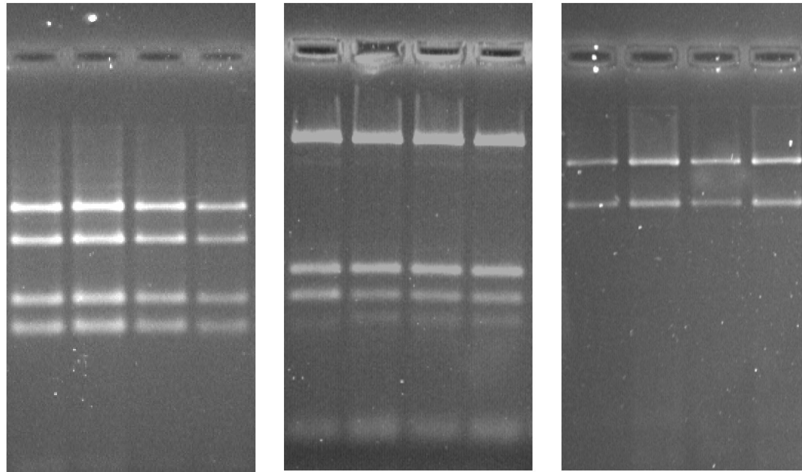


Plate 4: ITS-RFLP digest of *P. palmivora* by Hinf1 (a) Msp1 (b) and Alu1 (c)

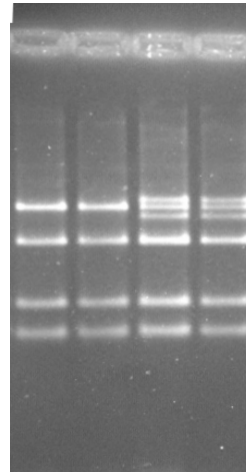


Plate 5a: Hinf1 digest of atypical ITS-RFLP

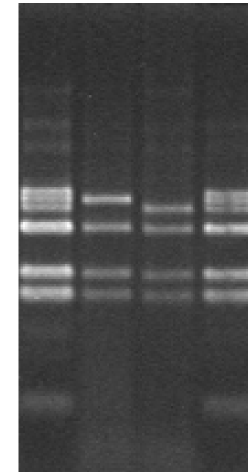


Plate 5b: Hinf1 digest of the 2 ITS clone types flanked by parental profiles

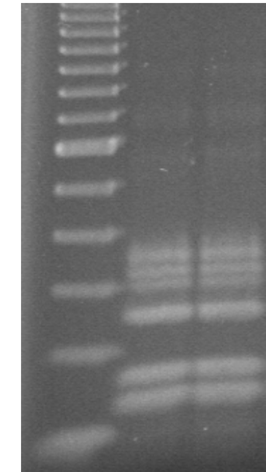


Plate 5d: Atypical Hinf1 ITS-RFLP reformed by mixing clone types

Typical ITS sequence	ATCAAAACTTAGTTGGGGGTCTCTTTCGGC_GGCGGCTGCTGGCTTCATTGCTGGCGGCTGCTGTTGGGAGAGCT
Clone type A	ATCAAAACTTAGTTGGGGGTCTCTTTCGGC GGCGGCTGCTGTTGGGAGAGCT
Clone type B	ATCAAAACTTAGTTGGGGGTCTCTTTCGGCAGGCGGCTGCTGGCTTCATTGCTGGCGGCTGCTGTTGGGAGAGCT

Plate 5c: Sequence alignment of typical and atypical *P. palmivora* cloned ITS region with 22/23bp deletion

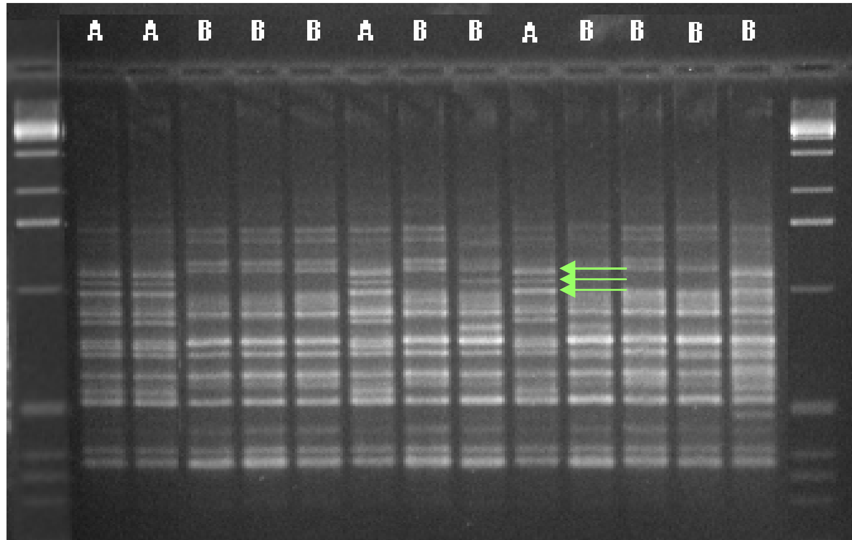


Plate 6: AFLP profiles with primer E of *P. palmivora* of Indonesia. AFLP profile A = cocoa type; B = coconut type

Plate 7: Molecular biology laboratory established in Manado

Plate 8a: Pathogenicity assessment on detached coconut nuts

Plate 8b: Typical premature nutfall symptoms caused by artificial inoculation with *P. palmivora*

Plate 9a: Pathogenicity assessment on detached cocoa pods

Plate 9b: Typical black pod symptoms (artificial inoculation)

Plate 10a: Pathogenicity assessment on coconut seedlings

Plate 10b: Typical budrot symptoms on coconut seedlings(artificial inoculation)

Plate 11: Disease symptoms on papaya (artificial inoculation)

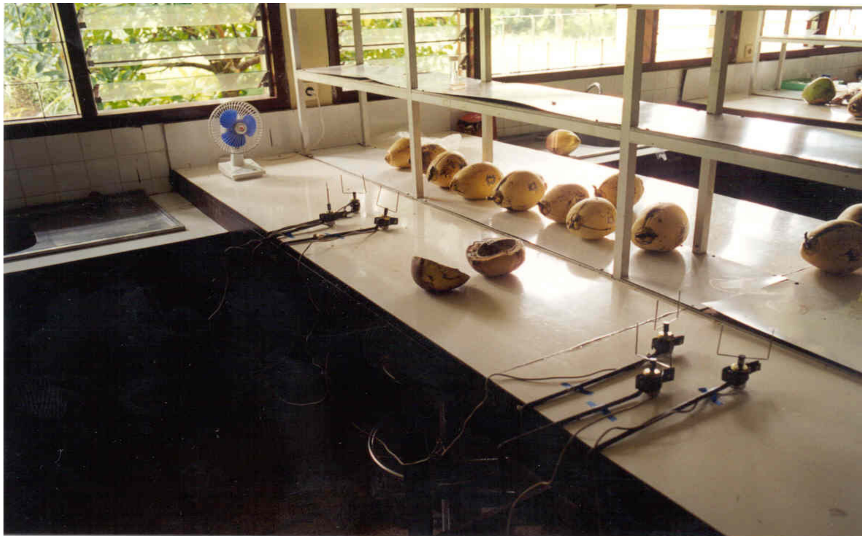


Plate 12a: Testing Roda Rods in the laboratory, Manado

Plate 12b: Testing Roda Rods in the field, Manado

Plate 12c: Baiting coconut nut husk cubes for *P. palmivora*

Plate 12d: Quantification of *P. palmivora* propagules

Plate 12e: Sampling rain water around BR, PNF and healthy palms for assessment of infectious *P. palmivora* propagules

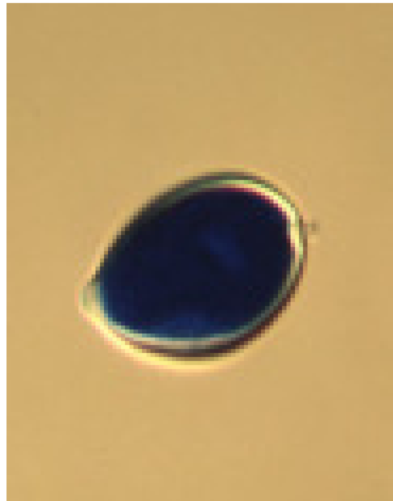
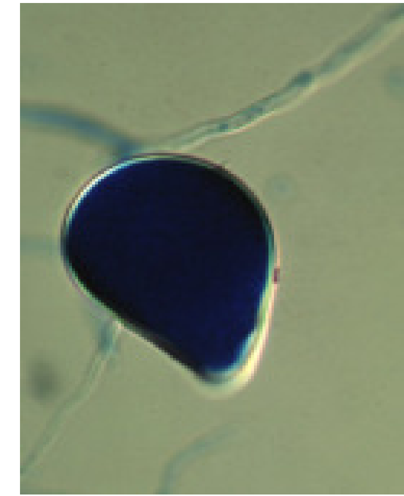


Plate 13a: Sporangia of *P. palmivora*

Plate 13b: Sporangia of *P. meadii*

Plate 13c: Sporangia of *P. capsici*



13d: Sporangia of *P. nicotianae*

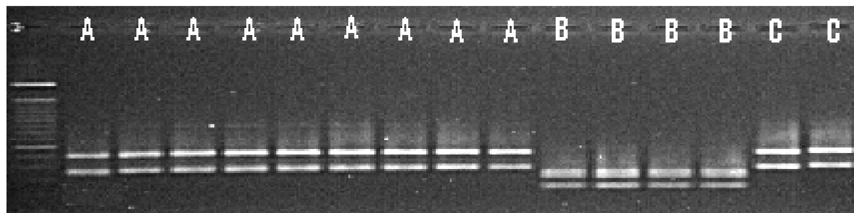


Plate 16: : Msp1 digests of *P. palmivora* of cocoa (a) and coconut (c), and *P capsici of cocoa* (b) of India

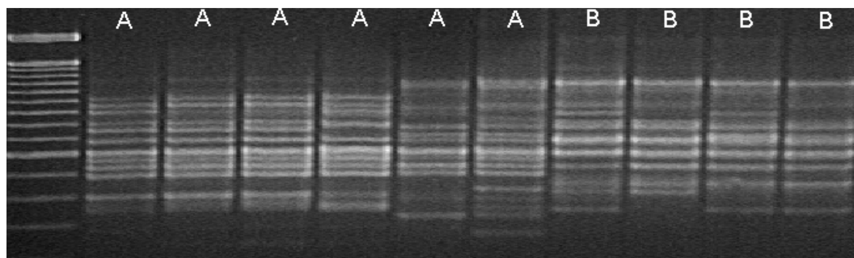


Plate 18: AFLP profiles with primer E of *P. capsici* (a) *and P. nicotianea* (b) of India

