MOLECULAR METHODS FOR DETECTING THE COCONUT LETHAL DISEASE (LD) PHYTOPLASMA IN TANZANIA

By

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ABBREVIATIONS

CsCl	caesium chloride
DNA	deoxyribonucleic acid
DAPI	4'-6'-diamidino-2-phenylindole-2HCl
LB	Luria bertani (medium or broth)
IPTG	isopropythio-β-D-galactosidase
bp	base pairs
kb	kilobases
kbp	kilobase pairs
l	litre
mg	milligramme
$MgCl_2$	Magnesium chloride
mAbs	monoclonal antibodies
min	minute
M	molar
ml	millilitre
mm	millimolar
Mw	molecular weight
ng	nanogram
NaCl	sodium chloride
OD	optical density
PCR	polymerase chain reaction
RNase	ribonuclease
rDNA	ribosomal DNA
rpm	revolutions per minute
sec	second (s)
SDS	sodium dodecyl sulphate
SSC	sodium chloride sodium citrate buffer
Tris	2-amino-2 (hydroxy methyl-1, 3-propanediol or tris (hydroxymethyl aminomethane)
UV	ultra violet light

v/v	volume to volume (expressed as a percentage)
w/v	weight to volume (expressed as a percentage)
λ	Lambda
µl	microlitre
µg	microgram
β	Beta
%	percentage
LD	lethal disease
LYD	lethal yellowing- type diseases
GVX	green valley X phytoplasma (= western X-disease)
APF	apple proliferation
AEY	American elm yellows
EEY	European elm yellows
PLP	Plum leptonecrosis
AYS	Aster yellows
ACLR	apple chlorotic leaf roll
FBP	faba bean phyllody
STOL	Stolbur of pepper
CAV	Catharanthus virescence
VAC	vaccinium witches'-broom
CP	clover phyllody
CST	Corn stunt spiroplasma
SC	*Spiroplasma citri*

ABSTRACT

Lethal disease (LD), a phytoplasma lethal yellowing-type disease of coconut palms, is the major threat to coconut cultivation in the coastal areas of Tanzania. Two molecular approaches have been developed for early and accurate disease diagnosis. Random fragments of LD phytoplasma DNA were generated as probes for pathogen detection. LD DNA extracted from infected coconut tissue was randomly fragmented and cloned into pUC 18. Selected recombinants were labelled with DIG-dUTP and used as probes in dot-hybridizations with total DNA from LD infected palms. The probes hybridized strongly to DNA from infected palms, but there was also a significant level of background hybridization to DNA from healthy palms. The second technique used oligonucleotide primers for conserved regions of the 16S rRNA gene and variable spacer regions between 16S and 23S rRNA genes in the polymerase chain reaction (PCR). Amplification of phytoplasma rDNA was primed from LD-infected palms in Tanzania, Kenya and Mozambique, and no amplification products were obtained from healthy coconut tissue

By use of these techniques infection could be reliably detected in the spear leaves and root tips of affected palms thereby avoiding destructive palm sampling. The pathogen was found in all meristematic tissues, with highest concentrations of phytoplasmas in the petioles of young unemerged leaves, the area below the growing point and the root tips in palms with moderately advanced disease symptoms. Root tips proved reliable for sampling when compared to spears, and are now recommended to be sampled together with the spears in routine, non-destructive sampling.

Phytoplasmas could be detected in symptomless palms one month before the onset of disease symptoms by use of DNA probes and two months before by PCR, when spear leaves were sampled monthly from 180 randomly selected palms for a year. Of the 24 palms which subsequently developed disease, LD was detectable in 25% prior to the onset of disease and in 46% at the time disease symptoms were visible. In 29% of these palms, phytoplasmas were not detected at all. No phytoplasmas were detected in any of the palms which remained healthy.

The genetic relatedness of the LD phytoplasma to twelve different non-coconut infecting phytoplasmas, two spiroplasmas, and phytoplasmas causing LYD in Kenya, Mozambique, Ghana, Florida and Jamaica were investigated. The LD DNA probe did not hybridize to any of the non-coconut infecting phytoplasmas and spiroplasmas. However, the probes detected a strong genetic relationship to all the LYD phytoplasmas. By use of PCR analyses, the phytoplasma causing LYD in Kenya was not found to differ from LD, but the pathogen causing LYD in Mozambique was found to be different. This appeared to be more closely related to the LYD phytoplasmas in West Africa.

Studies on auchenorrhynchous insects in LD infected coconut fields revealed a strong relationship between seasons and insect flight into the fields. They also showed that local environmental conditions have a strong influence on vector populations, and may be indirectly responsible for the differences in disease incidence observed in different regions of the country. The flight pattern of auchenorrynchous insects in general and of *Diastrombus mkurangai* in particular, coincided with the pattern of disease spread, implying that this species is the most probable vector of LD. A good correlation obtained when the disease incidence data was regressed on the numbers of *Diastrombus mkurangai* and *Meenoplus* spp, but not on the total number of trapped auchenorrhynchous insects provided additional evidence for implicating these species as vectors of LD.

More than 5000 individual insects were analysed by PCR in attempts to identify the insect vector or vectors for LD. PCR products of the right size were amplified from a few individuals of the species *Diastrombus mkurangai* and *Meenoplus* spp, and were shown to be LD phytoplasma by RFLP analysis.

The techniques have provided a quicker and more reliable means of detecting LD phytoplasmas in coconut tissues and in putative insect vectors than the conventional methods. Possible improvements on the techniques are suggested and the prospects of utilising them to find a sustainable method of disease control discussed.

Chapter 1: GENERAL INTRODUCTION

1.1 THE COCONUT PALM IN TANZANIA

Coconut palm (*Cocos nucifera* L.) is the most important perennial oil crop that supports the livelihood of most farmers in the coastal areas of Tanzania. It provides coconut water for drinking and food in the form of coconut meat and coconut milk. Fresh nuts, oil and copra are sold for cash income. Mature fronds are woven into thatching material for shelter. Coconut wood is converted into timber for furniture and other uses. Other products include baskets from leaves, mats and brushes from coir, shell flour from shells, and husks and shells are used for fuel. Nut production alone, which is essentially for subsistence and sale as fresh nuts, was estimated during the early 1980s to provide for about 40% of the total National vegetable oil supply (Agrar und Hydrotechnik GmbH, 1980). In addition to all these uses, coconut palms conserve the environment as an agroforestry crop. It is a tree of many uses, and as correctly stated (Ohler, 1984), "a tree of life" for the coastal communities.

A total of 22 million palms grow on 240,000 ha along the coastal belt of mainland Tanzania and the islands of Zanzibar, Pemba and Mafia. The crop is predominantly a small-holder one, with more than 95% of all palms being grown by farmers who own about 4 ha, and it is therefore of importance to the rural economy. However, coconut production and productivity has been on the decline since the early 1970s for various reasons. Some of the main reasons include old palm stands, slow rate of replanting, low genetic potential of the local East African Tall variety, poor management practices, and pests like the coconut bug (*Pseudotheraptus wayi* Brown), the rhinoceros beetle (*Oryctes monoceros* L.), the coconut mite (*Aceria guerreronis*), and termites (*Microtermes* spp). Moreover, a destructive lethal yellowing-like disease known as Lethal Disease (LD) has caused extensive damage to plantations on the mainland for more than 30 years, and is now present on the island of Mafia. This disease has been extensively investigated since 1979 when a project for promotion of the coconut industry in Tanzania (National Coconut Development Programme) was set up.

1.2 DISEASES OF THE COCONUT PALM IN TANZANIA

1.2.1 Minor Diseases

The coconut palm does not generally suffer from many diseases in Tanzania. Nursery diseases and diseases of young palms in the field are of localised importance. Among these are the leaf blotch and leaf spot fungal diseases caused respectively by *Helminthosporium palmarum* and *Cercospora* spp. These do not cause economic damage to mature palms, and on the susceptible young palms can be effectively controlled with fungicide sprays. Other nursery problems which can cause considerable, though localised losses, are the wet and dry bud rots. Wet bud rot or blast is characterised by a damp rot of the spear leaf which spreads to and kills the growing point. Subsequently all the leaves dry off. Dry bud rot occurs as a dry, woody rot of the spear leaf and the growing point. It causes halted growth and eventual death of the plant. The causal pathogens for these two diseases have not been identified. However, dry bud rot is known to be transmitted by two jassid species, *Nesodryas antiope* and *Sogatella kordofan (Homoptera- Delphacidae)*(Julia and Mariau, 1982), while the wet bud rot is transmitted by leafhoppers (*Recilia mica* Kramer) (Renard *et al*, 1982). Both diseases are controlled by application of systemic insecticides, but can also be effectively controlled by good nursery hygiene, which involves keeping surrounding areas free of weeds, where the insects breed.

A disease of young palms, that caused considerable losses of trees during the early 1970s, was the lethal bole rot caused by the fungus *Marasmiellus cocophilus* Pegler (Bock *et al.*, 1970). This disease is characterised by a primary dry red-brown rot of the bole, followed by frond wilt and rot of the crown and subsequent death. It was reported to affect seedlings and palms younger than 8 years, and was particularly damaging in Kisarawe and Kilwa districts to the south. It has not been encountered since 1979.

Physiological stress resulting from extended dry periods often manifests itself as disease-like symptoms (Schuiling and Mpunami, 1992). Symptoms include nutfall, drying up of open inflorescences, drying up of leaves with frequent breaking of the midribs, and drooping of the central leaves. Total recovery does occur after the rains, although some palms die completely during extended dry spells.

1.2.2 The Lethal Disease (LD)

The single most important disease of coconut palms in Tanzania is the lethal disease (LD). It was first reported affecting coconuts near Bagamoyo early in this century (Stein, 1905). The disease was then known as Herzfaule (heartrot). Further reports of the same disease followed in subsequent years at Saadani near Tanga, and Magogoni near Dar-es-Salaam respectively (Vosseler, 1907), as well at Ras Msimbati in the far south (Anonymous, 1913). The disease spread further as coconut plantations expanded until it reached epidemic proportions by 1960 (Schuiling *et al.*, 1992a). A map of the coastal regions of Tanzania showing the coconut growing areas and the distribution of lethal disease is in appendix 3.

LD is a destructive yellowing-type disease that has been associated with phytoplasmas or mycoplasma-like organisms (Schuiling *et al.*, 1981, Nienhaus *et al.*, 1982). Symptoms of the disease, reported by Schuiling *et al.* (1981), are: premature nutfall, bronzing of successively younger leaves, blackening of young emergent inflorescences, drying of older inflorescences, rot and collapse of the spear leaves, and decay of the root system, with subsequent sudden death. Plate 1.1 shows some of the typical symptoms of LD. Affected palms die within 4-6 months from the onset of symptoms. The disease affects palms at all ages, including transplants as young as 18 months.

Plate 1.1: Typical lethal disease symptoms on mature coconut palms in Tanzania. (A) The three palms in the foreground show an advanced stage of disease, and those in the background are healthy. (B) Necrosis and rot of the spear leaf (C) Flower sheath of the young unemerged inflorescence forced open to reveal blackening. (D) Standing dead poles left after the palm crown fall off.

Similar symptoms have been reported for other yellowing diseases of the coconut palm which are also associated with phytoplasmas. These include Lethal Yellowing (LY) in the Caribbean, Florida and Mexico (Plavsic-Banjac *et al.*, 1972; Parthasarathy, 1974; Thomas, 1979; Robert and Zizumbo, 1990), Cape St. Paul Wilt (CSPW) in Ghana (Johnson and Harries, 1976; Dabek *et al.*, 1976), Kaincope disease in Togo (Nienhaus and Steiner, 1976; Dabek *et al.*, 1976; Dollet and Gianotti, 1976), Kribi disease in Cameroon (Dollet *et al.*, 1977), and Awka or bronze leaf wilt in Nigeria (Bull, 1955; Ekpo and Ojomo, 1990). The yellowing diseases also occur in Kenya (Nienhaus, 1984) and Mozambique (Mpunami and Seguni, 1996).

LY has been known in the Caribbean for a long time, is quite widespread, and has been extensively investigated (Eden-Green, 1993). According to Howard (1983), LY first occurred on the Caribbean islands of Cayman in 1834. It next occurred more or less simultaneously on Jamaica, Cuba and Haiti during the late 1800s, and after some time exploded into epiphytotics in the separate localities. It eventually spread to the Dominican Republic, Bahamas, Florida, and Texas, and is now spreading rapidly in Mexico. It has caused extensive destruction to coconut plantations in the region, and also to other palm species in Florida where they are grown as ornamentals. All the other yellowing diseases which have occurred after LY, have been similarly destructive where they occur, and are collectively referred to (LD included) as lethal yellowing- type diseases (LYD) (Schuiling *et al.*, 1992a; Eden-Green, 1993).

Despite symptomatological similarities with LY, LD differs with respect to epidemiology, susceptibility of coconut varieties and insect vectors. Whereas LY is characterised by a 'rapid jump spread' pattern in the Caribbean (McCoy, 1976; Romney, 1983; Howard, 1983), jump spread is rare with LD (Schuiling *et al.*, 1992a). The vector of LY has been reported to be a cixiid planthopper, *Myndus crudus* van Duzee (Howard *et al.*, 1983), but this insect has not been found in association with coconuts in Tanzania (Schuiling and Mpunami, 1990). Furthermore, while some coconut varieties, such as the Malayan and Sri Lanka Green dwarfs are considered highly resistant to the Caribbean LY (Been, 1981), all are susceptible to

LD in Tanzania (Schuiling *et al.*, 1992b; Kullaya *et al.*, 1995). These differences have led to speculation that different pathogens might be involved for each disease (Schuiling *et al.*, 1992b), and this has recently been confirmed by genomic studies of the respective phytoplasmas (Harrison *et al.*, 1994a; Tymon, 1995).