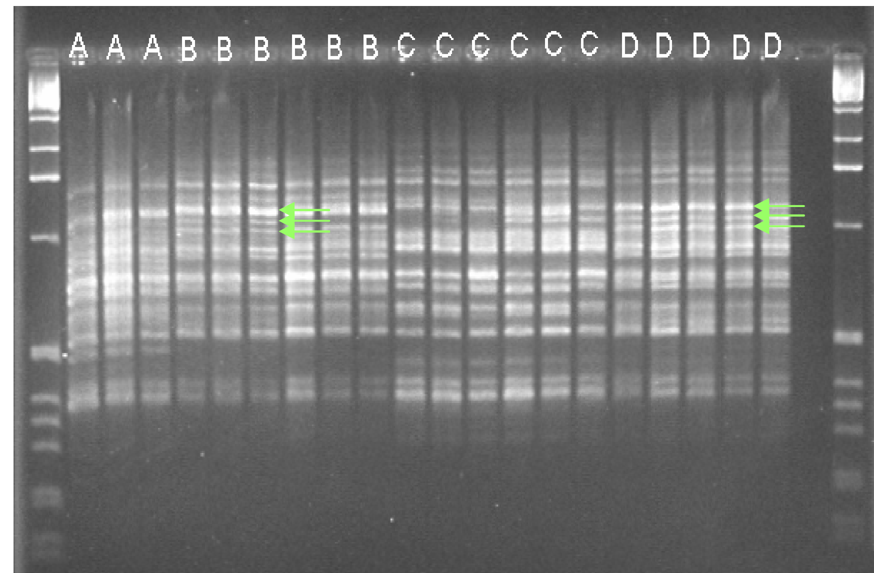


Plate 17: Comparison of AFLP profiles of *P. palmivora* of coconut and cocoa of India and Indonesia, a, b, c & d, respectively

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10. APPENDIX

DFID Contract R6766 Epidemiology and population structure of *Phytophthora* species causing diseases of coconut in Indonesia

ECONOMIC CONSEQUENCES OF *PHYTOPHTHORA* DISEASES IN COCONUT AND MECHANISMS FOR RESEARCH DISSEMINATION

Report on a visit to Manado, Indonesia

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Plant Pathologist

20 July 1997

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SUMMARY

Purpose

Budrot and premature nutfall are the major plant disease problems affecting coconut in Indonesia. The project aims to determine the genetic nature and interrelationships of populations of *Phytophthora* species causing these diseases and to develop and use characterisation tools to determine mechanisms of disease spread within pure and mixed coconut plantations. Training of Indonesian plant pathologists is integral to the programme.

The aim of this socio-economics component, funded under an 'add-on' contract, was to gauge the impact of the disease at the micro and macro level, then to suggest how the research might generate technologies of control and how well these may be taken up by farmers, and to determine linkages to be established with other institutions to develop and deliver effective extension messages resulting from the BALITKA research.

Summary of findings

Phytophthora bud rot is primarily associated with the PB 121 variety of coconut planted under a number of coconut rehabilitation programmes in Indonesia. The total number of PB121 palms at risk in Indonesia is around 80,000 ha (total coconut area in Indonesia is 3.5 million ha), of which about 10 to 12% have already been affected. The risk of the lethal disease spreading further in the future is probably reducing as no PB 121 has been planted in affected areas since 1989, and it is thought that palms are not at risk of bud rot infection once they achieve a height of 10 metres (around 15 years of age).

The immediate solution to bud rot is to replace palms that die with a variety that is not at present susceptible - cv. 'Khina' is used and has been resistant to date. However, this carries a penalty of lost production until the new palm comes into bearing. For those farms still susceptible to attack, research should endeavour to make prediction of the likely spread of the disease easier and to target dissemination mechanisms, so that the farmer can take appropriate action to replant or limit spread at an earlier stage. Future rehabilitation programmes will need to take full account of the risks posed to monocultures by diseases of this kind and the possible impact of mixed systems on disease spread and economic returns. The economic impact of nutfall has not yet been fully gauged and survey data is required to determine the link between this syndrome and bud rot and the economic losses caused.

[1] Report previously submitted to CPP Programme Manager in draft format, subject to approval by BALITKA - this has now been received.

How will the results of research be transmitted to and taken up by farmers? The results of the chemical treatment of trees at risk using phosphonic acid suggest that farmers are reluctant to spend cash on disease prevention, especially when the results claimed are not guaranteed and without clearer knowledge of the risks of disease spread. To improve the dissemination of research information to farmers, close involvement of the project with the agencies concerned in coconut rehabilitation programmes, notably TCSDP and Dinas Perkebunan, should ensure the progressive transfer of information and be of considerable value in selection of field trial sites and in obtaining pertinent information from farmers. The Research Assessment Institutes (BPTP) also offer a useful site for managed field trials, particularly on the impact of intercrops.

1. EXTENT OF DISEASE

1.1 National

Although previously present on local coconuts in Indonesia at levels which did not generally have any major economic impact, *Phytophthora* bud rot was first identified as a significant problem in extensive new plantings of the hybrid coconut PB 121 in North Sulawesi in 1984. PB 121 was introduced as part of a number of coconut rehabilitation schemes including the World Bank-assisted Smallholder Coconut Development Programme (SCDP) and Nucleus Estate & Smallholder (NES) project and the government funded PRPTE. These programmes aimed to rehabilitate and improve the productivity of smallholder production, replanting old stands of (local tall) coconut with a hybrid variety that had the potential to increase yields by 50% to 75%. Starting in 1978 and finishing in 1989, the SCDP programme supported replanting of about 170,000 ha, out of a total coconut hectarage of 3.7 million, or 5% of the total coconut area.

However, *Phytophthora* bud rot has since proved extremely damaging to these hybrids and of this area around 80,000 ha are considered vulnerable to the disease (35,000 ha are planted to other varieties, and the rest are planted on acid peat soils which are considered to be less conducive to bud rot (notably on the eastern side of Sumatra)). On the basis that the credit scheme was primarily targeted at holdings of 1-2 ha, this represents 40-80,000 farmers. The areas that have suffered most from *Phytophthora* bud rot have been North and Central Sulawesi (Table 1).

Table 1. Bud rot disease in SCDP plantings, per province

Province	Area infected (ha)
N. Sulawesi	3,157
C. Sulawesi	1,814
S. Sulawesi	890
Maluku	664
Lampung	349
D.I Aceh	390
Total	7,264

source: Survey in 6 provinces, Dinas Perkebunan, 1992

From a survey by Dinas Perkebunan in 1992, the equivalent in palms of over 7,000 ha of PB121 had been affected by bud rot, adding in other program planting, a total number of palms equivalent to more than 11,500 ha have been affected.

The SCDP programme was replaced by the Tree Crop Smallholder Development Project (TCSDP) in 1993. This programme will provide seedlings (at 380 Rp) and free advice, but no longer provide credit. This programme is more broad-based, but in N Sulawesi will

primarily be concerned with coconut. The SCDP has now changed from PB121 to the more resistant Khina[2] hybrid variety.

Premature nutfall involves loss of immature bunches and thus reduction in yield. As yet, there are few reliable figures as to actual losses to this disease and most farmers cannot distinguish such losses from physiological shedding. Nutfall is a problem in both Talls and hybrids.

1.2 North Sulawesi

In North Sulawesi out of a total of about 270,000 ha of coconut, 18,000 ha have been planted by the SCDP between the years 1979 to 89, of which PB 121 planting is about 14,000 ha. Of these, approximately 12% (233,000 palms) have been killed by *Phytophthora* bud rot. Out of 24 geographical units (UPP) in the SCDP project area, 10 have suffered 10% or more death, and the most serious attack has been in Kauditan (48%), Dimembe (35% affected) and Bolaang (40% affected).[3] Within the areas that PB 121 has been planted, it is known that the disease is prevalent in higher rainfall areas and in areas of high humidity, notably on lower slopes and valleys.

Very many farmers have thus been adversely affected by the disease and look unlikely to be able to repay their creditors for the replanting programme. In the smallholder systems concerned, previous credit schemes have been secured by land title, so there are a significant number who could be at risk of losing their land if creditors should foreclose.

2. FARMING SYSTEMS

2.1 Production systems

Typically, coconut farmers are smallholders using little or no purchased inputs. Coconut is usually their main or only source of cash income. Harvesting is carried out every 3 months, allowing a regular cash income. The smaller farmers may do much of the harvesting themselves, but most farmers also employ labour to harvest and process copra on a 33% crop sharing arrangement (bagi tiga). The average size of farms in North Sulawesi is about 4.5 ha. All coconut holdings in North Sulawesi, with the exception of one government owned estate, are smallholdings.

Most of the coconuts are grown as pure stand, especially in the Minahasa area around Manado. As one travels further eastwards, there appears to be more intercropping with annual crops such as corn and soybean, and perennials such as banana, cocoa and pineapple.

Coconut is particularly favoured as a crop as the market infrastructure exists for easy sale, and the timing of harvesting or the making of copra are not critical, allowing a degree of flexibility that other crops, such as rice, do not offer. The farmers perceive coconut as being a secure crop, offering a reliable income. Many of the farmers thought that it was also the most profitable crop. Farmers were not interested in replacing their coconut palms with an alternative crop.

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[2] Khina - is a composite of a number of hybrids of Palu or Tenga talls x NYD, developed at BALITKA and evaluated from the late '70s.

[3] See Appendix 1 for breakdown of geographical spread of *Phytophthora* in North Sulawesi

Farmers growing coconut are able to secure credit from the copra buyers sometimes as much as one or two months in advance of harvest, but usually not more than two weeks. The credit system is a means by which the copra buyers secure regular customers.

Coconut is a “cash lean” crop. This means the farmer actually needs very little cash to operate the coconut farm. The harvesting and processing into copra can be paid for on a share basis of the proceeds, and copra processors are often willing to extend credit to facilitate the farmer in harvesting. The only cash expenditure necessary is for circle weeding. Little or no fertiliser is applied. Labour costs in N Sulawesi are relatively high, at around 7,000 Rp/day compared with 2,500 Rp in Java.