LD occurs throughout the coastal belt of mainland Tanzania but not on the islands of Zanzibar and Pemba. Over the last thirty years this disease has destroyed nearly 40% of the coconut population (Schuiling *et al.*, 1992a). The incidence of disease differs significantly among the affected regions on the mainland. It is widespread in the southern regions, where it has killed about 56% of palms since 1965, while only 8.5% have been affected in the northern regions (Schuiling *et al.*, 1992a). These differences are difficult to reconcile because environmental conditions including moisture, temperature, soils, flora and insect fauna on palms appear to be similar. Schuiling *et al.* (1992a) investigated the history, distribution and epidemiology of LD and suggested that a possible explanation could be the genotypic differences within the local coconut population, in relation to the origin of the coconut groves. In the northern part of the country coconut has been grown for more than 150 years, and is a long established crop, presumably exposed to disease for a long time. This may have enabled an endemic balance to become established between the coconut and LD, with a reasonable level of resistance building up within the northern population. On the other hand, coconuts in the south are a fairly recent crop, having been planted after 1890, and all originated predominantly from one source, the neighbouring Mafia Island. Another possible explanation for the differences could be that different strains of the pathogen may be involved, or possibly different insect vectors.

Lack of a quick, specific and sensitive method for detection of the pathogen in the field has been the main factor limiting our understanding of the aetiology and epidemiology of LD, making it difficult to develop a sound control programme. Kaiza (1987) injected tetracycline into the trunks of LD-affected palms at different stages of disease progression, and demonstrated remission of symptoms in palms at a relatively early stage of disease. This provided evidence for the association of LD with a phytoplasma. Staining of LD-infected tissue with a fluorochrome, 4'-6-

diamidino 2-phenylindole 2HCl (DAPI) and a linking of the fluorescence in those tissues to the presence of phytoplasmas shown by electron microscopy (Deutsch and Nienhaus, 1983) confirmed the phytoplasmal actiology of LD. DAPI staining was subsequently adopted as a technique for field screening of LD. This technique has since been used routinely to identify infected palms (Schuiling and Mpunami, 1990). It cannot, however, identify the pathogen, and confirmation of infection still requires visual observation of phytoplasmas in infected tissues by electron microscopy. Although electron microscopy is a sensitive method for detecting LD phytoplasmas, the procedure is time consuming due to the very low concentration and uneven distribution of the phytoplasmas in palm tissue (Deutsch and Nienhaus, 1983). Furthermore, the electron microscope is very expensive to maintain, hence the technology is not sustainable.

1.3 THE PHYTOPLASMAS

1.3.1 Introduction

Phytoplasmas are non-culturable, pleomorphic, cell wall-less plant pathogenic prokaryotes. They are the causal agents of 'yellows type' diseases, formally thought to be of viral origin. They were first discovered to be plant disease causing pathogens in 1967 (Doi *et al.*, 1967). Since then, they have been found to cause disease in more than 300 plant species and in a number of arthropods (McCoy *et al.*, 1989). Phytoplasmas induce diverse disease symptoms in their hosts, which include leaf yellowing, leaf mottling, leaf dwarfing, leaf roll, flower dwarfing, flower virescence, witches'-broom, epinasty, wilting, internode shortening, stunting and decline (Nienhaus and Sikora, 1979; Bove, 1984; McCoy *et al.*, 1989). Despite the diverse nature of the symptoms, the phytoplasmas, have been grouped into two main types. The floral reversion type cause virescence (greening of floral tissues) and / or phyllody (leaflike petals and sepals) in their herbaceous hosts. The decline type do not induce floral reversion but cause a general decline of infected plants. Both groups, however, produce symptoms like stunting, chlorosis, shoot proliferation, and

TABLE 1.1 BIOLOGICAL CHARACTERISTICS OF SOME PLANT PATHOGENIC PHYTOPLASMAS

Pathogen	Host range		Disease symptoms	Disease group	Insect vector(s)	Cell morphology	Genome size	References
	Species	Commom name						
Lethal yellowing (LY)	*Cocos nucifera L.*	Coconut palm	Nutfall, yellowing of successively younger fronds, blackening of emergent inflorescences, rot of spear leaves and roots, sudden death	Decline	Planthopper, *Myndus crudus* van Duzee (*Homoptera: Cixiidae*)	Pleomorphic- spherical, ovoid, elliptical, and elongated filamentous bodies. Average dia: - 295 nm for nonfilaments, - 600-800 nm for ovoid forms - 142 nm for filamentous forms. Filamentous forms up to 2,000 nm long.	ND	Plavsic-Banjac *et al.*,1972; Parthasarathy, 1974; Thomas, 1979; Howard *et al.*, 1983; Howard, 1983; Harrison *et al.*, 1992, 1995. Waters & Hunt, 1980
	Veitchia merrilli	Manila palm						
	Phoenix dactylifera	Date palm						
	Phoenix canariensis	Canary Island date palm						
	Phoenix reclinata Jacq	Senegal date palm						
	Phoenix sylvestris L.	Silver date palm						
	Phoenix rupicola	Cliff date palm						
	Aiphanes lindeniana	-						
	Pritchardia pacifica	Fiji fan palm						
	Pritchardia affinis	Kona palm						
	Pritchardia thurstonii	Thurston palm						
	Trachycorpus fortunei	Windmill palm						
	Pandanus utilis Bory	Screw pine						
	Allagoptera arenaria	Seashore palm						
	Arenga engleri Becc	Dwarf sugar palm						
	Syagrus schizophylla	Arikury palm						
	Borassus flabellifer	Palmyra palm						
	Caryota mitis Lour.	Clustering fishtail palm						

Table 1.1 (cont.)

	Chrysalidorcarpus cabadae	Cabada palm							
	Corypha elata Roxb	Gedang palm							
	Caryota rumphiana	Giant fishtail palm							
	Dictyosperma album (Bory)	Princess palm							
	Gaussia attenuata	Puerto Rican gaussia palm							
	Howea belmoreana Becc.	Belmore sentry palm							
	Hyophorbe verschaffeltii	Spindle palm							
	Latania sp. Comm.	Latan palm							
	Livistona rotundifolia	Footstool palm							
	Chelyocarpus chuco	-							
	Neodypsis decaryi	Triangle palm							
	Naunorrhops ritchiana	Mazari palm							
	Veitchia montgomeryana	Montgomery palm							
	Veitchia macdanielsii	Sunshine palm							
	Veitchia arecina	Arecina palm							
	Ravenea hildebrantii	-							
Awka (bronze leaf wilt) - Nigeria	*Cocos nucifera*	Coconut palm	similar to LY	Decline	NK		ND	ND	Bull, 1955; Ekpo and Ojomo, 1990.

Table 1.1 (cont.)

Cape St. Paul wilt - Ghana and Keincope disease in Togo	*Cocos nucifera*	Coconut palm	similar to LY	Decline	NK	Pleomorphic- Spherical, 100-800 nm diam. Ovoid, 1000nm Filamentous forms, 15-90 nm diam, & up to 1500 nm long Beaded chains, and 'budding' forms also present.	ND	Johnson and Harries, 1976; Dabek *et al.*, 1976. Dabek, 1977; Dollet & Giannotti, 1976.
Kribi disease - Cameroon	*Cocos nucifera*	Coconut palm	similar to LY	Decline	NK	Pleomorphic- Spherical forms, 50-150 nm diam., Elongate forms, 800-1400 nm; Trefoil shapes with necks or 'budding'	ND	Dollet *et al.*, 1977
Lethal disease (LD) in Tanzania and Lethal yellowing- like diseases (LYD) in Kenya and Mozambique	*Cocos nucifera* L.	Coconut palm	Nutfall, bronzing of successively young leaves, blackening of inflorescences, rot of spear leaf and roots, sudden death	Decline	NK	Pleomorphic Spherical, Elongate and cylindrical bodies	ND	Schuiling *et al.*, 1981. Nienhaus *et al*, 1982. Deutsch and Nienhaus, 1983; Nienhaus, 1984, Mpunami and Seguni, 1996.

Table 1.1 (cont.)

Peach X-disease, Western - X	*Prunus persicae* *P. virginiana* *P. cerasus* L. *P. avium* L. *Apium graveolens* L.	Peach Choke cherry Sour cherry Sweet cherry Celery	Yellow - red blotches on leaves, early defoliation, no fruits on peach, small green cherries Sour cherry on mahaleb rootstock wilt and die	Decline	Leafhoppers, *Colladonus montanus, & Fiebriella florii* for western-X disease, and *Paraphlepsius irroratus, & Scaphytopius acutus* for Eastern (Canada)-X	Pleomorphic-Spherical and ovate bodies, 120-360 nm diameter, elongate cylindrical bodies, 200nm dia, and 720-3060 nm long. Max. 5400nm long	640 kbp	MacBeath *et al.*, 1972 Jones *et al.*, 1974; Granet and Gilmer, 1971; Kirkpatrick *et al.*, 1987; Lee *et al.*, 1992b; Lee and Davis, 1992; Neimark and Kikpatrick, 1993.
Witches,-Broom disease of lime	*Citrus aurantifolia* L (Swingle) *Catharanthus roseus*	Small-fruited acid lime Vinca	Witches'-brooms Leaf yellowing	Decline	Dodder , *Cuscuta campestris* Yunker (Susp)- Leafhopper, *Hishimonus phycitis*	Pleomorphic-	720 kbp	Bove, 1986; Bove *et al.*, 1988; Garneir *et al.*, 1991; Zreik *et al.*, 1995
Pigeon pea witches'-broom	*Cajanus cajan* *Catharanthus roseus*	Pigeon pea Periwinkle	Proliferation of shoots from axillary buds, leaf stunting, flower distortion, short internodes, witches'-broom, no fruit set.	Floral reversion	NK	Pleomorphic-Spherical to filamentous types, 200-800 nm, diameter.		McCoy *et al.*, 1983; Harrison *et al.*, 1991
Aster yellows - Severe western - Dwarf western - Eastern - Tule lake western - Oenothera virescence	*Callistephus chinensis* Nees *Lactuca sativa var. capitata* *Catharanthus roseus* *Nicotiana rustica* *Daucus carota* *Lycopersicon*	China aster Lettuce Vinca Tobacco Carrots Tomato	Spindling side shoots, chlorosis, witches'-brooms, long internode, dwarfed foliage, leaf distortion, phyllody, severe stunting,	Floral reversion	31 leafhopper species, including: *Macrosteles fascifrons* *Cicadula sexnotata* *Aphrodes bicinctus* *Macrosteles severini* *Circulifer tenellus*	Pleomorphic-	Severe AY - 1185 kbp Dwarf AY 1185 kbp T.lake AY	Markham, 1982b; Ahrens and Seemuller, 1992; Whitcomb and Black, 1982; Lee and Davis, 1992; Granados and Chapman, 1968

Table 1.1 (cont.)

- Beet leafhopper transmitted virescence	*esculentum* *Beta vulgaris L.* *Allium cepa* *Avena sativa* L. *Triticum aestivum* L *Trifolium spp* *C. coronarium* *Apium graveolens* L. *Medicago sativa* *Zinnia elegans* Jacq. *Oenothera hookeri*	Sugar beet Onion Oats Wheat Clover Chrysanthemum Celery Alfalfa/ Lucerne Zinnia Chinese cabbage Evening primrose	proliferation, flower dwarfing, virescence.				1185 kbp EAY-850 kbp OAY - 870 kbp BLTVA-675 kbp	Neimark and Kirkpatrick, 1993
Rice yellow dwarf	*Oryza sativa* L.	Rice	Chlorosis, leaf yellowing, dwarfed growth	Decline	Green rice leafhoppers *Nephotettix cincticeps* Uhler *N. virescens* Distant *N. nigropictus* Stal	Pleomorphic-	ND	Nakashima *et al.*, 1991 Nasu *et al.*, 1967
Maize bushy stunt	*Zea mays* L. *Zea mays L. subsp. Mexicana* Schrader	Corn Teosinte	Chlorosis of whorl leaves, old leaf tip reddening, Shoot proliferation, Severe stunting,	Decline	Corn leafhoppers, *Dalbulus maidis* De L. & Wolcott *D. elimatus* (Ball) *D. gelbus* De Long *D. guevarai* De Long *D. tripsacoides* *D. quinquenotatus* De L.	Pleomorphic	ND	Davis *et al.*, 1988b; Nault, 1980; Nault & Bradfute, 1979; Madden & Nault, 1983; Madden *et al.*, 1984

Table 1.1 (cont.)

					D. longulus De Long *Balbulus tripsaci* *Graminiella nigrifrons*			Nault *et al.*, 1984
Elm yellows	*Ulmus americana* L. *U. rubra* Muhl. *U. alata* Michx. *U. serotina* Sarg. *U. crassifolia* Nutt. *U. parvifolia* Jacq. *U. minor* Mill. *U. laevis* Pall. *Catharanthus roseus*	N. American elm Red elm Winged elm - - Chinese elm Europ. field elm Europ. white elm Vinca	Defoliation, phloem necrosis, chlorosis, and death of American elms. -Same symptoms plus witches,-broom in red elms. -Witches'-brooms and stunting in European elms. -Chlorosis, stunting and epinasty in vinca.	Decline	White-banded elm leafhopper, *Scaphoideus luteolus*	Pleomorphic-Spherical and oval, 200-1,000 nm, diameter, filamentous forms up to 2200 nm long	ND	Braun and Sinclair, 1976, 1979; Baker, 1949; Maurer *et al.*, 1993 Wilson *et al.*, 1972.
Ash yellows	*Fraxinus americana* L *F. pennsylvanica* Marsh. *F. nigra* Marsh. *F. quadrangulata* Michx. *F. berlandieriana* *F. americana* X *F.quadrangulata* *Catharanthus roseus* *Daucus carota* 35 lilac taxa, e.g.	White ash Green ash Black ash Blue ash Arizona ash Hybrid ash Vinca Carrots	Slow growth, witches'-brooms, chlorosis, dieback, deliquescent branching, basal epicormic sprouts, clumped foliage on shoots (abortive) premature death.	Decline	Dodder species, *Cuscuta subinclusa* Dur. & Hilg. *Cuscuta campestris* Yunker	Pleomorphic-Elongated, ovoid, and spherical forms predominant. Few filamentous forms. Spherical forms 100-300 nm diam. Filaments up to 1000 nm long.	ND	Sinclair *et al.*, 1989; Matteoni and Sinclair, 1985; Hibben & Wolanski, 1971; Hibben *et al.*, 1991

Table 1.1 (cont.)