2.2 Returns to coconut

The net annual margin (excluding capital costs) will be about Rp 500,000 for local tall and Rp 700,000 for hybrid (see appendix 2 for cost breakdown). About 1/3 of production cost is paid to labour for harvesting in the form of a share of the crop, a further 10% goes to pay for the making of copra. Weeding usually has to be paid for with cash. In those farms that depend on employed labour, a reduction in cash income from sale of nuts due to diseased palms can lead to a reduction in the money spent on weeding. The result is that the plots become overgrown, and this may in turn further aggravate the conditions under which *Phytophthora* flourishes and hamper harvest operations.

Prices for coconuts and copra decrease the further from Manado the farm is located. Copra in Manado area fetches Rp 850 to Rp 900 per kg. In Bolaang area (100 km from Manado) the price is Rp 660 per kg.

Farmers sell to village buyers and the village buyers sell-on to collectors who sell-on to factories. The prices offered to the farmers tend to be the same within a locality and it is not possible to shop around. It appeared that most farmers would stay with a single buyer, as they were able to get credit from this buyer and a relationship had been established. This credit is usually without interest, but does involve a rather lower price.

2.3 Intercropping

Intercropping is now being actively encouraged by Dinas Perkebunan, as a means of diversifying away from coconuts and in order to increase returns per hectare. This has replaced the previous recommendation for the use of cover crops under the coconuts. The TCSDP has recently started promoting the use of intercrops rather than cover crops, while access to this credit previously required use of cover crops only. Annuals such as corn, beans, soybean are popular intercrops, and in suitable areas, pineapple. For “industrial crop” perennials, clove was the traditional crop of the region, while coffee and cacao have both been developed to a minor extent, largely by individual farmers (Table 2)

Table 2: Areas of other perennial crops recorded in North Sulawesi

CROP	Area (ha)	Yield per ha
Clove	43,500	0.2 tonne
Coffee	2,000	1 tonne
Cacao	4,000	0.1 tonne (young plt)
Oil palm	0	-

Clove was a popular crop in the seventies, but the price now discourages farmers from even picking and vanilla has replaced clove in some cases (although price declines are in turn influencing vanilla production and alternatives such as paddy and pineapple are being explored). Cocoa has been widely planted, but there seems to be no organised marketing in the Province (in part because this crop is not a government priority for the region) and farmers interviewed said that they had not found a buyer for their crop. The Crop Assessment Institute (BPTP - see page 9) have trial plots with black pepper, vanilla and rice, of which vanilla is regarded as the most promising. One farmer interviewed was considering having cattle under his coconut. Intercrop choice is determined by farmer preference, market potential and location.

2.4 Agronomic practices

A possible link between maintenance of a plot and incidence of *Phytophthora* bud rot was suggested, based on reporting of two farms which were fertilised and regularly weeded but which did not have any bud rot, despite being situated in areas with the disease. However, results from trials at BALITKA and observations by Dinas Perkebunan contradict this, as more palms became infected in weeded areas than where weeds remained. It is understood that TCSDP has done trials in West Sumatra to look at nutrients and susceptibility to *Phytophthora*, but did not find any correlation.

Other points noted were that farmers often tend to plant at 7m x 7m, rather than the recommended 8.5 x 8.5 or 9 x 9m (partly to satisfy credit requirements for a minimum number of palms). This results in crowns meeting at 3-4 years, with a closed canopy by 8 years, in turn creating high humidity in the crowded crowns and a greater risk of inter-palm spread.

3. WHAT DO FARMERS DO WHEN PALMS ARE DISEASED?

3.1 Cut and burn infected tree

Most farmers appeared to recognise the bud rot symptom of the disease, but few reported nut-fall as being symptomatic. There is no curative treatment for affected palms once severely affected. Farmers have been reluctant to immediately cut and burn the infected palms because of heavy fruiting on these palms, which may in turn have favoured disease spread to other palms. However, once the palm stops fruiting, most farmers are quick to cut it down (and sometimes split the stem) and burn it - as per advice of Dinas Perkebunan. An exception to this was Lolayan, Bolaang, where the dead palms were left like pillars, and interestingly this was where the greatest incidence of the disease was seen.

3.2 Prophylactic treatment

Phosphonic acid can be injected into healthy palms to protect them against disease. Mr. Dominique Boutin, the TCSDP agronomist, said the use of FOLI-R-FOS 400 AS injected into the trunk @ 20 ml per tree would afford protection to the tree for 2 years. If root infusion is used, the treatment apparently lasts 6 months to a year.

The cost of the fungicide is about Rp 50,000 per ha. If application is conservatively costed at Rp 15,000 per ha (Rp 100 per tree), then the total cost is equivalent to the annual production from 10 palms. This means that a farmer is not likely to spend money to protect all his palms unless he thinks that he will definitely lose 10 palms in the next two years.

Many of the farmers interviewed had used Aliette (another commercial product with the same active ingredient) provided free of charge by the project (around 250,000 palms have been treated). However, farmers were not agreed on its efficacy, a finding that is probably at least in part due to initial applications not having been followed by necessary further applications. One man's plot in Loloyan, Bolaang, which TCSDP consultant's had visited, claimed the fungicide had been ineffective in preventing the spread of the disease, while others in Dimembe and Kauditan observed no effect from a previous one-off treatment. Some farmers also felt that the root infusion technique was ineffective.

Although the TCSDP had given the fungicide free to many farmers and assisted in its application, many farmers were unwilling to spend their own money on a preventative programme they were not sure would be effective. Furthermore, no financial assistance in the form of credit is offered to farmers to encourage the use of the fungicide. All the evidence from discussions with farmers was that they had not bought or applied fungicide outside government project inputs. Although Aliette injection had been recommended by the TCSDP, fungicide treatment was not considered effective enough to be recommended by Dinas Perkebunan! Overall, prophylactic fungicide treatment does not appear to be a viable solution to the *Phytophthora* problem for the smallholder.

3.3 Replanting coconut

Dinas Perkebunan sell seedlings at Rp 600 each, but most farmers reported using their own nuts from their own talls to replant. The cost of digging a hole and planting is Rp 350 per plant. In replacing palms, farmers have planted local Talls, as they have said that there has not been any hybrid available. The preference is usually to replace a diseased hybrid with a local palm, as the local Tall is known to be less susceptible to *Phytophthora*. However, the Tall takes 5 years to bear fruit, increasing the period of lost income.

Farmers in the Minahasa area generally seem to be less quick to replant, and are not so dependent on coconut as the sole source of livelihood, also, they appear to show less interest in intercropping. Further along the coast, farmers seem more willing to plant an intercrop and will replant coconuts quickly to replace palms that have died.

In the Minahasa area, 9 farmers were interviewed. Two had lost 50% of their palms to the disease, 4 had lost more than 20% to 30% and the rest less than 10%. One of those who had lost 50% had a full-time job and had done little replanting. He planned to run cattle under the coconut at a later date. The other had replanted under the coconut with cocoa. Lack of money to weed the property had meant the cocoa had become overgrown and neglected and little income was being obtained from the farm. The farmer hoped to sell the land for real estate! The farmers with 20% or more diseased palms had all been under-planted with annual crops - mainly corn and cassava, and had all replanted coconuts where there was sufficient light.

Further West along the coast, farmers were much faster to replant when a tree was diseased. Even if only 3 or 4% had died, they would immediately replant coconut, where there was sufficient light, and extend their annual intercrops into the area to compensate for the loss of income. Almost all farmers felt that the income lost from coconuts could not be made up by the added income from intercrops.

In Loloyan village in Bolaang, the disease had devastated more than 80% of the plots. Three farms were visited to see how the farmers had coped. The first farmer had extended the paddy area into the coconuts, and planted corn and soybean under the coconut. A second farmer had lost the entire crop and had not even bothered to uproot the leafless palms, that stood like lines of pillars. He had replanted under the dead palms with talls and let his workers intercrop soya and corn, from which the owner took no money in return for their nursing the new coconut. The farmer had accepted that he would get no income for 5 years. A problem for all the farmers was to know at what point they should replant the entire area. When 20% of the crop was diseased, or 50% or 80%? In reality, it was not until 75% of the crop was diseased that they assumed that they would lose all their hybrid palms, and only at that point would they under-plant the entire field with talls.

3.4 Farmer's dilemma

It is difficult for farmers to know at what speed the disease will spread. If it spreads fast, they would do best to replant under their existing coconut at an early stage of infestation, so ensuring an income when all the coconut palms die. In this respect they would prefer a tree that will fruit quickly - such as Khina hybrid In the absence of Khina, they are replanting with Local Tall (Kelapa Dalam) which doesn't fruit until the fifth year after planting and is not as high yielding as the PB 121 (although some farmers had a different opinion - see below).

As most farmers hope the disease will not spread too fast, the replanting of the whole plot tends not to happen until most of the palms have died, so causing a serious depression in production. The resultant loss of income is in part made up by the planting of intercrops.

4. HYBRID v. LOCAL TALL

4.1 Advantages & Disadvantages

The increased productivity of the hybrid over the local tall is disputed. Some farmers see the hybrid as better, but many farmers thought it was better to have the local tall.
These arguments are basically:

Pro Hybrid

Tall palms (Kelapa Dalam) are more expensive to climb for less nuts. A tall tree can cost Rp 350 per tree to climb as against a shorter hybrid at Rp 250.

The hybrid produces 70 to 80% more nuts, although copra yield per nut is 2/3. The resultant increase in production is about 18 to 20%.

Hybrid yields more precociously, giving an earlier cash return on the planting.

Against hybrid

Because hybrid yields less copra per nut, the operations to harvest, collect and make copra can cost more as these are done on a per nut basis.

Preference for a big nut and high yield per fruit, which saves labour. The local Tall (Kelapa Dalam) fetches a slightly higher price per kg of meat than does the hybrid (Rp 415 as against Rp 405)[4].

[4] Prices given by Fabrik Murni Jaya, Tenga in November 96, for fresh coconut meat.

Not as hardy to drought as the tall variety.

4.2 Farmer's preference:

Farmers were asked what characteristics they preferred in a coconut. Generally, these were firstly the size of nuts and secondly the number of nuts. Those that had suffered heavy losses due to *Phytophthora* were concerned to have an early maturing variety.

4.3 Current promotion

BALITKA had developed a series of local hybrids known as Khina at the same time as cv. PB 121 was being introduced. Khina has the advantage of larger nuts than PB121, being early maturing, and is apparently less susceptible to *Phytophthora*. However the longevity of this resistance is difficult to determine as at least one parent involved, Nias Yellow Dwarf, is known to be susceptible to bud rot in N. Sulawesi. This hybrid is now being taken up by the Tree Crop Smallholder Development Project (TCSDP), successor to the SCDP.