	Syringa microphylla *Syringa tomentella* *S. villosa* *S. vulgaris*					Elementary bodies, 100-200nm diam.		
Blueberry stunt	*Vaccinium corymbosum* L	Highbush blueberry	Malformed and sterile floral parts, witches'-brooms, small mottled leaves stunted growth.	Floral reversion	Sharp-nosed leafhopper *Scaphytopius magdalensis* Prov	Pleomorphic-Spherical to oval, 160-700nm, daim.	ND	Chen, 1971; Schaper & Converse, 1985; Chiykowski, 1981 Lee and Davis, 1992;
Clover yellow edge	*Trifolium pratense* *T. repens*	Red clover Ladino clover	Chlorosis, leaf yellowing	Decline	Leafhoppers, *Aphrodes bicinctus* Schrank *Paraphlepsius irroratus*	Pleomorphic	ND	Lee & Davis, 1992

ND, Not determined; NK, Not known; Susp, Suspected vector

The phytoplasmas resemble the non-helical animal mycoplasmas both morphologically and ultrastructurally (hence the old trivial name mycoplasma-like) by their small size, generally rounded and pleomorphic shape, lack of a cell wall, and being bound by only a plasma membrane (Nienhaus and Sikora, 1979; Bove, 1984; Lee and Davis, 1992). These characteristics form the basis for grouping phytoplasmas in the class *Mollicutes*, a distinct group of wall-less prokaryotes that is phylogenetically related to Gram-positive bacteria (Whitcomb and Black, 1982; Razin, 1985; Weisburg *et al.*, 1989). Another characteristic feature of phytoplasmas which groups them with *Mollicutes* is possession of a very small genome (600-1050 kbp) (Lim and Sears, 1989; Neimark and Kirkpatrick, 1993; Zreik *et al.*, 1995), and a low content of guanine and cytosine (G + C) nucleotides, ranging between 23-30 mol %. (Sears *et al.*, 1989; Lim and Sears, 1991; Tully, 1993).

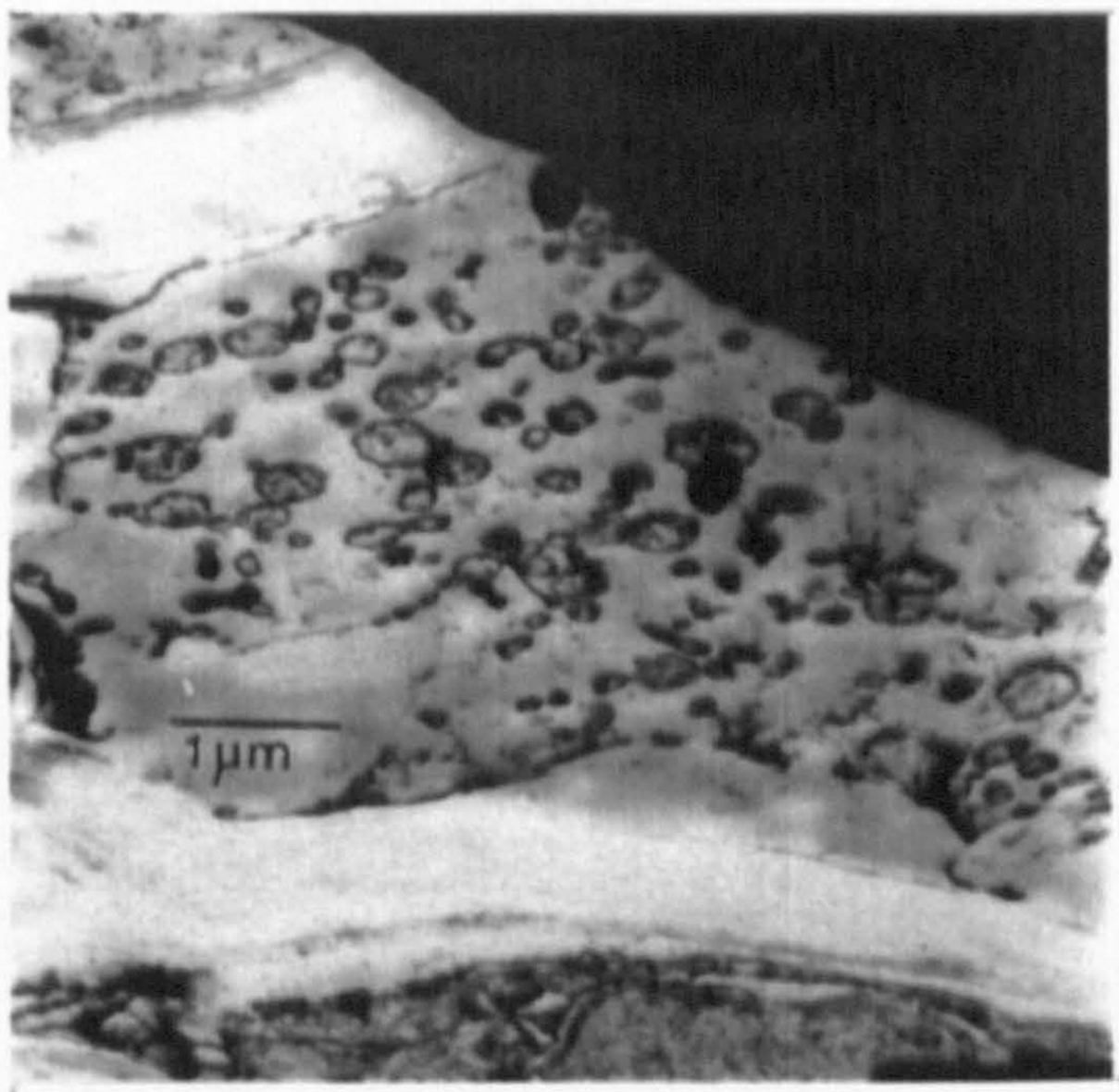
1.3.2 Morphology, Ultrastructure, and Localisation of Phytoplasmas in Infected Tissue

Phytoplasmas appear in different forms when observed in ultrathin sections of infected tissue under the electron microscope. They occur either as spherical, ovoid, large globular, or filamentous bodies inside the host cell (Maramorosch *et al.*, 1970; Davis and Whitcomb, 1971; Dabek *et al.*, 1976; Dollet *et al.*, 1977; Nienhaus and Sikora, 1979; Plate 1.2). The proportions of these bodies varies depending on the disease, but generally many of them are spherical (Davis and Whitcomb, 1971). In addition, small dense bodies 50-100 nm in diameter, and referred to as elementary bodies, are often seen. These are presumed to be packed with ribosomes, as a possible explanation for their granular appearance. Spherical bodies range between 50-1000 nm diameter, with intermediate bodies of 350-500 nm diameter comprising the modal class. The large, globular bodies are up to 1100 nm in diameter and transparent. Filamentous bodies have been found in sieve tubes of plants, but seldom occur in insects. These range between 46-300 nm diameter, and may be as long as 2000 nm. In some cases, they have been seen migrating through sieve pores on the sieve plates (Maramorosch *et al.*, 1970; Davis and Whitcomb, 1971). In the sieve tubes of palms, cells of the lethal yellowing phytoplasma have been seen to range

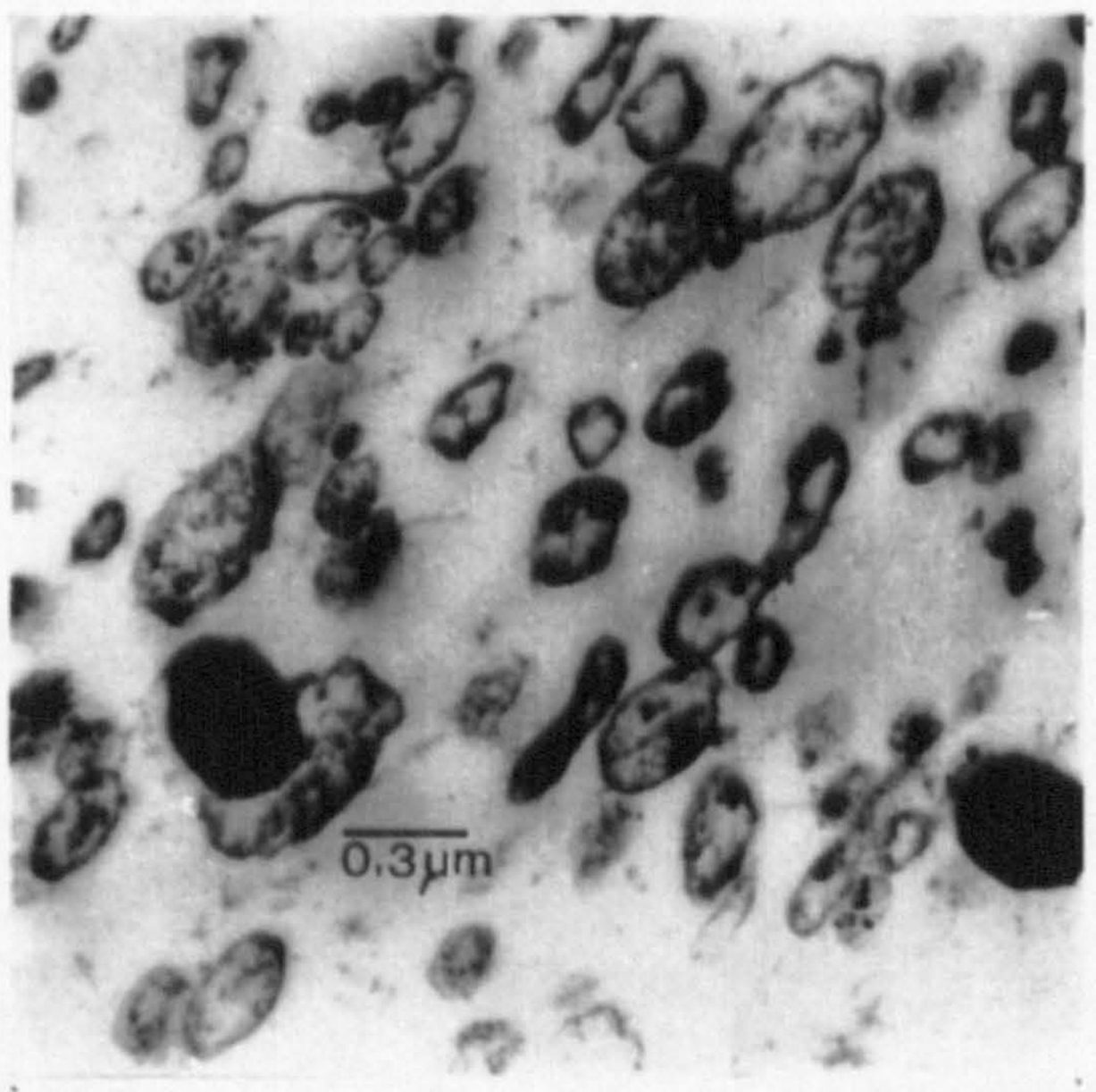
between 142-295 nm diameter and 1000-1600 nm length (Waters and Hunt, 1980). The large variation in sizes within the different forms may be influenced by methods used to prepare tissue for electron microscopy. The phytoplasmas change structurally in form as the disease in the plant progresses (Nienhaus and Sikora, 1979). They occasionally reveal characteristics like budding and binary fission (dumb-bell shape), and sometimes show chains of small spherical bodies (Dabek *et al.*, 1976; Dollet *et al.*, 1977). It is speculated that these formations represent some mechanism of multiplication inside the host cells.

Similar to mycoplasmas, each phytoplasma cell is bound by a single, 7-12 nm thick membrane, which appear as two electron dense layers with a lucent layer in-between. The central core of a phytoplasma cell contains fibrillar, presumably DNA material, that condenses when the tissue is fixed (Plate 1.2). RNA-containing ribosome granules are either scattered throughout the whole cell for small spherical bodies, or clustered about the peripheral zone of the large globular bodies (Maramorosch *et al.*, 1970; Davis and Whitcomb, 1971). With a diameter of 10-15 nm, phytoplasma ribosomes are smaller than host ribosomes, and therefore readily identified. Vacuoles, which are only occasionally found in filamentous bodies, are also frequently encountered in the large globular bodies (Nienhaus and Sikora, 1979). Membrane-bound inclusion bodies have been described in the cytoplasm of some phytoplasmas, but these have been shown to be caused by sectioning through two closely positioned organisms (Chen *et al.*, 1989).

Phytoplasmas are confined to the sieve elements of the phloem in plants in which they cause disease. The number of phytoplasmas in ultrathin sections can reach densities of 100 per cell. In such cases the infected cells are completely filled with the organisms. In the insect vectors, phytoplasmas have been found in the salivary glands, alimentary canal, haemolymph, fat body cells, malpighian tubules, brain and the ventral ganglia (Maramorosch *et al.*, 1970; Davis and Whitcomb, 1971).