5. DEVELOPING EXTENSION MESSAGES

The question of how the research results will be taken up by farmers depends on what the results are, how complex they are to implement, how much they will cost the farmer and how effective they are expected to be.

5.1 Prophylactic fungicide treatment

The development of chemical control measure using phosphonic acid has not been widely adopted as a control measure despite its widespread support and advocacy by the TCSDP project. In fact there seems a divergence between the TCSDP who consider it very effective, and farmer experience, which differs. The difference might be due to improper application or use at the wrong time. Nevertheless, the farmers do not view this as effective and therefore show little willingness to undertake chemical protection with their own resources.

Farmers have very little cash resources and would be reluctant, or find it difficult, to take further credit. Farmers are already debt-bound to the TCSDP project for the original planting costs. They see the project as being morally bound to provide solutions if they want them to continue to repay their loans. All the farmers interviewed that had more than 10% death of their trees had ceased loan repayment to the project.

5.2 Institutions to develop and deliver messages

5.2.1 BPTP - Institute for Technological Assessment

A newly formed institution called the Balai Pengkajian Teknologi Petani (BPTP), or Institute for technological assessment has been formed with headquarters in Central Sulawesi. Manado is one sub-station, employing 40 professional staff, of whom 6 are researchers and 7 extensionists.

It was understood that the function of this institute is to develop and test extension messages and then pass them on to the Dept for Industrial Crops (Disbun).

In 1997, BPTP plans to:

Begin work on coconut farming systems research, looking at the different inter-crops that can be grown with coconut.

Undertake a replanting practices programme: The Provincial Governor plans to institute a programme to replant 3,000 ha per year, including the use of talls and other varieties, chosen according to the type of farm. This would be a joint programme between Dinas and BPTP using a loan scheme.

Diversify product use of coconut: BPTP plan to run programmes in nata da koko (fermented juice drink); domestic ketchup production; desiccated coconut; uses for the shell, and production of gula merah (brown sugar from the sap)

BPTP operates a number of research farms, containing a variety of crops where it is proposed that field-based research could be carried out.

It is not possible after two years operation to evaluate the competency of BPTP to convert research findings into deliverable extension messages. Valuable work could be done in co-operation with BALITKA on susceptibility of various intercrops on their research stations.

5.2.2 TCSDP - Tree Crop Smallholder Development Project

This project is directly involved with farmers who planted PB121 under the forerunner project (SCDP). In the case of *Phytophthora* diseases, it would be extremely useful for the project to work directly with TCSDP. The project has a full complement of staff in all the districts and have experience in data collection and recording disease. Further, they have an interest in aiding farmers to overcome the problems resulting from the susceptibility of PB 121, especially as many farmers with infected palms are unable to repay their loans.

The planting programme will start using Khina as the planting stock in 1997. It is planned to establish 235,000 plants (1,560 ha) in the first year.
Attached in Appendix 2 is a summary of some of the data that TCSDP has collected on the incidence of *Phytophthora* disease in North Sulawesi. This data is broken down at the UPP level by farmer's plots. The fact that all the palms planted under the TCSDP have been recorded and that there has been a close monitoring of the disease should give researchers highly useful information on which to work.

6. IMPORTANCE OF RESEARCH

6.1 Diminishing Importance of Disease?

At first sight, the problem of *Phytophthora* is a diminishing problem. The TCSDP view is that the disease is becoming of less immediate importance as more resistant material is planted and as stands of PB 121 mature. Bud rot is at present largely limited in its distribution to the PB 121 variety and planting of this variety stopped after 1989. The number of palms recorded as infected by TCSDP in North Sulawesi in 1992/93 was 193,000. By 1995/96 this had risen to 233,000, an overall increase of 38,500 or 2%. Moisture availability in the crown is essential to the growth of *Phytophthora* and spore spread is more likely in the high relative humidities near the ground. Once palms reach 8 to 10 metres high (age 12 plus), there will be greater air movement through the crown, the distance from soil inoculum sources to the crown increases and disease losses should reduce. As the last planting of PB 121 took place in 1989/90, the number of palms at risk is reducing each year. According to this theory, more than 55% of the coconut planted is now over 12 years old, and so the risk of disease in this area will be low. By the year 2002 all the PB 121 will be over 12 years old and the disease will no longer occur on this variety.

6.2 Need for better understanding

PB 121 was originally introduced to improve yields of coconuts and so incomes of smallholders. The need to develop high yielding hybrids remains, as the coconut industry is faced by increasing competition from oil palm. Oil palm is no substitute for coconut though, which does not lend itself to dispersed smallholder production, as does coconut. The Khina varieties appear to be much more resistant to *Phytophthora* but widespread planting carries the attendant risk of disease proneness in new environments. The disease thus poses a continuing concern to improvement of the industry and to the potential benefits to be gained from incorporation of exotic germplasm.

6.3 Premature nutfall

The impact of premature nutfall is considered negligible on local and exotic talls in Indonesia, but dwarf varieties are highly susceptible, including Nias Yellow Dwarf, Malayan Yellow Dwarf and the Cameroon Red Dwarf. Losses in N. Sulawesi can be serious on dwarf varieties in the rainy season. The valuable characteristics of dwarf varieties such as precociousness and low harvest height may thus be at least in part counteracted by potential losses to nutfall if such varieties are used in coconut improvement programmes.

6.4 National Context

Coconut is considered a very important crop as many smallholders depend on it for their livelihoods. Various substitute crops have been considered, but these alternative crops do not offer the flexibility or security that coconut offers. The government policy is therefore to raise yields through planting hybrids, encourage intercropping to raise productivity per hectare and to try and develop alternate uses for coconut and coconut by-products.

6.5 International context

Developing a better understanding of the means of transmission of *Phytophthora*, so leading to control measures, will help other countries affected by the disease. Coconut remains an ‘orphan’ crop in terms of research for yield improvement and oil markets continue to be threatened by other tropical oils, notably palm oil. Similar problems have arisen with the introduction of dwarf x tall hybrids to rehabilitate the industry in Papua New Guinea, although in that case rhinoceros beetles are also implicated. Research on resistance may also lead some countries to source exotic planting material from Indonesia. Bud rot is also of significance in west Africa and research outputs may be applicable there.

6.6 Research Development

This programme provides a good opportunity to improve the field pathology skills of the researchers at BALITKA and is an opportunity for them to apply molecular biological tools in a field context and to obtain experience of working directly with farmers in gathering data and researching the problem.

7. RESEARCH AREAS

On-going research into *Phytophthora* bud rot must therefore include the following elements, so as to enable better risk assessment on the part of the farmer or extensionist on the likelihood and rate of spread, as well as trying to develop control measures:

Age: The age palms can become infected and the age palms are no longer at risk

Micro-environmental characteristics: that increase the likelihood of infection e.g. humidity in canopy.

Agronomic practices: From survey data, determine the association between soil fertility, agronomic practices and disease incidence.

Rate, patterns and mechanisms of spread determined by mapping and associated population characterisation.

Determine **epidemiological association** between premature nutfall and bud rot.
The role of **inter-crops** as possible hosts.

Mark Holderness & Peter Oldham
11 April 1997

Appendix 1

	Geographical Extent of Phytophthora Bud Rot Disease in North Sulawesi										
		1992/93		**Phytophthora**			**1995/96**		**Phytophthora**		
	UPP	**No. trees**	***SAMPLE:***					***SAMPLE:***			
		originally	***No. trees***	***Terserang***	**Total**			***No. trees***	***Terserang***	**Total**	
No.	**Area**	**planted**	***sampled***	***Affected***	**affected**			***sampled***	***Affected***	**Affected**	
1	KAUDITAN										
	Airmadidi	71,015	28,825	5,296	13,048	18%		10,717	2,715	17,991	25%
	Bitung	97,060	13,485	710	5,110	5%		18,906	1,561	8,014	8%
	Kauditan	74,080	6,167	1,027	12,337	17%		8,039	3,897	35,911	48%
2	DIMEMBE										
	Wori	39,262	13,184	1,366	4,068	10%		15,248	2,017	5,194	13%
	Likupamg	87,620	12,558	838	5,847	7%		16,027	1,510	8,255	9%
	Dimembe	98,154	45,758	16,105	34,546	35%		**49,972**	18,429	36,198	37%
3	TUMPAAN										
	Tomasian	112,941	5,376	55	1,155	1%		5,376	58	1,218	1%
	Tombariri	80,468	17,824	1,764	7,964	10%		17,824	1,764	7,964	1%
	Tumpaan	85,051	25,651	1,488	4,934	6%		25,901	1,665	5,467	6%
4	TENGA										
	Tenga I	96,401	12,892	780	5,833	6%		**13,000**	965	7,156	7%
	Tenga II	95,240	12,103	218	1,715	2%		12,103	218	1,715	2%
	Tab. Utara	31,850	**4,392**	566	4,105	13%		4,392			
5	BELANG										
	Tombatu	73,633	5,138	154	2,207	3%		7,749	216	2,052	3%
	Belang	60,319	1,300	24	1,114	2%		1,300	24	1,114	2%
	Kotabunan	90,926	7,317	380	4,722	5%		7,514	495	5,990	7%
6	BOLAANG										
	Poigar	88,770	24,611	2,819	10,168	11%		24,703	2,819	10,130	11%
	Bolaang	104,462	23,027	9,224	41,845	40%		23,027	9,224	41,845	40%
	Lolak	62,971	12,637	1,899	9,463	15%		12,637	1,899	9,463	15%
7	LIMBOTO										
	Batudaa	70,797	2,869	91	2,246	3%		4,283	121	2,000	3%
	Limboto	79,310	2,507	49	1,550	2%		2,768	51	1,461	2%
	Suwawa	77,007	14,657	649	3,410	4%		20,300	1,791	6,794	**9%**
8	TIBAWA										
	Tibawa	104,570	11,163	644	6,033	6%		11,163	644	6,033	6%
	Kwandang	84,277	12,726	250	1,656	2%		12,726	250	1,656	2%
	Tilamuta	57,599	21,789	3,583	9,472	16%		21,789	3,583	9,472	6%
		1,923,783	337,956	49,979	194,548	10%		347,464	55,916	**233,093**	12%
	Source:	**INDONESIA PHYTOPHTHORA 1996 DATA SERANGAN PENYALAIT**									
		(Busuk Pucuk) Tanaman Kelapa Hybrida PB 121 Bagian Proyek Pengembangan									
		Budidaya Propinsi Sulawesi Utara						TCSDP			