A



B

Plate 1.2: Electron micrograph (A) showing lethal disease phytoplasma cells inside an infected phloem sieve cell, and a magnified portion of the phloem cell (B) showing the morphological features of the phytoplasma cells. Cells are pleomorphic and each is bound by a single plasma membrane (photographed by Nienhaus *et al.*, 1982).

1.3.3 Phytoplasma Transmission and Pathogenicity

The natural transmission of phytoplasma from plant to plant is either by insect or dodder. Arthropods are the most important vectors. All known vector species belong exclusively to the order *Homoptera*, and predominantly, to the suborder *Auchenorrynchia* (leafhoppers, planthoppers, froghoppers). Other vectors belong to suborder *Sternorrhyncha* (aphids and psyllids), and specifically to psyllids. No aphid vectors of phytoplasma are known (Purcell, 1982; Markham, 1982b). Even within the two suborders, transmission of phytoplasmas seem to be a specialised function of only a few species. About 150 species of *Auchenorrynchia*, the majority of which are leafhoppers (family *Cicadellidae*), as well as some planthoppers (Fulgorids) and froghoppers (Cercopids) have been implicated as phytoplasma vectors (Nielson, 1979). The cixiid planthopper, *Myndus crudus* van Duzee, the vector of coconut lethal yellowing (Howard *et al.*, 1983) is one example of non-leafhopper vectors. Within the suborder *Sternorrhyncha*, only a few species of psyllids have been shown to be phytoplasma vectors (Nienhaus and Sikora, 1979; Purcell, 1982).

The vectors, whose mouthparts are adapted for piercing and sucking without causing much damage, acquire the phytoplasmas from phloem tissue during feeding. The acquisition period in leafhoppers varies between 3 h and 5 days. The phytoplasmas then circulate and multiply inside the insect (incubate) before being transmitted to other plants during feeding. The length of incubation period varies, depending on the pathogen, the vector, temperature, and many other factors, but is usually between two and six weeks (Markham, 1982b). Long incubation periods have been reported and are related to phytoplasma migration in the vector or to the influence of environmental conditions. In a review on multiplication of phytoplasma in leafhoppers, Maramorosch *et al.* (1970) indicated that these organisms systemically invade and multiply in various organs of the vectors. The phytoplasmas have been detected extra- and intracellularly in a number of vector organs, including the salivary glands, alimentary canal, adipose tissue, brain, ventral ganglia and the haemolymph. However, it is in the salivary glands where they multiply to fairly high concentrations, and from where they get injected into plants when the insect feeds (Purcell, 1982).

Transmission by grafting, and through the parasitic seed plant, dodder (*Cuscuta spp)* are alternative methods for phytoplasma transmission (Maramorosch *et al.*, 1970; McCoy *et al.*, 1989). Grafting of symptomatic material to healthy tissue, and subsequent development of symptoms in the latter, have been used in some plant species to implicate phytoplasmas as possible disease pathogens, and for propagation of these organisms in the propagation host; usually periwinkle (*Catharanthus roseus*) (McCoy *et al.*, 1989). This type of transmission has been used for multiplication of phytoplasma in periwinkle for purposes of purification, because the organisms can multiply in periwinkle to high numbers. Similarly, transmission of phytoplasmas to periwinkle has been useful for comparison of types of symptoms induced by various phytoplasmas (Garnier *et al.*, 1991). Dodder has been particularly useful for transmission of phytoplasmas from woody to herbaceous hosts because of its ability to form vascular connections between taxonomically disparate plant species. Maramorosch *et al.* (1970) observed that during the transmission process, the phytoplasmas may multiply in the *Cuscuta* tissue and cause development of disease symptoms. In such instances *Cuscuta* serves as an alternate host, rather than a passive transmission agent.

Mechanical transmission has been unsuccessful, because of the extreme fragility of the phytoplasma membrane during extraction (Nienhaus and Sikora, 1979). There has been no recorded case of seed transmission of any phytoplasma although Harrison *et al.* (1995) have shown their presence in coconut embryos.

1.3.4 Phytoplasma Taxonomy

Taxonomy strives to find genetic characters that allow inference of evolutionary relationships among organisms (Macleans *et al.*, 1993). Based on such characters, the phytoplasmas have been grouped in the class *Mollicutes*, which belong to the division *Tenericutes* (wall-less bacteria), in the *Prokaryote* kingdom (Razin, 1989). The taxonomic position of phytoplasmas within the class *Mollicutes* has not been resolved yet, because these organisms have been difficult to characterise. However, the description and eventual taxonomic placement of new unclassified organisms will

provide not only new insights into the systematics of wall-less prokaryotes, but will also contribute fundamental knowledge of the biology, ecology, and host range of the class as a whole (Tully, 1989).

In any classification system, the most basic taxonomic unit or rank is the species. One strain of a species must be established as the "type strain" and this organism functions as a reference culture of properties described for the species name. According to the regulations in the Bacteriological Code (Lapage *et al.*, 1975), a type strain of a new species must be deposited in a recognised culture collection; and for the epithet of the named species to be accepted, a description of the properties of the organism must be validly published.

Until recently, a formal classification of phytoplasmas had not been established. Conventional methods of taxonomy which were used to classify other related *Mollicutes* were based on phenotypic characters, including morphological, biochemical and physiological traits, such as cell size and shape, genome size and composition, nutritional requirements, and serological relationships. Classification of phytoplasmas by such a standard approach has been hampered by the inability to culture them *in vitro*. However, various classification systems have been produced based on such characteristics as disease symptoms, plant host range, and specificity of pathogen transmission by insect vectors.

Identification and classification of phytoplasmas based on disease symptoms relies on their ability to interact with their hosts producing characteristic syndromes (McCoy *et al.*, 1989; Lee and Davis, 1992). Induction of 'yellows' type symptoms in the host is one typical feature of phytoplasmas, and based on this they have been grouped into two main types, those causing floral reversion and the decline type (section 1.3.1). Generally, symptoms alone cannot distinguish between phytoplasma types because some of them are able to induce both types of symptoms in different hosts. In addition, the host range of some phytoplasma diseases are not known due to the difficulty of determining the vectors and the transmission characteristics. Thus, classification based on symptoms alone has not been reliable. However, there are

exceptions. For example, Lee and Davis (1992) noted that some phytoplasma diseases in N. America have been grouped into two mutually exclusive categories based on flower symptoms; the floral reversion type (eastern aster yellows, western aster yellows, clover phyllody, clover proliferation), and those inducing reduction of flower size and colour (peach X-disease, clover yellow edge). Genomic classification of these phytoplasmas based on dot hybridizations with DNA probes have also produced the same categories, except that clover proliferation is genomically different from other members in the group. This has led to the conclusion that use of a combination of characteristic symptoms and genetic studies in the classification of phytoplasmas might lead to identification of genes responsible for inducing symptoms.

Host range and specificity of transmission by particular insect vectors are other biological factors which have been used traditionally to identify and classify phytoplasmas (Lee and Davis, 1992). Host range alone fails to provide unique taxonomic information for classification purposes when the phytoplasmas induce similar disease symptoms in similar hosts. An example is provided by a disease complex involving aster yellows (AY), Canadian clover phyllody, and beet leafhopper transmitted virescence (BLTVA). These organisms have been distinguished by use of both hosts and vector relationships, but not hosts alone. BLTVA has been distinguished from AY because it is transmitted only by the beet leafhopper (*Circulifer tenellus*), but not by the AY vector, *Macrosteles fascifrons* (McCoy *et al.*, 1989). Similarly, LD of coconuts in Tanzania has been provisionally differentiated from LY in the Caribbean and Florida, due to differences in host range and insect vectors (Schuiling *et al.*, 1992b), and the difference has been verified by genomic analysis of the 16S rRNA gene of the two causal organisms (Harrison *et al.*, 1994a).

The development of specific and sensitive classification procedures such as serological techniques and nucleic acid hybridizations has been delayed by difficulties in obtaining both antigens and DNA of these nonculturable pathogens. In recent years, assays using specific antibodies and DNA hybridisation probes have provided rapid and reliable means of phytoplasma classification (Chen *et al.*, 1992b; Lee and

Davis, 1992). Production of antiserum to partially purified immunogens has been of limited value for determining serological relationships due to high background resulting from contaminating host antigens (Clark *et al.*, 1983; Sinha and Benhamou, 1983). However, with the advent of hybridoma technology, specific monoclonal antibodies have been produced which can distinguish between different phytoplasma strains, and determine serologically related groups (Lin and Chen, 1985; Chen and Jiang, 1988; Clark *et al.*, 1989). Due to the high specificity of monoclonal antibodies, they can only be used to differentiate closely related strains, and this limits their usefulness for taxonomical purposes.

Since development of the first cloned phytoplasma DNA probes (Kirkpatrick *et al.*, 1987), rapid progress has been made in the use of molecular methods for phytoplasma classification. Pioneering work on classification has been based on the use of dot hybridizations with cloned DNA probes to group phytoplasmas into a system of genomic clusters. By use of this technique, clusters of closely related phytoplasma strains have been established, including the aster yellows cluster (Lee and Davis, 1988; Davis *et al.*, 1990), apple proliferation cluster (Bonnet *et al.*, 1990), ash yellows cluster (Davis *et al.*, 1992), clover proliferation cluster (Lee *et al.*, 1991), X-disease cluster (Lee *et al.*, 1992b), and coconut lethal yellowing cluster (Harrison *et al.*, 1994a). Each cluster consists of phytoplasma strains that share extensive sequence homology with one another and are distinct from strains in other strain clusters.

Differentiation amongst members of the same cluster into strains has been achieved through restriction fragment length polymorphism (RFLP) analysis of phytoplasma chromosomal DNA by using specific DNA probes to type strains. For example, clover proliferation and potato witches'-broom phytoplasmas have been shown to be closely related strains of the same phytoplasma, but differentiated from each other using DNA probes to the former (Lee *et al.*, 1991). Similarly, members of the aster yellows strain cluster have been classified as either Type I, Type II, or Type III by use of probes to the Maryland aster yellows strain (Lee *et al.*, 1992a). Furthermore, probes to severe strain of western aster yellows have been used to differentiate

virescence inducing phytoplasmas (Kuske *et al.*, 1991a); and the X-disease phytoplasmas have been differentiated using probes to the peach-X and Western-X phytoplasmas respectively (Lee *et al.*, 1992b).

One limitation of serological and nucleic acid hybridizations using randomly cloned DNA fragments for classification has been their inability to reveal phylogenetic or taxonomic positions of phytoplasmas in relation to each other, and to other micro-organisms (Seemuller *et al.*, 1994). A new approach to phytoplasma classification that is now accepted has been based on analysis of the highly conserved genes coding for the 16S rRNA, and the spacer region between 16S and 23S rRNA (Schneider *et al.*, 1993; Lee *et al.*, 1993a; Seemuller *et al.*, 1994; Kirkpatrick *et al.*, 1994a). According to Razin (1989) ribosomal RNAs are preferred for purposes of *Mollicute* classification because they are present in all organisms (highly conserved), and they serve the same functions in all organisms, which implies that they have undergone the same selection pressure. Furthermore, their primary structure is known to change slowly with time, so they keep a good record of phylogenetic changes over long genealogical times. Most important of all, they can be easily isolated and sequenced. The 16S rRNA gene also has the advantage of possessing both conserved and variable regions which can be used for phylogenetic and taxonomic classifications at various levels, including intrageneric differentiation (Seemuller *et al.*, 1994).

The ability to rapidly amplify and analyse phytoplasma 16S rRNA genes using the polymerase chain reaction (PCR) has resulted in the identification of several major taxonomic groups. By using a primer pair based on the 16S rRNA gene, Lee *et al.* (1993a) have amplified about 80% of the gene sequence of 40 different phytoplasma strains from different continents. The amplified sequences have been compared by RFLP analyses and used to classify the phytoplasmas into distinct 16S ribosomal RNA (16Sr) groups and subgroups (Table 1.2). The outstanding result from this classification system is that some of the 16Sr groups match exactly the groups delineated in DNA hybridisation assays using cloned chromosomal DNA probes.

Table 1.2 Classification of phytoplasmas

Common name	16S rRNA group and sub-group[a]	Strain cluster[b]
	Group I	**Aster yellows**
Tomato big bud	16SrI-A	Type (I)
Western aster yellows	16SrI-A	
Periwinkle little leaf	16SrI-A	
Eastern aster yellows (Canada)	16SrI-A	
Maryland aster yellows (AY1)	16SrI-B	Type (II)
Western dwarf aster yellows (DAY)	16SrI-B	
Western severe aster yellows (SAY)	16SrI-B	
Tulelake aster yellows (TLAY)	16SrI-B	
Chrysanthemum yellows	16SrI-B	
Ipomea obscura witches'-broom	16SrI-B	
Hydrangea phyllody	16SrI-B	Undesignated
Clover phyllody	16SrI-C	Type (III)
Paulownia witches'broom	16SrI-D	Undesignated
Blueberry stunt	16SrI-E	Undesgnated
	Group II	
Peanut witches'-broom	16SrII	-
UDI	16SrII	-
	Group III	**Peach X-disease**
Canada peach X-disease	16SrIII-A	Peach X (I)
Western X-disease	16SrIII-A	Peach X (II)
Clover yellow edge	16SrIII-B	Peach X (III)
	Group IV	**Lethal yellowing-type**
Coconut lethal yellowing	16SrIV	LYD
Coconut lethal disease	16SrIV	LYD
Cape St Paul wilt	16SrIV	LYD
Awka wilt	16SrIV	LYD
	Group V	**Elm yellows**
American elm yellows	16SrV	
European elm yellows	16SrV	
	Group VI	**Clover proliferation**
Clover proliferation	16SrVI	
Potato witches'-broom	16SrVI	
Beet leafhopper transmitted viresc.	16SrIVI	
	Group VII	**Ash yellows**
Ash yellows	16SrVII	
	Group VIII	
Loofah witches'-broom	16SrVIII	-
	Group IX	-
Pigeon pea witches'-broom	16SrIX	
	Group X	-
Apple proliferation	16SrX	

[a] Based on restriction fragment length polymorphisms (RFLP) analyses of their rDNA sequence (Lee *et al.*, 1993a), or [b] hybridizations using cloned phytoplasma DNA.

In concurrent but independent studies, Schneider *et al.* (1993) have also used PCR and RFLP analyses to amplify and study the 16S rDNA from 52 other phytoplasma isolates. Their proposed system of classification is similar to that of Lee *et al.* (1993a), but they have also shown that most of the phytoplasmas pathogenic to herbaceous plants cluster together, separately from the cluster formed by those infecting woody hosts.

Seemuller *et al.* (1994) have recently sequenced the 16S rDNAs from phytoplasmas representing many of the groups and subgroups proposed by Schneider *et al.* (1993), and revealed that the DNA sequences show a classification pattern similar to that shown by RFLP of PCR products. The sequence data has also shown that all the phytoplasmas examined belong to one homogenous group that evolved monophyletically from a common ancestor. In another study, Gundersen *et al.* (1994) have investigated phylogenetic interrelationships among phytoplasmas and their relationships to other mollicutes by sequencing and comparing the 16S rDNA. Their conclusions are similar to those of Seemuller *et al.* (1994). Sequencing and comparisons of the 16S / 23S spacer regions of various phytoplasmas (Kirkpatrick *et al.*, 1994b) has also produced a classification pattern which is similar to that based on the sequence of the 16S ribosomal gene. All these studies have contributed to the framework upon which the current system of phytoplasma classification is based.

The molecular techniques involving both DNA amplification and sequence analysis have provided confirmatory evidence that phytoplasmas belong to the class *Mollicutes*. Comparison of 16S rRNA gene sequences of different phytoplasmas and other *Mollicutes*, has revealed that they are more closely related to *Acholeplasma laidlawii* and to other *Mollicutes* in the genus *Anaeroplasma* than to the phytopathogenic *Spiroplasma* species, or to the vertebrate-associated *Mycoplasmas* (Lim and Sears, 1989; Kuske and Kirkpatrick, 1992a; Namba *et al.*, 1993b; Gundersen *et al.*, 1994; Seemuller *et al.*, 1994; Zreik *et al.*, 1995). From these comparisons, phylogenetic trees which show the level of relatedness of selected phytoplasmas to other members of class *Mollicutes*, and establish their phylogenetic positions in the class have been constructed. A sample phylogenetic tree of some

phytoplasmas and related *Mollicutes* is shown in Fig.1.1.

Similar to the 16S ribosomal gene sequences, comparisons of the 16S/23S spacer region sequences have demonstrated that phytoplasmas are phylogenetically more related to the *Acholeplasma / Anaeroplasma* group than to *Spiroplasmas* and *Mycoplasmas* (Kirkpatrick *et al.*, 1994b). Sequence data from the two conserved ribosomal protein genes (rp122 and rps3) (Lim and Sears, 1992), and the characteristics of the phytoplasma cell membranes (Lim *et al.*, 1992) have also confirmed this phylogeny. It has been based on the apparent evolutionary distance of these organisms from other *Mollicutes*, coupled with their phytopathogenic properties and their habitat in the plant phloem sieve tubes, that they are considered not to be mycoplasmas, or mycoplasma-like organisms (MLO), but a distinct group, the *Phytoplasmas* (Tully, 1993).

1.3.5 Detection and Diagnosis

Diseases caused by phytoplasmas have been routinely diagnosed by host range and symptomatology because these organisms produce characteristic symptoms in their hosts (Section 1.3.1). Symptom remission following tetracycline treatment has been considered essential in proving that these organisms cause the plant disease being investigated (Nienhaus and Sikora, 1979). This approach has been used to confirm the phytoplasmal aetiology of coconut diseases, including LD (Kaiza, 1987), Kaincope disease (Steiner, 1976b), and LY (McCoy, 1972).

Until recently, host range, and transmission to indicator hosts by dodder, graft, or insect vector, were used for the detection of phytoplasmas. Such tests were complemented by observation of the phytoplasmas in ultrathin-sections of infected phloem tissue in the electron microscope (Maramorosch *et al.*, 1970; Nienhaus and Sikora, 1979; McCoy *et al.*, 1989; Lee and Davis, 1992; Hansen and Wick, 1993).

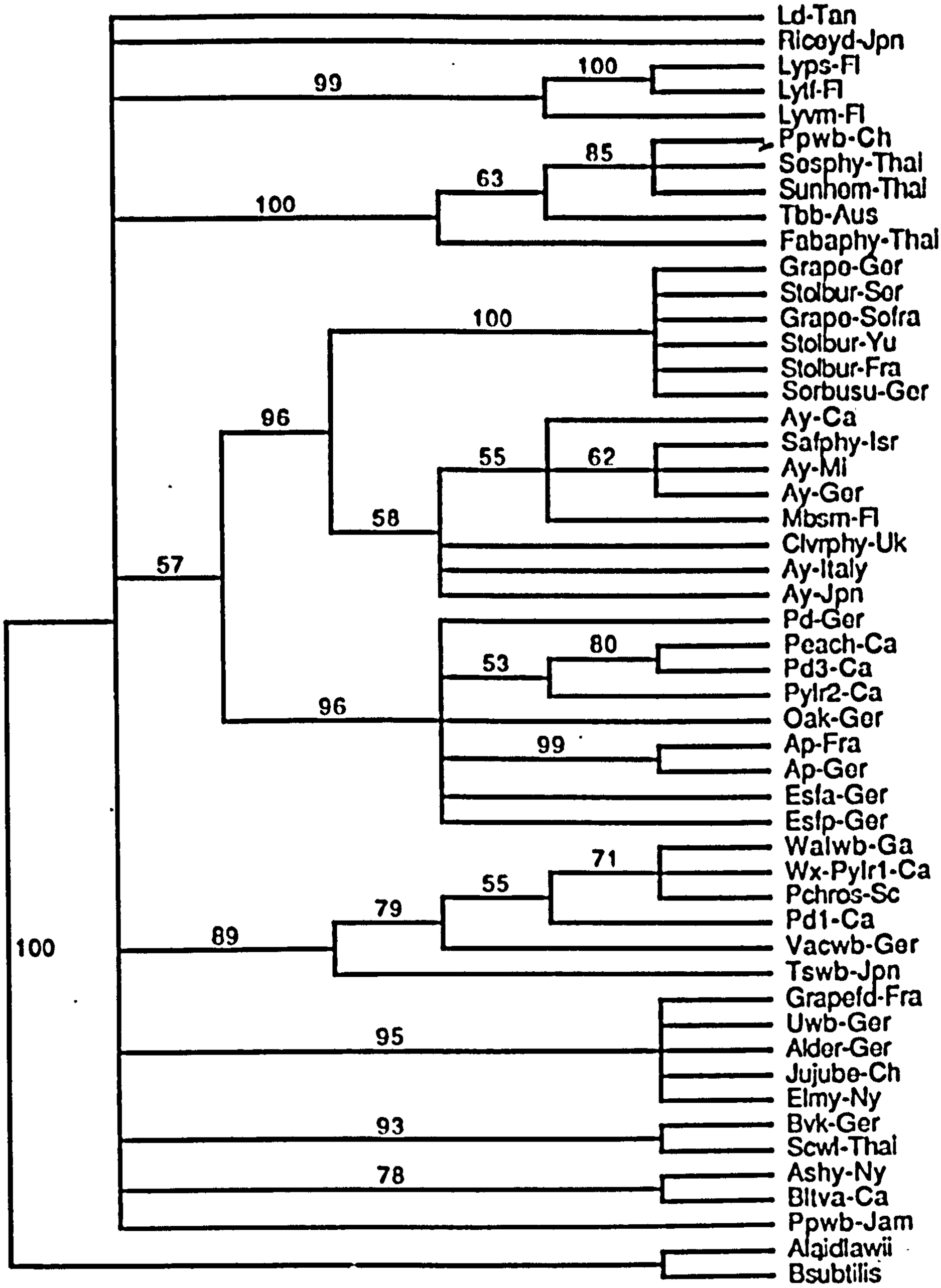


Figure 1.1: Phylogenetic tree of phytoplasmas from diverse geographical regions based on analysis of the spacer region sequences between 16S / 23S ribosomal RNA genes. The cladogram was generated by Kirkpatrick *et al.*, 1994. Individual taxa are labelled on the right hand side and are followed by the location where the isolate was collected. *A. laidlawii* and *B. subtilis* were used as outgroups. Numbers on the tree indicate the number of times the taxa formed the same clade after random addition of all taxa 100 times.

However, these techniques are not practical for rapid diagnosis. Preparation of samples for the electron microscope is time consuming, and only a small amount of tissue can be studied in thin sections. False negatives are more likely to occur because the organisms are not distributed uniformly in the host tissue. In addition, the cost of purchasing and maintaining the electron microscope limits its use in many developing countries. Furthermore, electron microscopy cannot differentiate between phytoplasmas in mixed infections because under the microscope all phytoplasmas look exactly the same inside the sieve cells of infected plants (Jones *et al.*, 1995).

Though not commonly used, light (optical) microscopy has been successfully used to detect phytoplasma in infected tissue after staining free-hand or microtome sections with the chromatic Dienes' stain (Deeley *et al.*, 1979; Matteoni and Sinclair, 1985). Phytoplasma infections are normally associated with accumulation of these organisms in the phloem sieve tube elements of their hosts. Staining the sections makes the cell contents readily visible, and enhances detection of the phytoplasmas inside the phloem sieve cells, on observation in a compound microscope.

Fluorescence microscopy, using the DNA-specific stain, DAPI (4'-6'-diamidino-2-phenylindole-2HCl is also useful for detecting phytoplasmas. This stain binds to DNA from any organism, and fluoresces under UV radiation (Russel *et al.*, 1975). Pieces of disease-infected tissue containing phloem vessels are fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7, sectioned longitudinally either by hand or by microtome, stained in a solution of DAPI (2-4 $\mu g\ ml^{-1}$ DAPI in 0.1 M phosphate buffer, pH 7) for 30 min, and examined with an epifluorescence microscope. By use of an exciter filter allowing peak transmission at 360 nm and a barrier filter with a short-wave cut-off at 420 nm on the microscope, the stained phytoplasma cells and cell nuclei emit a blue-white fluorescence. Differences in location and intensity of fluorescing spots allows for distinguishing between host DNA and the phytoplasma DNA, because the latter only accumulate inside the phloem sieve cells.

The DAPI technique has been used for detection of several phytoplasmas including

the agents for LD (Deutsch and Nienhaus, 1983; Schuiling and Mpunami, 1990), aster yellows, clover proliferation, and potato witches'-broom (Hiruki and da Rocha, 1986). Other diseases include blueberry stunt disease (Schaper and Converse, 1985), ash yellows (Sinclair *et al.*, 1990), and Parry's disease of pear (Davies *et al.*, 1992). Schaper and Seemuller (1982) have also used the DAPI technique to detect phytoplasmas in apple and pear trees infected by apple proliferation and pear decline respectively. They observed degeneration of sieve tubes during fall and winter months in association with complete disappearance of phytoplasmas from stems of affected trees, while roots retained these organisms. This led to the conclusion that phytoplasmas survive the adverse winter conditions in the roots and spread to shoots during the following spring. Schaper and Converse (1985) have observed a similar tendency in blueberries, and emphasised the importance of assaying roots rather than shoot and petiole samples because the phytoplasmas often colonised roots while the aerial parts were still symptomless.

The main disadvantage of optical and fluorescence microscopic methods is that, like electron microscopy, they cannot specifically identify the pathogen. Therefore, their use is restricted to investigating known phytoplasmas, or generally associating a disease with a phytoplasma infection.

Serological and nucleic acid techniques which have been recently developed for the detection of phytoplasmas have made their detection more realistic (Hansen and Wick, 1993). Assays using specific antibodies and DNA hybridisation probes have provided rapid and reliable means of phytoplasma detection and diagnosis (Chen *et al.*, 1992b; Lee and Davis, 1992).

The production of polyclonal and monoclonal antibodies against phytoplasma immunogens has facilitated sensitive detection of these organisms in infected hosts. However, polyclonal antibodies have been of limited use due to the problems of low titer and cross-reaction with antigens to healthy plants (Clark *et al.*, 1983; Sinha and Binhamou, 1983; Lin and Chen, 1986). These problems have been overcome with the production of monoclonal antibodies (mAbs). mAbs have been produced to

several phytoplasmas and their sensitivity and specificity ascertained (Chen and Jiang, 1988; Clark *et al.*, 1989; Jiang *et al.*, 1989; Lin and Chen, 1985; 1986). The mAbs which are produced by the hybridoma techniques, are highly specific and sensitive, because each react only to one epitope of the selected antigen. They have, therefore, been used successfully in the detection and differentiation of closely related phytoplasma strains (Clark *et al.*, 1989), and in the detection of phytoplasma in individual insect vectors (Boudon-Padieu *et al.*, 1989). Due to the high sensitivity and specificity of mAbs, their utilisation in enzyme-linked immunosorbent assay (ELISA), and immunosorbent electron microscopy (ISEM) has become one of the most reliable methods for diagnosis of phytoplasma infections (Lee and Davis, 1992). The main limitation to production of mAbs, however, is that it requires availability of pure immunogens, and phytoplasmas are difficult to purify. Thus, effective mAbs have been produced only when the phytoplasma can be purified from alternative hosts, such as Madagascar periwinkle (*Catharanthus roseus*), in which the phytoplasmas often multiply to high titres. Purification of the phytoplasmas from Madagascar periwinkle also avoids contamination with antigens associated with the host. Antigens for production of mAbs have also been purified from insect vectors (Lin and Chen, 1985).