Appendix 2

COST OF PRODUCTION & RETURNS (Rupiah) for coconut in North Sulawesi

	Kelapa Dalam	**Hybrid**
Production		
Nuts per tree	12	25
Harvest per year	4	4
No. nuts = 1 kg of copra	5	7
Trees per ha	140	140
Total nuts per annum	6,720	14,000
Annual labour costs		
Circle weeding		
Fertilising		
Fertiliser		
Ditch maintenance		
Sub-total	151,000	151,000
Harvest		
Harvesting cost per nut	47	40
Transport nuts from field	10	10
Make copra/nut	17	12
Total harvesting cost/ha/annum	497,280	871,000
Production		
kg of copra produced	1,344 Kg	2,000 Kg
Price per kg of copra	850 Rupiah	850 Rupiah
Price per nut	167 equivt price	120 equivt price
Total Value	**1,142,400**	**1,700,000**
Total cost	**648,280**	**1,022,000**
MARGIN	**494,120**	**678,000**
Margin as % value:	**43%**	**40%**

Appendix 3

Contacts made:

BALITKA

Dr. David Allorerung	Director
Dr. Amrizal	Deputy Director
Ir. Sonny Warokka	Pathologist
Ir. Arie Lolong	Pathologist
Ir. Mustafa Djafar	Economist
Dr. Novarianto Hencky	Plant Breeder
Ir. Jacqueline Motulo	Pathologist

Dinas Perkebunan

Ir Arie Sumarab	Dinas Perkebunan, Crop Protection, N. Sulawesi

Tree Crop Smallholder Development Project

Ir Jocelien Makalew	Crop Protection Officer, TCSDP
Ir Sinaulin	Head of UPP, Kauditan, TCSDP
Mr. Dominique Boutin	Agronomist, CIRAD / TCSDP, Jakarta

Balai Pengkajian Teknologi Petani (Manado IPPTP Kalasey)

Ir Arifuddin Lantja	Director, IPPTP
Ir G.H. Joseph	
Ir Rita Novarianto	

Appendix 4

Itinerary for P. Oldham: (all November 1996, accompanied by M. Holderness 9-16)

Date		Activity	project
Sat/Sun	9th	*London to Manado*	
MANADO			
Mon	11th	BALITKA office,	PHYT
Tues		(PTPB), Dinas Perkebunan Provincial Office, Tree Crops Smallholder Development Project TCSDP provincial office	PHYT
Weds		Visit farmer Kem, Airmadidi	PHYT
Thurs		Visit farmers in Airmadidi & Kauditan & Unicotin desiccated coconut factory. Meet D Boutin, TCSDP.	PHYT
Friday		Visit Farmers in Mibembe & Kema	PHYT
Saturday		BALITKA (MH departs p.m.)	PHYT
Sunday		Write up notes	PHYT
Monday	18th	BALITKA & TCSDP plan next visit *Fly to Jakarta*	PHYT
Tuesday		Visit Bogor	DOUE
KALIMANTAN			
Wednesday		*Fly to Kalimantan* Visit Prov Office of Dinas Perkebunan in Pelangka Raya *Drive to Sampit*	DOUE
Thursday		Visit District Office of Dinas Perkebunan in Sampit *Travel to Semuda* meet 2 farmers	DOUE
Friday		Visit 3 farmers	DOUE
Saturday		Visit 3 processors Visit farmer across the river	DOUE
Sunday		*Travel Kalimantan to Jakarta to Manado*	DOUE
MANADO			
Monday	25th	Visit Field:	PHYT
Tuesday		Visit Field Bolaang; Oil Factory @ ;	PHYT
Wednesday		BALITKA	DOUE
Thursday	28th	BALITKA : round up meeting *Fly Manado to Singapore*	DOUE

User manual of selected molecular methods in the characterisation for *Phytophthora* diseases of coconut in Indonesia

Prepared for: **Mr Arie Lolong,**
BALITKA, Manado,
North Sulawesi.

By: **Julian Smith**
CABI Bioscience UK Centre [Egham]
Egham, TW20 9TY
United Kingdom.

Methods covered: **Rapid extraction of fungal DNA**
ITS-RFLPs
AFLPs

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1. INTRODUCTION

- The DNA methods described has been selected as it does not require phenolchloroform which is highly toxic and produces waste that demands specialised disposal.

- The PCR based methods use specific primers that should enable comparisons on results between laboratories (CABI Bioscience UK Centre [Egham] and BALITKA, Manado).

- ITS-RFLP has been shown to be highly appropriate for species determination between *Phytophthora* spp., having advantages over morphological approaches due to the paucity of robust morphological characters in this genus.

- AFLP is probably the most appropriate genomic fingerprinting method available, combining high levels of discrimination with robust reproducibility. The method described has been adapted from a method that uses polyacrylamide gels and specialised electrophoretic equipment for use with standard agarose gels.

- Collectively, these methods have been chosen on the basis of their efficacy and robustness and will optimise the likelihood of achieving the objectives of the project. Special thought has been given to avoid the use of hazardous reagents and thus minimise associated problems of waste disposal.

- The text does not attempt to provide an exhaustive manual for laboratory work, but has selected basic aspects of molecular biology that are frequently misunderstood and yet are fundamental to day-to-day working.

- No theory is provided on the methods described. This information is available in the CABI Bioscience manual on PCR and Modern Methods.

- A procedural outline of each method is provided.

- Information on the solutions used and how they are made is provided in the appendix.

2. WHAT IS A MOLECULAR LABORATORY AND HOW DOES IT OPERATE

a) Laboratory requirements

There is nothing exceptional required of a laboratory to make it appropriate for molecular techniques. Rational, common sense thinking will dictate what is the best set-up for you to achieve the below requirements.

- The facility (or a working zone) should be dedicated solely to molecular methods, and spatially separated from other laboratory practices such as isolation work from dirty materials and sub-culturing where spores will be released.

- Air currents should be minimised in the area of the work bench to reduce airborne dust. Specific attention needs to be drawn to air conditioning appliances. Ceiling fans should not be present above or near the work bench.

- Work surfaces should be laminated or tiled for ease of cleaning.

- Where a near-dust free environment can not be achieved procedures will need to be undertaken in a sterile flow cabinet.

- It is highly desirable to have a maintained day and night air temperature of 20-25°C, to ensure the longevity of molecular reagents and uniformity of molecular processes.

- The facility should be as near to self-sufficient as possible to reduce the need to use additional resources in adjoining rooms. This is particularly true for fridge and freezer.

- Where electricity is unreliable a back-up generator is highly desirable and essential for the maintenance of freezers if prolonged breaks are expected.

- The minimum capitol equipment to perform the described methods include:

  Fridge (+5°C)
  Freezer (-20°C)
  Incubator
  Autoclave
  Sterile flow cabinet
  Distil for deionised water
  pH meter
  Weighing balance
  PCR machine
  Electrophoretic equipment
  Power pack
  UV transilluminator
  Camera.

b) Working procedures

Having the equipment to perform molecular methods is not a guarantee of success. Success relies on the operation of the lab, attention to detail, attention to routines; it comes from you, from you implementing and carrying out good working procedures. All research procedures are 2/3 preparation 1/3 doing. This can be frustrating as it appears that progress is slow, but the preparation, the anticipation of events is essential: volumes of solutions required, equipment required and especially the time required.

The below are minimum working standards that need to operate:

- Be aware of the hazards of the chemicals that you are using and take all necessary precautions inline with hazard data sheets. Always wear protective clothing and laboratory gloves when working. This is for your own safety, but and will also reduce the risk of contaminating solutions with human DNA which will effect subsequent work.

- The facility must be scrupulously clean:

  Working surfaces need to be washed with industrial alcohol or 4% solution of bleach once ever week.

  Floors need to be swept at the end of each day so dust generated can settle overnight.

  Glassware must be washed as soon after use as is practical.

  Spilt chemical on weighing are cleaned up immediately.

- Weighing balance is level prior to use (see air-bubble is central) and checked for accuracy against standard weights once every month.
- When weighing out a chemical or measuring a reagent, never return any excess to the original container. Discard in accordance with hazard data recommendations.
- Pipettes (Gilsons) are checked for accuracy at regular intervals (see appendix: Calibrating a pipette).
- pH meter is calibrated on a once weekly basis if in regular use, or prior to starting a specific piece of work. This will require the regular renewal of the pH buffer solutions. Note that the pH meter is a sensitive piece of equipment and must be maintained in accordance with the manufacture's recommendations. The electrode is particularly sensitive and must be kept immersed in water when not in use.
- Solutions must be clearly labelled with information on components, concentration, pH and date of making.
- Each autoclave run should include a thermolog device, such as autoclave tap, to ensure the process was successful.
- It is essential to know the reliability of the equipment, as sub-standard performance can often be compensated for when the extent of the problem is known e.g. weighing balance and pipettes.

3. OVERVIEW ON MAKING SOLUTIONS

a) Types of solutions

Solutions expressed as molarity

- Molarity (molar solution) is designated by a capitol M e.g. 1.0M NaCl. This should not be confused with the designation mol which describes an amount of a mole present e.g. primer x was used at 50pmol per reaction. In this example the reaction is of a known volume and this pmol value can be converted to a molarity (see appendix: PCR components).
- The concentration of a solution is described by its molarity. A 1 molar (1.0M) solution contains the molecular weight (mw) of the chemical when dissolved in water to give a final volume of 1 litre (1000ml).

  e.g. Preparing molar solutions of sodium acetate (mw = 136.1g).

1.0M solution	=	136.1g in 1000ml total volume
1.0M solution	=	13.6g in 100ml total volume
3.0M solution	=	40.8g in 100ml total volume

- Preparing a molar solution from a chemical supplied as an aqueous solution requires consideration of the % saturation of the solution and its specific gravity. These details are provided on the label.

  e.g. Preparing molar solutions of hydrochloric acid from a saturated 37% solution (mw = 36.46g; specific gravity = 1.19 (1.0l = 1.19kg)).