Sensitive and specific detection of phytoplasmas has been enhanced by recombinant DNA technology that made it possible to clone phytoplasma DNA. By use of cloned random fragments of phytoplasma DNA extracted from infected insects (Kirkpatrick *et al.*, 1987; Davis *et al.*, 1988b) as probes in DNA hybridisation analyses, phytoplasmas have been reliably detected in their plant hosts and insect vectors. DNA probes have also been produced using extracts from phytoplasma-infected plants (Table 1.3). These have been used extensively in dot hybridization assays to detect and identify phytoplasmas in infected hosts (Table 1.3). In addition to specific and sensitive detection, these probes have provided an easier means of quantifying phytoplasmas in infected tissue (Kirkpatrick *et al.*, 1987; Harrison *et al.*, 1992).

Table 1.3 Cloned random DNA probes and PCR primers developed for specific detection of phytoplasma diseases

Phytoplasma disease	Source of DNA	Cloned DNA probes	PCR primers
Coconut lethal yellowing	*Veitchia merrillii*	Harrison *et al.*, 1992	Harrison *et al.*,1994b
Coconut lethal disease	*Cocos nucifera*	This thesis	Rohde *et al.*, 1993
Maize bushy stunt	Vector, *Dalbulus maidis*	Davis *et al.*, 1988	
Eastern aster yellows	*Catharanthus roseus*	Lee and Davis, 1988	Schaff *et al.*, 1990
Western aster yellows	*Apium graveolens*	Kirkpatrick *et al.*, 1991	Deng and Hiruki, 1991a
Western X-disease	*Colladonas montanus*	Kirkpatrick *et al.*, 1987	
Canada peach X	*Catharanthus roseus*	Lee *et al.*, 1992	
Periwinkle little leaf	*Catharanthus roseus*	Davis *et al.*, 1990	
Clover proliferation	*Catharanthus roseus*	Deng & Hiruki, 1990; Lee *et al.*, 1991	Deng and Hiruki, 1991a
Ash yellows	*Catharanthus roseus*	Davis *et al.*, 1992	
Elm yellows	*Catharanthus roseus*	Lee *et al.*, 1993b	Lee *et al.*, 1993b
Pigeon pea witches broom	*Catharanthus roseus*	Harrison *et al.*, 1991	
European elm witches'-broom	*Catharanthus roseus*	Maurer *et al.*, 1993	
Apple proliferation	*Malus domestica*	Bonnet *et al.*, 1990; Kollar *et al.*, 1990	Ahrens & Seemuller, 1992
Flavescence doree	*Catharanthus roseus*	Daire *et al.*, 1992; Davis *et al.*, 1993	
Rice yellow dwarf	*Oryza sativa*	Nakashima *et al.*, 1992	Namba *et al.*, 1993a
Chrysanthemum yellows	*Chrysanthemum coronarium*	Bertacini *et al.*, 1990b	Bertaccini *et al.*, 1992
Appricot chlorotic leaf roll	*Catharanthus roseus*	Kollar *et al.*, 1990	Ahrens & Seemuller, 1992
Walnut witches'-broom	*Catharanthus roseus*	Chen *et al.*, 1992a	

The DNA probes have also been used to study the ecology and distribution of phytoplasma-induced diseases, like western X of stone fruit (Kirkpatrick *et al.*, 1990), and in the evaluation of genetic relatedness among phytoplasmas (Lee *et al.*, 1991; 1992a; 1992b; Deng and Hiruki, 1991b; Harrison *et al.*, 1994a). The sensitivity of phytoplasma detection by dot hybridization assays has been reported to exceed serological detection using ELISA, since the ^{32}P-labelled probes readily detect phytoplasmas in small groups and in individual insect vectors (Davis *et al.*, 1988b; Kirkpatrick *et al.*, 1987).

Greater sensitivity in phytoplasma detection has been attained through amplification of phytoplasma genomic DNA sequences by use of polymerase chain reaction (PCR) assays (Ahrens and Seemuller, 1992; Deng and Hiruki, 1991a). The PCR assay which allows a target DNA fragment present in a sample to be copied and extensively amplified, has so far provided the most sensitive means of phytoplasma detection (Lee and Davis, 1992). It is particularly useful for detection of phytoplasmas because the major obstacles to diagnosis of phytoplasma-induced diseases has been the relatively low titres and uneven distribution of these organisms in plant hosts. By use of PCR to amplify 16S ribosomal DNA (rDNA) sequences, detection of different phytoplasma strains from low titre plant hosts like chrysanthemum (Bertaccini *et al.*, 1992) and coconut palms (Rohde *et al.*, 1993, Harrison *et al.*, 1994b) has been enhanced (Table 1.3). Various phytoplasma strains have also been readily identified and differentiated by use of specially selected primer pairs in PCR assays (Schaff *et al.*, 1992; Lee *et al.*, 1993b; Namba *et al.*, 1993a). Furthermore, PCR has been used to assay individual insect vectors and study how they interact with phytoplasmas (Vega *et al.*, 1993; Harrison *et al.*, 1995).

It is anticipated that by use of techniques that have proved useful in the diagnosis of other phytoplasma-associated diseases, a solution could be found to the problem of diagnosing LD infections.

1.4 THE OBJECTIVES OF THIS STUDY ARE:

1. To develop a reliable detection method for the phytoplasmas associated with the lethal disease of coconut palm in Tanzania.
2. To study the basis of the actiological and epidemiological differences observed among the affected regions in the country by:

a) Determining if there are a number of phytoplasma strains associated with the disease in regions where the disease incidence differs significantly.

b) Screening different Homopterans found in association with coconut palms, in an attempt to identify potential insect vectors of LD, in order to establish the number of insect species involved in the different regions.

Chapter 2: MATERIALS AND METHODS

2.1 MATERIALS, CHEMICALS AND MEASUREMENTS

2.1.1 Chemicals, Enzymes and Cloning Vectors

Chemicals used in this study were all purchased from either Sigma, Bochringer Manheim, or BDH. Enzymes were from Gibco BRL, Life Technologies Inc., UK, Promega, UK, and Bochringer Mannheim, UK. The bacterial plasmid pUC18 was from Pharmacia, LKB Biotechnologies. The LigATor kit for direct cloning of fragments generated by PCR was from R and D Systems Europe Ltd, UK. The Quiaquick gel extraction kit was from Quiagen GmbH, Germany.

2.1.2 Optical Density Measurements

Optical density measurements were taken on purified DNA extracts using a Hewlett Packard 8452A diode array spectrophotometer. The concentration of DNA in the extract was then calculated based on the fact that one absorbency unit at 260 nm wavelength is proportional to a concentration of 50 $\mu g\ ml^{-1}$. Purity of the extracts was monitored by calculation of the A260/280 ratio. Extracts were considered clean when the ratio was between 1.6 - 1.9. In the absence of a spectrophotometer, the concentration of DNA was estimated from 1% (w/v) agarose electrophoresis gels of sample specimens (section 2.3.3.1).

2.1.3 Measurement of pH

Adjustments of buffer pH was done with a Corning pH meter 240 at Rothamsted Experimental Station, whereas at NCDP, a WTW (Wissenschaftlich-Technische Werkstatten) pH 522 meter was used.

2.2 PLANTS

2.2.1 Healthy and Disease-Infected Coconut Tissue for Production of Probes

Three East African Tall (EAT), and one New Guinea Brown Dwarf (NGBD) coconut palms (ages 4 - 10 years) showing early to moderately advanced symptoms of lethal disease (Plate 1.1, Schuiling *et al.*, 1981) in the National Coconut Development Programme (NCDP)'s Kifumangao variety screening trial, (Kisarawe district) were the source of phytoplasma DNA used in molecular cloning. The palms were cut down in January 1993, and immediately on felling, palm heart tissue consisting of the apical meristem and the surrounding immature leaf bases (Plate 2.1) were excised from the crowns. These were then transported to Dar-es-Salaam where they were wrapped in newspaper, chilled overnight at 4 C, and next day transported at ambient temperature to Rothamsted Experimental Station, UK. Similar tissues were excised from a symptomless (apparently healthy) 5-year old Cambodia Tall (CBT) palm. On arrival, the tissues were trimmed to remove necrosis, wrapped in paper towels and stored in a cold room at 4 C, for up to 2 weeks; during which DNA was extracted from fresh tissue. The remaining tissue was chopped into small pieces and frozen at -20 C.

For additional palm DNA, crowns were excised from three EAT palms sampled at Mpeketoni at the northern Kenya coast, two EAT palms from Sotele in southern Tanzania, and three EAT palms from Chambezi in central Tanzania. These were similarly processed and transported to Rothamsted, where they were prepared for DNA extraction as described above.

2.2.2 Meristematic Palm Tissue and Leaves Sampled for Detection of Phytoplasma at NCDP

Several palms showing disease symptoms at different stages of progression were sampled as described above from Sotele, Kifumangao, and Miteja in southern Tanzania. Others were sampled from Chambezi in central Tanzania, and Kigombe in the north. The excised palm heart tissues were brought to the laboratory at NCDP,

trimmed, and immediately processed for DNA extraction. Where immediate extraction was not possible, they were kept at 4 C and processed within three days.

Samples of spear leaves were collected monthly for one year from 180 randomly selected EAT palms. Thirty palms (15 bearing, and 15 non-bearing) were located at each of the six trial sites in the disease affected regions. Two sites (Kifumangao and Sotele) were in the high incidence, two (Chambezi and Kigamboni) in moderate incidence, and two (Kigombe and Pongwe) in the low incidence areas. DNA extracted from these leaves was used to determine the earliest time at which incubating infections could be detected.

DNA was also extracted from embryos of ten nuts which were collected from LD-infected palms at Kigombe, in the north. Furthermore, spear leaves were sampled from eight seedlings which were raised from nuts collected from LD-infected palms at Kifumangao, and DNA extracted for phytoplasma assay.

2.2.3 Source of Other Phytoplasmas

Twelve different phytoplasmas and 2 spiroplasmas maintained in Madagascar periwinkle (*Catharanthus roseus*) in the screenhouse were kindly provided by Dr. M. Clark of Horticulture Research International, East Malling, U.K. They included six phytoplasmas which infect woody hosts; green valley X (GVX), apple proliferation (APF), American elm yellows (AEY), European (Morvan) elm yellows (EEY), plum leptonecrosis, ex-Italy (PLP), and apple chlorotic leaf roll, ex-Spain, (ACLR). The other six phytoplasmas were from herbaceous hosts, and include; faba bean phyllody, ex-Sudan (FBP), stolbur of pepper, ex-Germany (STOL), *Catharanthus* virescence (CAV), aster yellows, strain chlorantie of rape, ex-France (AYS), *Vaccinium* witches-broom (VAC), and clover phyllody, ex-UK (CP). The two spiroplasmas were corn stunt (CST), and *Spiroplasma citri* (SC). In addition, lethal yellowing phytoplasma DNA isolated from *Veitchia merrillii*, and an isolate from coconut originating in Jamaica were kindly provided by Dr. N. Harrison of the University of Florida, Institute of Food & Agricultural Science at Fort Lauderdale.

Healthy *Vinca* plants were maintained in the screen house at Rothamsted Experimental Station.

2.3 ISOLATION OF DNA

The concentration of phytoplasmas in infected coconut tissue is generally low, and their distribution uneven (Deutsch and Nienhaus, 1983; Thomas, 1979). Furthermore, one of the objectives of this thesis was to develop a detection technique suitable for use in a less-well equipped laboratory with limited funds. It was therefore necessary to evaluate different extraction procedures, in order to select the most appropriate for routine use. Hence, four different DNA extraction procedures in addition to the one adopted to purify DNA for probe making were tested.

2.3.1 Buffers Used for DNA Isolation

a) Potassium phosphate buffer, pH 7.4

100 mM K_2HPO_4

31 mM KH_2PO_4

300 mM Sucrose

0.15 % Bovine serum albumin, fraction V

2.0 % (w/v) Polyvinylpyrrolidone [PVP-40]

30 mM Ascorbic acid

10 mM EDTA

b) DNA extraction buffer (DEB)

100 mM Tris-HCl, pH 8.0

50 mM EDTA

500 mM NaCl

10 mM 2-β-Mercaptoethanol)

1 ml of 20 % (w/v) SDS, added just before use.

c) DNA resuspension buffer

50 mM Tris-HCl, pH 8.0

10 mM EDTA, pH 8.0

d) 1 x TE buffer, pH 8.0

10 mM Tris-HCl, pH 8.0

1 mM EDTA, pH 8.0

e) 1 x TAE buffer

40 mM Tris acetate

1 mM EDTA

f) Cetyl trimethyl ammonium bromide (CTAB) buffer,

2 % (w/v) CTAB (Sigma)

1.4 M NaCl

0.2 % 2-β-Mercaptoethanol

20 mM EDTA

100 mM Tris-HCl, pH 8.0

g) DNA extraction buffer, pH 7.4 (Ahrens and Seemuller, 1992).

125 mM Potassium phosphate

30 mM Ascorbic acid

10 % (w/v) Sucrose

2 % (w/v) Polyvinylpyrrolidone (PVP-15)

0.15% Bovine serum albumin (BSA)

h) DNA extraction buffer, pH 7.5 (Rohde *et al.*, 1993)

50 mM Tris-HCl, pH 7.5

1 mM EDTA

0.1% Triton X-100

i) CTAB Buffer (for extraction of insect DNA)

2 % (w/v) CTAB

100 mM Tris-HCl, pH 8.0

20 mM EDTA, pH 8.0

1.4 M NaCl

1 % (w/v) PVP-40

1 % (w/v) 2-β-Mercaptoethanol

j) Acidified 5 M Potassium acetate

60 ml 5 M Kac

11.5 ml glacial acetic acid

28.5 ml sterile distilled H_2O

Unless otherwise stated, all buffers, reagent solutions, glassware, plasticware, tubes and tips were sterilised for 20 min at 121°C before use.