Therefore	1000ml sat HCl	=	1190g
	1ml sat HCl	=	1.19g
	1ml contains 0.37 x 1.19	=	0.44g HCl
	1.0M HCl	=	36.46g HCl in 1000ml water (total volume)
	1.0mol HCl is contained in 36.46/0.44	=	82.8ml saturated HCl
	82.8ml sat HCl in 1000ml total volume	=	1.0M solution
	8.3ml sat HCl in 100ml total volume	=	1.0M solution
	41.5ml sat HCl in 100ml total volume	=	5.0M solution

Solutions expressed as a percentage

- A percentage solution is very straightforward to work out and describes the gram weight of a chemical dissolved in 100ml of a solution.

  e.g. 10% solution of SDS is 10g SDS dissolved in solute to give 100ml.

Solutions expressed as rations

- Ratios are often used when mixing a number of solutions and describe mixing known volumes of each solution.

  e.g. Solution X comprises solutions A, B and C mixed at a ratio of 10:5:1. This means for every 10ml of solution A, 5 ml of solution B and 1 ml of solution C was added.

Solution expressed as specific activity

- Enzymes and antibiotics are frequently described as having Xunits of activity per mg or ul.

- From solutions such as restriction enzymes:

  e.g. Hinf 1 is required at 20U per enzymatic digestion. If the container describes the enzymes as having an activity 10U/ul then 2ul is to be added to your solution.

- From solids such as antibiotics:

  When Xunits are to be added then you need to establish how many units are present in 1mg. The required information will be available on the container:

  e.g. If 100U are to be added to 100ml and the activity specified as 10,000 units in 700mg then:

1mg	=	14.2U/mg
therefore to add 100U (100/14.2)	=	7.0mg is required.

b) Setting the pH of a solution

- Check that the pH meter is calibrated and functioning properly by use of pH buffer tablets.

- Add the required chemical components to half the final intend volume of solute (normally water). i.e. if the final volume is to be 100ml add 50ml of solute at this stage.

- Depending on the size of the pH change and the buffering capacity of the solution you intend to set the pH of, select either 1.0M or 5.0M HCl or NaOH for pH decreases or increases, respectively. See section on molarity for detail on making molar solutions.

- Add acid/alkali drop wise until the required pH is obtained. Finally, make up the solution to the required volume.

- Wash the electrode and return it to the water.

c) Using stock solutions

- A stock solution is a solution of known specification (molarity, percentage etc.) from which a measured volume is used to make a second solution.

- It is often a practical approach to solution preparation. For example consider the preparation of 100ml of TE buffer.

  TE buffer (10mM Tris; 1mM EDTA; pH8.0).

Tris (mw = 121.1g)	=	0.121g
EDTA (mw = 372.2g)	=	0.0372g
Water to give a total volume of 100ml		

  It is not practical to weigh-out 0.0372g of EDTA and a far better approach is to use stock solutions:

Stock solutions		
1.0M Tris (pH 8.0)	=	1ml
0.5M EDTA	=	200ul
Water	=	98.8ml

4. DNA EXTRACTION

a) Preparation and harvest of culture

- Filter V8 broth through fine sieve (1mm gauge) and then through a muslin cloth. Autoclave and dispense as 10ml aliquots into sterile universals.

- Inoculate with *Phytophthora* mycelium plug, and incubate at 30°C (room temp) for 48-72hrs.

- Dissect out agar plug from mycelial growth and transfer mycelial growth in to a 1.5ml eppendorf. Suspend mycelium in 500ul of TE.

- Mycelium can be stored frozen at -20°C or processed immediately for DNA extraction.

b) DNA extraction method

- Spin down mycelium at 13,000rpm for 5 mins. Remove supernatant.

- Add 300ul of extraction buffer and macerate mycelium by use of a grinder and drill. Work with four samples at a time and store the macerated sample in the freezer until all samples have been processed.

- Add 150ul of 3M Na acetate (pH5.2), mix by inversion and place in freezer (-20°C) for 10 mins.
- Spin down sample at 13,000rpm for 10 mins. Transfer supernatant to new eppendorfs.
- Add an equal volume of ice-cold isopropinol. Mix by inversion and place in freezer (-20°C) for 10 mins. Watch for precipitated DNA!
- Pellet the precipitated DNA by centrifugation at 13,500 for 5 mins. Pipette of supernatant.
- Wash pelleted DNA with 300ul of 70% ethanol. Centrifuge at 13,000rpm for 5 mins, discard supernatant, and dry DNA in a desiccator.
- Dissolve DNA in 100ul of TE. This may take some time and may even require warming to 50°C for 5 mins: use PCR programme labelled Heat 1.
- Store DNA at -20°C.

5. QUANTIFICATION OF DNA ON AN AGAROSE GEL AND INFERENCE ON QUALITY

- A good knowledge of the concentration and quality (integrity) of DNA in each of your stock DNA extracts is vital for all subsequent work. A sufficient index can be gauged by visualising your DNA stained with ethidium bromide on an agarose gel.
- Defrost the stock DNA thoroughly and make a 1:10 dilution by taking 5ul of the stock DNA and adding to 45ul of water (PCR grade).
- Load stock and 1:10 diluted DNA (5ul DNA plus 5ul loading buffer) on a agarose gel (1.5% (w/v) LE agarose - see appendix). Always include a 1kb ladder as this provides a standard of known DNA concentration. It is the positive control to which your extracted DNA can be compared. Stain DNA with ethidium bromide and visualise under a UV transilluminator.
- Has the 1kb ladder stained properly? If not then the ethidium bromide is out of date and needs to be made a fresh. Until the 1kb ladder is effectively stained conclusions can not be drawn about the concentration of your DNA sample.
- If the 1kb ladder is clearly visible then the chromosomal DNA of your sample(s) should be visible as a discrete band at about 1cm distance from the well, having a mobility similar to the largest fragments of the 1kb ladder
- A very bright streaked band with a defined leading edge is typical of electrophoresis of DNA that is too concentrated. This may be apparent in the undiluted sample, and should be resolved as a discrete band in the 1:10 dilution. If the sample is smearing at a 1:10 dilution then a 1:100 dilution maybe needed.
- If dilution fails to resolve a discrete band and a smear is present at low concentrations and appears not to have a defined leading edge then the DNA has been degraded by DNAs activity and is of no use. Repeat the DNA extraction.
- Ribosomal DNA is often apparent ‘further up’ the gel as a number of discrete bands or a smear. Absence or presence of ribosomal DNA is not a concern.

- Stock DNA samples should be dilution to a DNA concentration where a 10ul aliquot gives a clear and discrete band on electrophoresis. At this concentration the DNA approximates to 100ng/ul and is the DNA from which subsequent work will be performed. It is termed your working stock solution.

7. ITS-RFLP

- ITS-RFLP is a two step process of PCR to amplify the ITS fragment followed by restriction analysis (RFLP) by endonuclease enzymes.

a) ITS-PCR

- PCR: For a 50ul reaction volume

Mastermix:				
	a)	dNTP	=	0.2mM
	b)	PCR buffer	=	1 x buffer
	c)	ITS 1 primer	=	50pmol
	d)	ITS 4 primer	=	50pmol
	e)	Tth enzyme	=	5U
	f)	Water (PCR grade)	=	30.75ul
	g)	DNA (1:100 dil. of working stock soln.)	=	5.0ng (5ul)

Add components a-e to water, mix and aliquot out 45ul volumes into PCR eppendorfs. Add 5.0ul DNA (1:100 dil of working stock soln.) to tubes and 2 drops of mineral oil.

PCR programme:		
	a) 94°C	4mins
	b) 94°C	1min
	c) 55°C	1min
	d) 72°C	1.5min
	e) Goto b) 34 times	
	f) 72°C	5mins

b) Restriction of ITS product

- Estimate concentration and size of amplified DNA on 1.5% (w/v) LE agarose gel (see appendix) with 100bp ladder as standard. ITS PCR should produce a single DNA fragment that in the case of *Phytophthora* is typically 900bp in length. For restriction analysis approximately 500ng of ITS DNA are required (see quantifying [DNA]).

- Digestion components:

DNA	=	500ng
Enzyme buffer	=	1 x buffer
Enzyme	=	20U
Water (PCR grade) to give final volume of 20ul		

Incubate at 37°C for 4-12hs (overnight)

- Restriction fragments resolved by electrophoresis on 1.5% (w/v) High Pure low EEO agarose (see appendix) with 100bp ladder. Stain DNA with ethidium bromide and visualise under a UV transilluminator.

8. AFLP

- AFLP can be considered as having four stages

a) Restriction and ligation

Reaction components:
a) Adapter (labelled AD) = 0.2ug
b) Pst 1 = 20U
c) T4 DNA ligase = 1U
d) ATP = 0.5mM
e) Buffer = 1 x buffer
f) PCR grade water to a total volume of 20ul

g) DNA (working stock solution) = 100-500ng

Perform reaction in 0.5ml eppendorfs. As with making the PCR master-mix, add components a-e to water, mix and aliquot out.

b) Precipitation of restricted/ligated DNA

- To each sample add: 80ul water
  50ul 7.5M ammonium acetate
  2 volumes (300ul) 100% ice cold ethanol

- Pellet precipitated DNA by centrifugation at 13,000rpm for 10 mins. Wash pellet in 70% ethanol, re-pellet by centrifugation at 13,000rpm for 5 mins and dry in the desiccator.

- Dissolve DNA preparation in 50ul TE. This is termed the restriction/ligation (R/L) AFLP stock DNA. Note that insufficient DNA is present to quantify the concentration.

c) Pre-amplification with Adapter-A

- PCR: For a 50ul reaction volume

Mastermix:
a) dNTP = 0.2mM
b) PCR buffer = 1 x buffer
c) Primer Adapter-A (labelled ADA) = 50pmol
d) Tth enzyme = 5U
e) Water (PCR grade) = 35.75ul

g) DNA (1:10 dil of R/L AFLP stock DNA) = 5.0ul (approx. 50ng)

Add components a-d to water, mix and aliquot out 45ul volumes into PCR eppendorfs. Add 5.0ul DNA preparation to tubes and add to drops of mineral oil.

PCR programme:
a) 94°C 4mins
b) 94°C 1min
c) 60°C 1min
d) 72°C 1.5min
e) Goto b) 34 times
f) 72°C 5mins

Remove 40ul of PCR product and store as frozen. Determine DNA concentration on remaining 10ul of PCR product by agarose gel electrophoresis (2.0% (w/v) LE agarose –

see appendix) with reference to 1kb ladder. A smear of amplified products should be visible which is of a uniform concentration between samples. This is termed the pre-amp stock DNA.

d) Amplification with AFLP primers.