2.3.2 Isolation of Total DNA for Preparation of Probes

DNA was extracted from fresh meristematic tissue, consisting of the bases of unemerged leaves, using a procedure which enriches for phytoplasma and mitochondria DNA (Harrison *et al.*, 1992). About 200 g of tissue were homogenised in a blender in cold (4°C) potassium phosphate buffer, pH 7.4, using 3 ml of buffer per gram of tissue. The homogenate was chilled on ice for 30 min, and squeezed through four layers of cheese cloth. The filtrate was clarified by centrifugation at 4°C, for 10 min at 3,000 x g, to remove starch and chloroplasts, then the phytoplasma-enriched preparations pelleted from the supernatant by centrifugation at 20,000 x g for 30 min at 4°C.

Nucleic acids were extracted from the phytoplasma-enriched pellets by the method of Dellaporta *et al.* (1983). Each pellet was resuspended in 15 ml of DNA extraction buffer (DEB), and the mixture incubated at 65°C for 15 min with occasional gentle agitation. Proteins and polysaccharides were precipitated by addition of 5 ml of

acidified 5 M potassium acetate pH 5.2, followed by incubation on ice for 30 min, then pelleted at 25,000 x g for 20 min. The aqueous phase was filtered through Kleenex tissue and nucleic acids precipitated with 0.6 volume of cold (-20°C) iso-propanol. The mixture was chilled overnight at -20°C, and the nucleic acids pelleted at 20,000 x g for 15 min. Each pellet was resuspended in 3 ml of DNA resuspension buffer overnight at 4°C, then extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) to remove residual proteins, and once with chloroform/isoamyl alcohol (24:1) to remove traces of phenol (Sambrook *et al.*, 1989). Nucleic acids were precipitated from the aqueous phase by addition of 0.1 volume of 3 M sodium acetate, pH 5.2 and 2.5 volumes of cold 95 % (v/v) ethanol. Tubes were chilled overnight at -20°C then centrifuged at 20,000 x g for 15 min to pellet nucleic acids. Pellets were each rinsed in 0.5 ml of 80 % (v/v) ethanol, allowed to air dry, and dissolved in 2 ml of 1 x TE buffer, pH 8.0, containing RNAse A at a concentration of 10 μg ml^{-1}, and incubated at 37°C for 1 h. A 0.25 volume of 5 M NaCl and 2.5 volumes of cold 95 % (v/v) ethanol were added to each tube to precipitate the DNA as described above. Pellets were again rinsed and dried as described, then dissolved in 1 ml of 1 x TE buffer, pH 8.0 and stored at 4°C, until required for further purification.

2.3.3 Separation of Phytoplasma DNA from Host DNA

Buoyant density gradient centrifugation in caesium chloride-bisbenzimide was used to separate phytoplasma DNA which has a higher adenine and thiamine (A + T) content from mixtures with palm mitochondrial DNA (Harrison *et al.*, 1991; 1992). Density gradients were prepared according to the methods of Kollar *et al.* (1990) and Harrison *et al.* (1992). 6.25 g of caesium chloride was dissolved in 3 ml of TE buffer, pH 8.0 and loaded into a 13.5 ml thick-walled polycarbonate ultracentrifuge tube. To this 400 μg of total DNA was gently added. 91 μl of a 10 mg ml^{-1} stock solution of bisbenzimide was slowly added to the mixture with gentle mixing to avoid rapid precipitation. The volume was made up to 7.0 ml with TE buffer, pH 8.0 (final concentration of CsCl, 892 mg ml^{-1}, and that of bisbenzimide 127 μg ml^{-1}). Gradients were centrifuged at 50,000 rpm (ca 229,000 x g) in a fixed angle, Kontron TFT 75.13 rotor for 28 h at 20°C. To decrease the

centrifugation time, a vertical Kontron TV-865 rotor was later used. For this, gradients were prepared in 5.1 ml quick seal polyallomer tubes, and respectively 4.55 g CsCl, and 65 µl of bisbenzimide stock solution were loaded per tube. Total DNA loaded was 400 µg, but centrifugation was at 55,000 rpm (ca 287,000 x g) for 24 h at 20°C.

DNA banding patterns after ultracentrifugation were visualised under long wave UV-light. The bands were separated by inserting a 19-gauge long bent needle from the top, for thick-walled tubes, or by piercing from the side of the thin-walled polyallomer tubes with ordinary 19-gauge needle fitted with a 1 ml syringe. Due to the low concentration of phytoplasma DNA in the extracts, no bands were attributable to phytoplasma after the first round of ultracentrifugation. Therefore, aliquots of 0.5 - 1 ml were withdrawn from every two gradients immediately above the uppermost visible host DNA band, pooled, and loaded onto a second gradient. The gradients were prepared as described before, but without further addition of bisbenzimide. After each centrifugation, the refractive index of every gradient fraction was measured by refractometry. By converting the refractive indices to density using a standard conversion table the buoyant density of each was determined. The refractive indices and densities of different preparations are shown in Appendix 1.

Gel electrophoresis in agarose (Section 2.3.4) was used to determine the concentration of the DNA in each gradient fraction/ band after the second round of ultracentrifugation. Since the results indicated that the phytoplasma DNA was present in very small quantities ($\leq$ 2 ng μl^{-1}) at this stage, the process of pooling and ultracentrifugation was repeated. This process was continued for three, or up to five rounds before a faint, putative phytoplasma DNA band was clearly resolved above the main band of host DNA in the gradients.

After the final ultracentrifugation, both the bands containing host mitochondria DNA and the putative phytoplasma-DNA were separately extracted 4 times with an equal volume of NaCl-saturated isopropanol to remove the bisbenzimide (Harrison *et al.*, 1991; 1992; Kollar *et al.*, 1990). The CsCl was diluted out with 4 volumes of sterile distilled water, and DNA precipitated with 0.6 volumes of cold (-20°C) isopropanol, plus 10 µl glycogen

carrier. Precipitation was overnight at -20°C, then tubes were centrifuged 15 min at 20,000 x g at 4°C to pellet the DNA. Pellets were washed with 80 % (v/v) ethanol, dried under vacuum and each dissolved in 15-20 µl of TE pH 8.0, and stored at -20°C. The putative phytoplasma DNA was later used for cloning.

2.3.4 Agarose Gel Electrophoresis of DNA

For preparation of a 1 % (w/v) agarose gel, 1 g agarose was weighed in an Ehrlemeyer flask, and the volume made to 100 ml with 1 x TAE buffer. The flask was plugged with a spongy stopper, and the slurry heated in a microwave oven for 2 min at high setting to dissolve the agarose. The solution was cooled to 60°C, ethidium bromide added to a final concentration of 0.5 µg ml^{-1}, then poured into a prepared gel mould, and a comb inserted. The comb was removed after the gel was set (30 min), and the gel mounted into the gel tank, then covered with 1 x TAE buffer to 1 mm depth.

The sample for loading into the gel slots was prepared by mixing a 2 µl aliquot from each density gradient band with 2 µl of gel loading buffer (0.25% (w/v) bromophenol blue + 40% (w/v) sucrose in water). For separation of DNA bands in samples prepared for other uses, different volumes were mixed with the gel loading buffer. For example, in analysing products of restriction enzyme digests and polymerase chain reactions, aliquots of 5 or 10 µl were used. In either case, known quantities of standard DNA were loaded on the same gel for comparison. Electrophoresis was carried out at 60 V for 1 h, and DNA visualised on a UV transilluminator.

2.3.5 Isolation of Total DNA from Plant Tissue for Phytoplasma Screening

Four different procedures were evaluated for extraction of DNA from small quantities of palm tissue for screening purposes. The first method tested was based on the miniprep procedure of Dellaporta *et al.* (1983). The second was the cetyl trimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1990), and the third was a modification of the procedure used by Ahrens and Seemuller (1992). The fourth extraction method was according to Rohde *et al.* (1993). For extraction from palm

meristems, the tissue was already frozen at -20°C. In the first three procedures, the frozen tissue was directly transfered into DNA extraction buffer for processing. Initially a comparison was made of different methods of pulverising the frozen tissue, and grinding with a mortar and pestle was found to be superior to either grinding with a coffee mill or blender. In all subsequent extractions, pulverisation was done with a mortar and pestle. Freezing in liquid nitrogen was omitted for the fourth method, where tissue was directly ground in cold DNA extraction buffer with a mortar and pestle in presence of acid washed sea sand.

Both the miniprep procedure of Dellaporta *et al.* (1983) and the CTAB procedure of Doyle and Doyle (1990) were modified and adopted for routine DNA extraction. The modification involved omission of freezing the tissue and use of liquid nitrogen. Thus, fresh tissue was directly ground into a paste with a mortar and pestle in presence of acid washed sea sand and preheated DNA extraction buffer.

2.3.5.1 *The Miniprep DNA extraction procedure (Dellaporta et al., 1983)*

The miniprep procedure of Dellaporta *et al.* (1983) was essentially the same as described above (section 2.3.2), except that there was no phytoplasma - enrichment step. Coconut meristematic tissue was diced into small pieces, and frozen in liquid nitrogen before being pulverised to powder with a mortar and pestle. The frozen powdered tissue was directly transferred into DNA extraction buffer and immediately incubated at 65°C, with SDS for cell lysis.

2.3.5.2 *The CTAB method (Doyle and Doyle, 1990)*

Five grams of diced, frozen coconut meristematic tissue were ground to powder in liquid nitrogen in a chilled mortar. For extraction of DNA of other phytoplasmas which were propagated in Madagascar periwinkle, twigs and midribs from freshly harvested *Vinca* leaves were similarly ground to powder. The powder was scraped directly into a tube containing 25 ml of pre-heated (60°C) CTAB buffer. After incubation at 60°C for 30 min, the lysate was cooled to room temperature, then

extracted with an equal volume of chroloform-isoamyl alcohol (24:1, v/v). The mixture was centrifuged for 10 min at 20,000 x g to separate the phases, and the aqueous phase re-extracted. Precipitation of nucleic acids from the aqueous phase was by addition of 0.1 volume of 3 M sodium acetate, pH 5.2 and 1 volume of cold isopropanol. Large cobwebs of nucleic acids were then spooled out with a glass hook. Alternatively, the mixture was chilled at -20°C for at least 2 h or overnight, then centrifuged at 20,000 x g for 10 min to pellet the nucleic acids. Pellets were each washed in 0.5 ml of 80 % (v/v) ethanol, and allowed to air dry or dried under vacuum. Each pellet was resuspended in 2 ml of 1 x TE buffer containing RNAse A at a concentration of 10 $\mu g\ ml^{-1}$ and incubated for 30 min at 37°C. The DNA was precipitated by addition of 0.25 volume of 5 M NaCl and 2.5 volume of cold absolute ethanol, followed by overnight chilling at -20°C. Mixtures were then centrifuged at 10,000 x g for 10 min to pellet the DNA. Pellets were washed in 80 % (v/v) ethanol and dried under vacuum or allowed to air dry. Each pellet was dissolved in 0.5 ml of 1 x TE buffer and a sample taken to determine DNA yield; either by measuring absorbency units in a spectrophotometer (A_{260} = 1 = 50 $\mu g\ ml^{-1}$ or by electrophoresis. These DNA preparations were stored at 4°C until required.

2.3.5.3 *The phytoplasma-enrichment procedure (Ahrens and Seemuller, 1992).*

25 g of diced, frozen meristematic tissue were ground to powder in liquid nitrogen in a pre-chilled mortar. The frozen powder was scraped into 125 ml of extraction buffer in a blender and the mixture pulsed for 30 s. The homogenate was centrifuged for 15 min at 3,000 x g at 4°C to pellet starch and chloroplasts, then the supernatant recentrifuged at 20,000 x g for 30 min. The phytoplasma-enriched pellets were each resuspended in 15 ml of preheated (60°C) CTAB buffer and incubated at 60°C for 30 min. The lysate was allowed to cool to room temperature, then extracted with an equal volume of Tris-neutralised phenol:chloroform:isoamyl alcohol (25:24:1). The mixture was centrifuged for 10 min at 20,000 x g to separate the phases, and the aqueous phase similarly re-extracted. The nucleic acids were precipitated from the aqueous phase by addition of 0.1 volume of 3 M sodium acetate, pH 5.2 and 1 volume cold isopropanol followed by chilling overnight at -20°C. The tubes were centrifuged at 10,000 x g for 10 min to pellet

the nucleic acids. Each pellet was resuspended in 2 ml of 1 x TE buffer containing RNase A at a concentration of 10 µg ml^{-1} and incubated at 37°C for 1 h. The DNA was precipitated with 0.25 volume of 5 M NaCl and 2.5 volume of cold absolute ethanol, then chilled overnight at -20°C and pelleted by centrifugation for 10 min at 10,000 x g. Pellets were washed in 80 % (v/v) ethanol, dried under vacuum, or air dried, then each resuspended in 0.5 ml of 1 x TE buffer, pH 8.0 and stored at 4°C until required.

2.3.5.4 *Rapid DNA extraction procedure (Rohde et al., 1993)*

5 g of diced, frozen coconut meristematic tissue was ground to powder with a mortar and pestle in 10 ml of cold extraction buffer, in presence of 1 g sea sand (40-100 mesh, acid extracted). The homogenate was extracted with an equal volume of Tris-equilibrated phenol:chloroform:isoamyl alcohol (25:24:1), and the phases separated by centrifugation for 10 min at 7,000 x g at room temperature. The aqueous phase was similarly re-extracted. Nucleic acids were precipitated from the aqueous phase by addition of 0.1 volume of 3 M sodium acetate and 1 volume of cold isopropanol, followed by chilling overnight at -20°C, then pelleted by centrifugation for 10 min at 20,000 x g at 4°C. Each pellet was resuspended in 2 ml of 1 x TE buffer containing RNase A at a concentration of 10 µg ml^{-1} and incubated for 1 h at 37°C. DNA was precipitated with 0.25 volume of 5 M NaCl and 2.5 volume of absolute ethanol, chilled overnight at -20°C, then pelleted by centrifugation for 10 min at 10,000 x g. Pellets were washed with 70 % (v/v) ethanol and vacuum-dried or allowed to air dry. Each pellet was resuspended in 0.5 ml of 1 x TE buffer, pH 8.0, and stored at 4°C.