- Dilute pre-amp stock DNA 1:100 with water (PCR grade), allowing for any variation observed in DNA concentration.
- Perform PCR as described for pre-amplification, substituting Primer Adapter-A for a AFLP primer (D, E, H or Q).
- Visualise 25ul of AFLP amplification by agarose gel electrophoresis (2% LE (w/v) agarose gel – see appendix).

9. MAKING SENSE OF YOUR RESULTS

a) ITS-RFLP data

ITS PCR amplification

- PCR amplification by primers ITS 1 and ITS 4 should produce a single DNA fragment that for *Phytophthora* spp. will be about 900bp in length. Do you have a single product and is it about 900bp in length, by reference to the 100bp ladder (Each band represents 100bp; the bright band is 600bp).
- If you have multiple bands is the 'non-expected' band present in the control PCR amplification. If so then contaminating fungal DNA is affecting your work. Repeat the PCR reaction and if necessary re-make the PCR components.
- If you have multiple bands that are specific to a single isolate then it is probable that the original *Phytophthora* isolate is mixed with another fungus. Hyphal tip sub-culture and repeat the DNA extraction.
- If the multiple bands is common to most DNA samples, but absent from the control PCR amplification then this indicates low specificity of the amplification. Try increasing the annealing temperature; reducing the [DNA]; reducing the [primer]. Perform these alterations 1 at a time so that you identify the problem.

RFLP analysis

- If the restriction enzyme recognises 1 restriction site then 2 DNA fragments will be visible following electrophoresis. If 2 restriction sites are identified then 3 DNA fragments will be seen, and so on.
- The sum of the digested ITS DNA fragments should equal the size of the undigested ITS DNA fragment.

  e.g. ITS band = digest bands A + B + C

- If the sum of the digestion products is less than that of the original ITS fragment it is probable that 1 or more very small (< 100bp) digestion products have been produced that are of insufficient size to for discrete bands on electrophoresis and are therefore not seen.

- If the sum of the digestion products is greater than that of the original ITS fragment it is probable that either 1) The isolate contains multiple ITS sequences or 2) The ITS product is derived from a mixed culture the fungi of which yield similar sized ITS fragments on amplification with ITS 1 and 4 primers but have different sequences. In both situations hyphal tip sub-culture and repeat DNA extraction and ITS-RFLP. If multiple ITS sequences are present in the single isolate then the initial result will be reproduced. If a mixed culture was present a different ITS-RFLP will be obtained, the fragments of which will now sum to the original ITS product size.

b) AFLP genomic fingerprinting data

Genomic fingerprinting can yield very complex data to analyse and when looking at many isolates can represent a significant challenge. The most usual objectives when analysing these data is to 1) Determine the number of distinct genomic profiles present within your study and 2) Determine the genetic diversity between these profile types.

- Accordingly, a rational approach is required with 3 distinct stages.

Stage 1 – Preliminary designation of a AFLP profile type

- From your gels undertake an initial screen of the isolates against. Identify the main features of the profiles obtained; those features that are common to all or most isolates and those features that are different.

- This is sometimes made easier by dividing the genomic profile for each isolate into 3 section (lower, middle and upper section).

- Assign a letter to the pattern observed in each section. Use the same letter for like profiles and different letters for dislike profiles.

- Combine the letters that describe each section to form a 3 letter code designation (e.g. AAC, BBB, BCE etc.).

- Group the isolates according to this 3 letter code.

Stage 2 – Confirmation of the AFLP profile types

- Repeat the AFLP for all the isolates, and perform the electrophoresis so that all isolates of the same code run together. This serves 2 purposes:

  By comparison to the first AFLP analysis it confirms the reproducibility of the AFLP profiles i.e. they should be very similar.

  It brings together all isolates previously identified as having the same AFLP code on a single gel. This markedly aids comparison of the profiles and it becomes a straightforward task to identify isolates assigned the wrong 3 letter code.

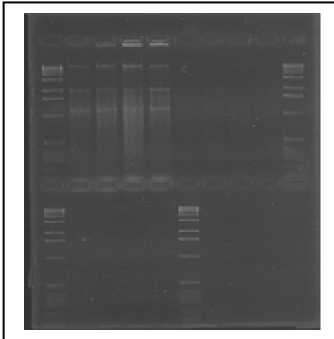
Stage 3 – Determination of total genetic diversity

- Take a single isolate representative of each of the codes identified and perform AFLP. Run on a single gel to capture the genetic diversity present within the population, and to enable pairwise comparisons of the profile types for the purposes of statistical analysis.

10. WORKED EXAMPLE: DNA EXTRACTION, ITS-RFLP AND AFLP

The objective of this section is to show what concentration of DNA is optimal for ITS-RFLP and AFLP. In this particular example the extracted stock DNA is at a concentration of 100ng/ul. In the earlier text, it is suggested that stock DNA be diluted to this concentration; the working stock solution.

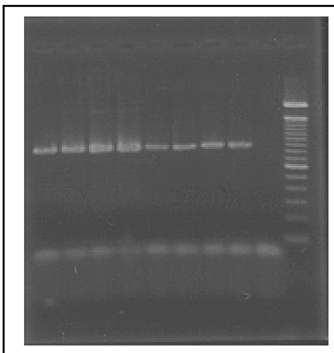
a) DNA extraction



Top lanes: 1 & 10: kb marker
Lanes 2 – 4: Stock DNA (100ng/ul)
Lanes 5 – 9: 1:10 dilution of stock DNA

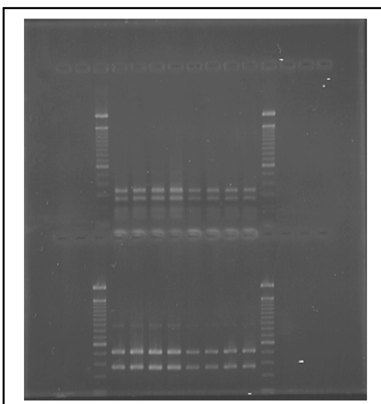
Bottom lanes: 1 & 5: kb marker
Lanes 2 – 5: 1:100 dilution of stock

b) ITS amplification



Top lanes: Lane 10: kb marker
Lanes 1– 4: 1:100 dilution of stock DNA
Lanes 5 – 8: 1:1000 dilution of stock DNA
Lane 9: PCR control

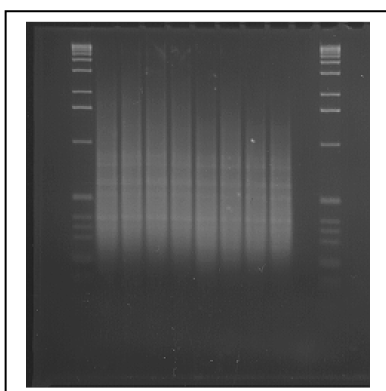
c) ITS-RFLP



Top: Hinf1 digestion products of ITS amplification (order of lanes as in ITS)

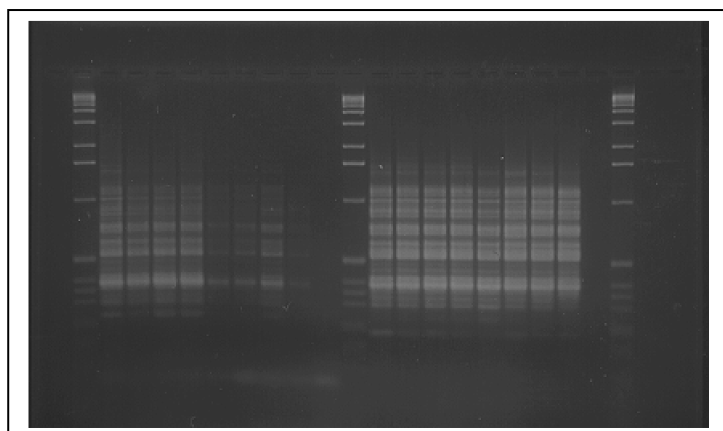
Bottom: Msp1 digestion products of ITS amplifiaction

d) AFLP Preamplification



Lanes 1 & 11: kb ladder
Lanes 2 – 4: Pre-amplification with restricted/ligated AFLP stock DNA
Lanes 5 – 9: Pre-amplification with 1:10 dilution of restricted/ligated AFLP stock DNA
Lane 10: PCR control

e) AFLP



Lanes 1, 11 & 21: kb ladder
Lanes 2 – 9: AFLP without pre-amplification using R/L AFLP stock DNA (2 – 5) and 1:10 dilution of R/L AFLP stock DNA (6 – 9)
Lanes 12 – 19: AFLP from 1:100 dilution of pre-amp stock DNA (amplified for R/L AFLP stock DNA and 1:10 dilution of R/L AFLP stock DNA, as used in lanes 2-9 above)
Lanes 10 & 20: PCR controls

11. APPENDIX

a) Conversion chart of measurements

Liquids

1l	=	1000ml
1ml	=	1000ul

Weights

1kg	=	1000g
1g	=	1000mg
1mg	=	1000ug

Molarities

1M	=	1000mM	=	1°M
1mM	=	1000uM	=	1^{-3}M
1uM	=	1000nM	=	1^{-6}M
1nM	=	1000pM	=	1^{-9}M
1pM	=		=	1^{-12}M

b) Common stock solutions

5.0M Hydrochloric acid

Saturated HCl (37%) = 41.5
Water = 58.5ml

IMPORTANT: Always add the water to the acid.

5.0M Sodium hydroxide

NaOH (mw = 40g) = 20g
Water to give a total volume of 100ml

1.0M Tris (pH 8.0)

Tris (mw = 121.1g) = 12.1g
Water to give a total volume of 100ml
Autoclave solution

Note: Dissolve Tris in 50ml water and pH with 5.0M HCl, then adjust volume to 100ml.

0.5M EDTA (pH 8.5)

EDTA (mw = 372.2g) = 18.6g
Water to give a total volume of 100ml
Autoclave solution

Note: Add EDTA to 50ml of water and adjust pH to 8.5 with 5M HCL. The EDTA will dissolve slowly as the pH changes.

c) Preparation of DNA extraction solutions

TE buffer (10mM Tris (pH8.0); 1mM EDTA)

Stock solutions
1.0M Tris (pH 8.0) = 1ml
0.5M EDTA = 200ul
Water = 98.8ml
Autoclave solution

Extraction buffer (200mM Tris (ph8.5); 250mM NaCl; 25mM EDTA; 0.5% SDS)

Tris (mw = 121.1g) = 2.42g
NaCl (mw = 58.14g) = 1.46g
EDTA (mw = 372.2g)) = 0.93g

SDS	=	0.5g

Water to give total volume of 100ml
Autoclave solution

Note: Add Tris to 50ml of water and adjust pH with HCl to pH8.5, and then continue to add remaining components.

3.0M Sodium acetate (pH 5.2)

Sodium acetate (mw = 136.1g)	=	20.14g

Water to give total volume of 50ml
Autoclave solution

Note: Dissolve Na acetate in minimum water (about 20ml) and adjust pH with 5.0M HCl.

70% Ethanol

Ethanol (high purity)	=	70ml
Water	=	30ml

d) Preparation of DNA ladders

1kb and 100bp ladder: Working concentration = 0.05ug/ul @ 10ul/well

Stock concentration = 1ug/ul
Therefore dilute stock 1:20 (50ul stock 1kb or 100bp to 950ul PCR grade water)
Dispense as 50ul aliquots in 0.5ml eppendorf and store at -20°C.

e) Ethidium Bromide: Working concentration = 0.5mg/l (0.5ug/ul)

Stock concentration = 15mg/ml
Therefore dilute stock 1:333 (16.6ul in 500mls of water

e) Preparation of PCR components

PCR grade water:

Filter sterilise about 20ml of HPLC grade water into glass universals and autoclave.
Dispense as 1ml aliquots in 1.5ml eppendorf and store at -20°C.

dNTPs: Working concentration = 2.5mM

Stock concentration = 100mM
Therefore dilute stock dNTPs 1:40 (100ul of each dNTP to 3.6ml of PCR grade water). Dispense as 200ul aliquots in 0.5ml eppendorf and store at -20°C.

Primers: Working concentration = 10pmol/ul or 10uM

Stock concentration = 100pmol/ul or 100uM
Therefore dilute each primer stock 1:10 (100ul of primer to 900ul of PCR grade water). Dispense as 200ul aliquots in 0.5ml eppendorf and store at -20°C.

Method of calculation based on ITS 1.

See number of moles provide by manufacturer. For ITS 1 this is 64.9nmol.

64.9nmol diluted in 1000ml	=	64.9fmol/ul	=	64.9nM
64.9nmol diluted in 1ml	=	64.9pmol/ul	=	64.9uM
64.9nmol diluted in 649ul	=	100pmol/ul	=	100uM

f) Preparation of AFLP components

Adapter AB: Working concentration = 0.2ug/ul

Adapter A and B have to be added together with equal molarity and then diluted to give 0.2ug/ul combined mass of Adapter-AB

Adapter-A: MW = 6386
Adapter-B: MW = 5194

100uM Adapter-A (stock solution)	=	0.52g/1l
100uM Adapter-B (stock solution)	=	0.64g/1l
100uM solution of Adapter AB	=	1.16g/1l
1ml of 100uM Adapter AB	=	1.16mg
1ul of 100uM Adapter AB	=	1.16ug

Hence, 100uM stocks of Adapter A and B to be diluted by 1.16/0.2 = 5.8

To make 1ml of Adapter AB

Adapter A	=	86.2ul
Adapter B	=	86.2ul
1M NaCl	=	150ul
Water (PCR grade)	=	677.5ul

Heat to 95°C for 10 mins and allow to cool slowly.

Aliquot 100ul into small eppendorf and use PCR program Heat 2 with the hot lid on. Store at -20°C.

7.5M Ammonium acetate

Ammonium acetate (mw = 77.08g)	=	28.9g

Water to give a final volume of 50ml
Autoclave solution

Note: No pH adjustment required.

g) Preparation of agarose gels and staining the gel

10 x TBE (0.8M Tris; 0.9M Boric acid; 25mM EDTA)

Tris (mw = 121.1g)	=	54.0g
Boric acid (mw = 61.83g)	=	27.0g
EDTA (mw = 372.2g)	=	4.65g

Water to give a total volume of 500ml

Note: No pH adjustment required.

Types of agarose gels and use

Agarose	Percentage (w/v)	Application	Ladder	Run time
LE agarose	1.5	DNA concentration	1kb	2hr
		ITS amplification	100bp	1hr
	2.0	AFLP preamplification	1kb	2hr
		AFLP amplification	1kb	5hr
LE agarose	1.5	ITS-RFLP	100bp	2hr

h) Taking the photograph

- You can not make a good photograph of a gel of poor quality, but it is easy to spoil a good gel by taking a bad photograph.

- DNA stained with Ethidium Bromide will fade when taken out of stain and placed under the UV transilluminator. Therefore, before proceeding to this step make sure you are in a position to take the photograph without delay.

- Ensure UV transilluminator plate is dust free.

- Ensure UV transilluminator plate is level and blot excess stain from around the gel. These 2 steps are to ensure the gel does not drift whilst the photograph is being taken. If drift has occurred a blurred photo will result.

- View gel and note intensity of ladders to verify that the gel has stained properly. If the ladder glows bright, then estimate the intensity of the your sample DNA. Set camera exposure accordingly:

For Polaroid 655 film only:

Band intensity	Camera exposure
Bright	30 seconds at F8
Average	30 seconds at F4.5
Faint	50 seconds at F4.5

Varnish photograph and place negative is tap water overnight to remove developers. Air dry negative for 24hrs and store in protective sleeve.

i) Calibrating a pipette

Verify the accuracy of a weighing balance by use of standard weights.

1ml Gilson:	10 x 1000ul	=	10g
	10 x 100ul	=	1g
200ul Gilson:	10 x 200ul	=	2g
	10 x 20ul	=	200mg
10ul pipette	10 x 10ul	=	100mg
	10 x 1ul	=	10mg

PATHOGENICITY ASSESSMENTS ON *PHYTOPHTHORA* SPP. FROM COCONUT BASED CROPPING SYSTEMS OF INDONESIA

VALIDATION OF INOCULATION METHODS

*** ACCUMULATED ANALYSIS OF VARIANCE AT OBSERVATION DATE 2***

CHANGE	D.F.	S.S.	M.S.	V.R.	F PR.
+ Replicate	3	0.5463	0.1821	0.53	0.663
+ Inoculum	2	25.8519	12.9259	37.56	<.001
+ Coconut age	2	3.9074	1.9537	5.68	0.005
+ Inoculum.Coconut age	4	32.9815	8.2454	23.96	<.001
Residual	96	33.0370	0.3441		
Total	107	96.3241	0.9002		

ASSESSMENT ON DETACHED COCONUT NUTS

*** ACCUMULATED ANALYSIS OF VARIANCE AT OBSERVATION DATE 3 ***

CHANGE	D.F.	S.S.	M.S.	V.R.	F PR.
+ Rep	4	22.4114	5.6028	7.75	<.001
+ AFLP	1	97.605	97.605	135.00	<.001
+ Host	3	35.8770	11.9590	16.54	<.001
+ Isolate	53	267.0592	5.0389	6.97	<.001
Residual	228	164.8406	0.7230		
Total	289	587.7940	2.0339		

*** ACCUMULATED ANALYSIS OF VARIANCE AT OBSERVATION DATE 3 ON AFLP 1 PROFILE TPYES ONLY***

CHANGE	D.F.	S.S.	M.S.	V.R.	F PR.
+ Rep	4	28.1687	7.0422	8.82	<.001
+ ITS-RFLP	1	18.9863	18.9863	23.79	<.001
+ Isolate	47	277.9576	5.9140	7.41	<.001
Residual	190	151.6583	0.7982		
Total	242	476.7709	1.9701		

ASSESSMENT ON DETACHED COCOA PODS

*** ACCUMULATED ANALYSIS OF VARIANCE AT OBSEERVATION DATE 3***

CHANGE	D.F.	S.S.	M.S.	V.R.	F PR.
+ Rep	4	3.2155	0.8039	1.59	0.177
+ AFLP	1	163.768	163.768	324.73	<.001
+ Host	3	12.5296	4.1765	8.28	<.001
+ Isolate	53	261.023	4.9250	9.77	<.001
Residual	228	114.984	0.5043		
Total	289	555.5216	1.9222		

ASSESSMENT ON COCONUT SEEDLINGS

*** ACCUMULATED ANALYSIS OF DEVIANCE AT OBSERVATION DATE 3***

CHANGE	D.F.	DEVIANCE	MEAN DEVIANCE	DEVIANCE RATIO	APPROX CHI PR
+ Rep	4	10.8399	2.7100	3.33	0.011
+ AFLP	1	53.2132	53.2132	65.36	<.001
+ Host	3	19.8519	6.6173	8.13	<.001
+ Isolate	53	331.8847	6.2620	7.69	<.001
Residual	228	185.6206	0.8141		
Total	289	601.4104	2.0810		

Dispersion parameter is estimated to be 1.84 from the residual deviance

*** ACCUMULATED ANALYSIS OF VARIANCE AT OBSERVATION DATE 3 ON AFLP 1 PROFILE TPYES ONLY***

CHANGE	D.F.	DEVIANCE	MEAN DEVIANCE	DEVIANCE RATIO	APPROX CHI PR
+ Rep	4	7.2215	1.8054	2.29	0.061
+ ITS-RFLP	1	0.7372	0.7372	0.94	0.335
+ Isolate	47	331.2015	7.0468	8.94	<.001
Residual	192	151.2685	0.7879		
Total	244	490.4288	2.0100		

Dispersion parameter is estimated to be 0.788 from the residual deviance

PNF INFECTION WITH AND WITHOUT WOUNDING

*** Accumulated analysis of variance ***

CHANGE	D.F.	S.S.	M.S.	V.R.	F PR.
+ Replicate	3	6.7500	2.2500	2.58	0.084
+ Wounding treatment	1	13.5000	13.5000	15.47	<.001
Residual	19	16.5833	0.8728		
Total	23	36.8333	1.6014		

CORRELATION BETWEEN INCIDENCE OF PNF ON BR AND NON-BUDROT INFECTED COCONUT PALMS

*** Analysis of variance based on proportion of coconut nuts/branch infected by PNF ***

SOURCE OF VARIATION	D.F.	S.S.	M.S.	V.R.	F PR.
Palm-Number stratum	4	3.2093	0.8023	1.16	
Palm-Number.Budrot stratum					
Budrot	1	0.7617	0.7617	1.10	0.353
Residual	4	2.7633	0.6908	6.03	
Palm_No.Budrot.Branch-number stratum					
Branch-number	8(1)	4.8033	0.6004	5.24	<.001
Budrot.Branch-number	7(2)	0.4418	0.0631	0.55	0.791
Residual	39(33)	4.4715	0.1147		
Total	63(36)	10.6319			