2.3.6 Isolation of DNA from Insect Specimens

The variation in the populations of homopteran insects suspected to be putative vectors of LD were studied at two trial sites, Chambezi and Kifumangao, representing the moderate and high disease incidence areas respectively. Thirty palms were selected at each site within the disease screening trials, and these involved tall (mature), as well as short (young) palms. The relative location of the selected palms in the farm was also critically considered, as some palms were located at the edge of

the field close to the bushes, while others were located in the middle of the field. The use of differing palm heights was to determine whether palm height influenced the flight of the insect vector, and hence the susceptibility to infection of palms of different height. The selection of the palms, particularly those near the edges of the field was intended to demonstrate whether infectious insects migrate into the field from the bush.

Insect traps were set up on each of the selected palms. A trap consisted of rectangular plywood, 4 x 6 inches with a hole in one corner. This was painted a bright yellow colour with oil paint. Both surfaces were coated with sticky insect adhesive, Oecotac™. The traps were enclosed with a wire mesh (chicken wire) to prevent the leaves from getting stuck to them. Each trap was hoisted high up in the canopy of the selected palm using a sisal string which formed a pulley system, and was tied to the lower part of the palm trunk (Plate 2.1).

Once a week all the traps were pulled down assisted by the ropes, and all trapped insects removed with a thin stick into insect bottles containing kerosene. The kerosene helped to dissolve any adhering Oecotac. The insects were then transferred to fresh bottles containing 70 % (v/v) ethanol, and transported to the laboratory for sorting. Insects collected from each palm were collected separately. In the laboratory, these insects were sorted into species with the aid of a binocular microscope, counted and recorded. The trapping of insects on the same selected palms was continued for a period of one year.

It was originally planned to extract DNA from the insects so collected, and screen them for phytoplasma infection by polymerase chain reaction (PCR). However, it was realised that the percentage of insects recovered intact after washes in kerosene and alcohol was small, as many were damaged in the process, such that they would be releasing inhibitors which could interfere with the screening process. Therefore, extraction of DNA from trapped insects was abandoned, but trapping continued in order to provide information on the relationship between vector flight and disease incidence in the field. Meanwhile, collection of insects from selected species for

Plate 2.1: A sample sticky trap for trapping insects in a disease infected coconut field. A sisal string (rope) was used to hoist the trap up into the canopy.

DNA extraction was done manually from the underside of leaves on palms showing typical LD symptoms by use of large conical flasks which did not damage the insects during collection. Different homopteran species were collected from four locations; Kifumangao, Chambezi, Kigombe and Miteja. The following species were included: *Diastrombus abdominalis, D. mkurangai, D. schulingi, Meenoplus* spp., *Phenice* spp, *Paraphenice* spp., *Elasmosceles cimicoides, Lydda woodi, Robigus* spp., *Amania angustifrons, Bandusia erythrostena, Zoraida fuligipennis, Zorabana* spp., *Diazanus* spp. and *Kamendaka* spp. More than 15,000 insects were collected, and DNA extracted according to the procedure of Dr. N. Harrison (personal communication). Insects used for DNA extraction were either freshly collected from the field, or frozen at -20 C immediately after collection. Single insects, or in groups of three, were hand crushed in microfuge tubes in 300 µl of pre-warmed (65°C) CTAB buffer, using disposable pestles made from blue Eppendorf pipette tips. Ground samples were incubated for 15 min at 65°C, cooled to room temperature, and extracted with an equal volume of chloroform: isoamyl alcohol (24:1). The mixture was centrifuged 15 min at 12,000 x g at room temperature to separate the phases, and nucleic acids precipitated from the aqueous phase with 0.6 volume of room temperature isopropanol for 30 min. Nucleic acids were pelleted by centrifugation for 15 min at 12,000 x g, washed in 70% (v/v) ethanol, air dried, and dissolved in 50 µl of TE, pH 8.0; then stored at 4°C until required for screening.

2.4 MOLECULAR CLONING

Cloning was performed by the standard procedures of Sambrook *et al.* (1989).

2.4.1 Preparation of Luria Bertani (LB) Medium and Broth

Luria Bertani (LB) medium and broth with ampicillin added to a final concentration of 50 µg ml^{-1} were prepared by combining 2.5 g NaCl, 2.5 g Bactotryptone, 1.25 g Yeast extract, and 3.75 g Bacto agar in a medium bottle, and the volume adjusted to 250 ml with distilled water. The mixture was sterilised by autoclaving and cooled to 50°C before

addition of 0.5 ml of a 25 mg ml^{-1} solution of ampicillin. The mixture was poured into 10 cm diameter sterile Petri dishes and allowed to solidify at room temperature. Plates were either dried overnight in an incubator at 37°C, or for 15 min in the oven at 55°C.

LB broth was prepared in the same way, except that Bacto agar was excluded from the mixture.

2.4.2 Preparation of *Eschierichia coli* Competent Cells

An overnight culture of *Eschierichia coli* (strain DH5α) was prepared on an LB medium plate (no ampicillin added as these cells are sensitive to it). A sample picked from this fresh plate, was used to inoculate 5 ml of LB broth (no ampicillin) and an overnight culture of the bacteria produced by shaking at 250 rpm at 37°C. The whole 5 ml of this culture was used to inoculate 250 ml of LB broth. The broth was shaken on a rotary shaker at 250 rpm at 37°C for about 1 h, during which the bacteria multiplied to OD 0.2. The OD was determined at time intervals by measuring the absorbency of a sample at 600 nm in a spectrophotometer. The culture was cooled, then subdivided into sterile centrifuge tubes, and the cells pelleted at 3,000 x g for 5 min at 0°C. All pellets were jointly resuspended in 125 ml of cold 50 mM $CaCl_2$ (hydrated calcium chloride [$CaCl_2.2H_2O$] was used to prepare the solution). The mixture was incubated on ice for 20 min, then pelleted at 3,000 x g for 5 min at 0°C. The pellet was resuspended in 20 ml of 50 mM $CaCl_2$ and maintained at 0°C for 4 h, then 7 ml of 50 % (v/v) glycerol added. Aliquots of the cells (200 µl) were immediately dispensed into cold 1.5 ml Eppendorf tubes, and stored at -70°C.

2.4.3 Enzyme Restriction Digestion of Phytoplasma DNA

A 20 µl reaction mixture was prepared by mixing all components in a microfuge tube on ice

Approximately 900-1,000 ng gradient-enriched phytoplasma-DNA (from Kifumangao).

2 µl restriction enzyme reaction buffer (10 x)

Sterile distilled water to make up volume to 20 µl

1 Unit restriction endonuclease *Eco* RI (Gibco BRL Life Technologies Inc., UK)

The tube was briefly spun to bring all tube contents to the bottom, then incubated in a water bath at 37°C for 1 h, to partially digest the DNA. The mixture was immediately cooled on ice, then chilled at -20°C. Meanwhile, a sample of the digest (100 ng) was loaded on a 1 % (w/v) agarose gel, electrophoresed and DNA visualised as described in section 2.3.4 to check whether digestion was successful. Results indicated that the digestion resulted in fragment sizes ranging from less than 500 bp to more than 20 kbp. 2 µl of 0.5 M EDTA, pH 8.0, was then added to stop the reaction, and aliquots withdrawn for use in ligation reactions.

2.4.4 Ligation of Digested DNA Fragments to the Digested Plasmid Vector

The *Eco* RI-digested bacterial plasmid, pUC 18 (Pharmacia, LKB Biotechnologies) used was already treated with bacterial alkaline phosphatase (BAP) to stop re-circularization of the vector during ligation. Fragments resulting from the partial digestion of phytoplasma DNA (section 2.4.4) were ligated into the bacterial plasmid by mixing equimolar amounts of the plasmid vector and digested DNA in a sterile microfuge tube. Sterile distilled water was added to a total volume of 7.5 µl, and the solution warmed to 45°C for 5 min to melt any cohesive termini that had re-annealed. The mixture was then chilled to 0°C, 1 µl of 10 x ligase buffer and 1 Unit (0.1 Weiss Unit) of T4 DNA ligase enzyme added, and the ligation reaction was incubated overnight at 15°C. 2 µl of the ligation reaction was used to transform competent DH5α *E. coli* cells.

Two additional ligations were set up to determine the optimum recombination ratio of vector to insert DNA. Thus, three different ratios, i.e. 1:1, 1:3, and 3:1, vector to insert DNA were ligated. For the 1:1 ratio (equimolar amounts), 100 ng of each were combined in a ligation reaction, whereas 300 ng and 100 ng, or 100 ng and 300 ng respectively were combined for the other ratios. The ligation reaction mixture was either in a total volume of 10 µl (1:1 ratio), or 20 µl (1:3, or 3:1 ratios), and each used only 1 Unit of enzyme.

2.4.5 Transformation of Competent Cells

Luria Bertani (LB) medium and broth with ampicillin added to a final concentration of 50 μg ml^{-1} were prepared just before use. The surface of each LB plate was spread with 40 μl of a 20 mg ml^{-1} solution (800 μg) of X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactocidase), and 4 μl of a 200 mg ml^{-1} solution (800 μg) of IPTG (isopropythio-ß-D-galactosidase). These solutions were left to soak in for 10 min, then the plates inverted and incubated at 37°C while preparing the ligation mixture.

Addition of X-gal and IPTG to the LB medium was to facilitate selection of recombinant colonies. Bacterial cells used in molecular cloning lack the gene for production of β-D-galactocidase enzyme. The plasmid pUC 18, contains the LacZα peptide sequence which complements the LacZ peptide synthesized in the competent cells so that the bacteria are able to produce a functional β-D-galactocidase enzyme. This enzyme cleaves X-gal to produce blue bacterial colonies, and IPTG ensures expression of the LacZα gene in cells containing the plasmid. By inserting phytoplasma DNA into the cut plasmid DNA, the LacZα peptide sequence would be interrupted, and this would prevent expression of the gene in the transformed bacteria. Consequently, colonies arising from transformed bacteria would be white instead of the usual blue colour.

E. coli, strain DH5α competent cells (section 2.4.2) were transformed. Five, 200 μl aliquots of these cells from the freezer at -70°C were each thawed in the palm of the hand, and as they just started to thaw were transferred to ice for 10 min. Each of the three aliquots was mixed with 2 μl (40 ng) of one of the ligation mixtures. The fourth and fifth aliquots respectively were mixed with 50 ng of the digested vector alone (pUC 18), and 50 ng of undigested vector (Bluescript KS) to serve as controls. Contents of each tube were gently mixed by inversion (no shaking or vortexing), then the tubes incubated on ice for exactly 30 min. Next, cells were given a heat shock for exactly 92 s in a water bath at 42°C, and the tubes immediately transferred onto ice and chilled for 2 min. 0.8 ml of LB broth was added to each tube and the contents mixed gently (no vortexing or shaking), then prewarmed to 37°C for 5 min in a water bath. The bacteria were then allowed to recover in the LB broth by gentle agitation on a rotary shaker (200 rpm) at 37°C for 1h.

This was to promote higher transformation efficiency. Two, 100 µl aliquots were then drawn from each tube and plated onto two of the already prepared LB medium plates. Cells were left to soak in for 10 min, then plates inverted and incubated overnight at 37°C. The remaining transformed cells were stored at 4°C for plating out within the next 2 days.

2.5 SCREENING OF RECOMBINANT COLONIES

2.5.1 Buffers and Solutions Used for DNA Blotting and Hybridizations

a) Miniprep solution 1

50 mM glucose

25 mM Tris- HCl, pH 8.0

10 mM EDTA, pH 8.0

b) Miniprep lysis solution 2

0.2 N NaOH

1% (w/v) SDS

c) Acidified 5 M Potassium acetate

60 ml 5 M Kac

11.5 ml glacial acetic acid

28.5 ml sterile distilled H_2O

d) Denhardt's solution

0.1% Ficoll

0.1% polyvinylpyrrolidone

0.1% bovine serum albumin

e) SSC solution (1 x SSC contains:)

150 mM NaCl

15 mM sodium citrate, pH 7.0

f) Depurination solution

0.25 N HCl

g) Denaturation buffer

0.5 N NaOH

1.5 M NaCl

h) Neutralization buffer (for colony hybridizations)

1.5 M NaCl

0.5 M Tris-HCl, pH 7.4

i) Neutralization buffer (for Southern blots)

3.0 M NaCl

0.5 M Tris-HCl, pH 7.2

All buffers and solutions were sterilised by autoclaving at 121°C for 20 min.

2.5.2 Generation of a Recombinant Plasmid Library

Following overnight incubation at 37°C of the culture plates, recombinant colonies were individually picked out onto grided LB-ampicillin plates using a toothpick (A sample grid is shown in Fig. 2.1). These were incubated overnight at 37°C for the colonies to enlarge to about 1.5 mm. A pick was then made of each individual colony into 200 µl of LB broth in sterile 96-well microtitre culture plates. The microtitre culture plates were incubated overnight at 37°C to multiply the bacteria, topped with 50 µl sterile glycerol per well, and frozen at -70°C, thereby generating a recombinant plasmid library. The grided LB-plates were also incubated overnight at 37°C to regenerate the colonies.

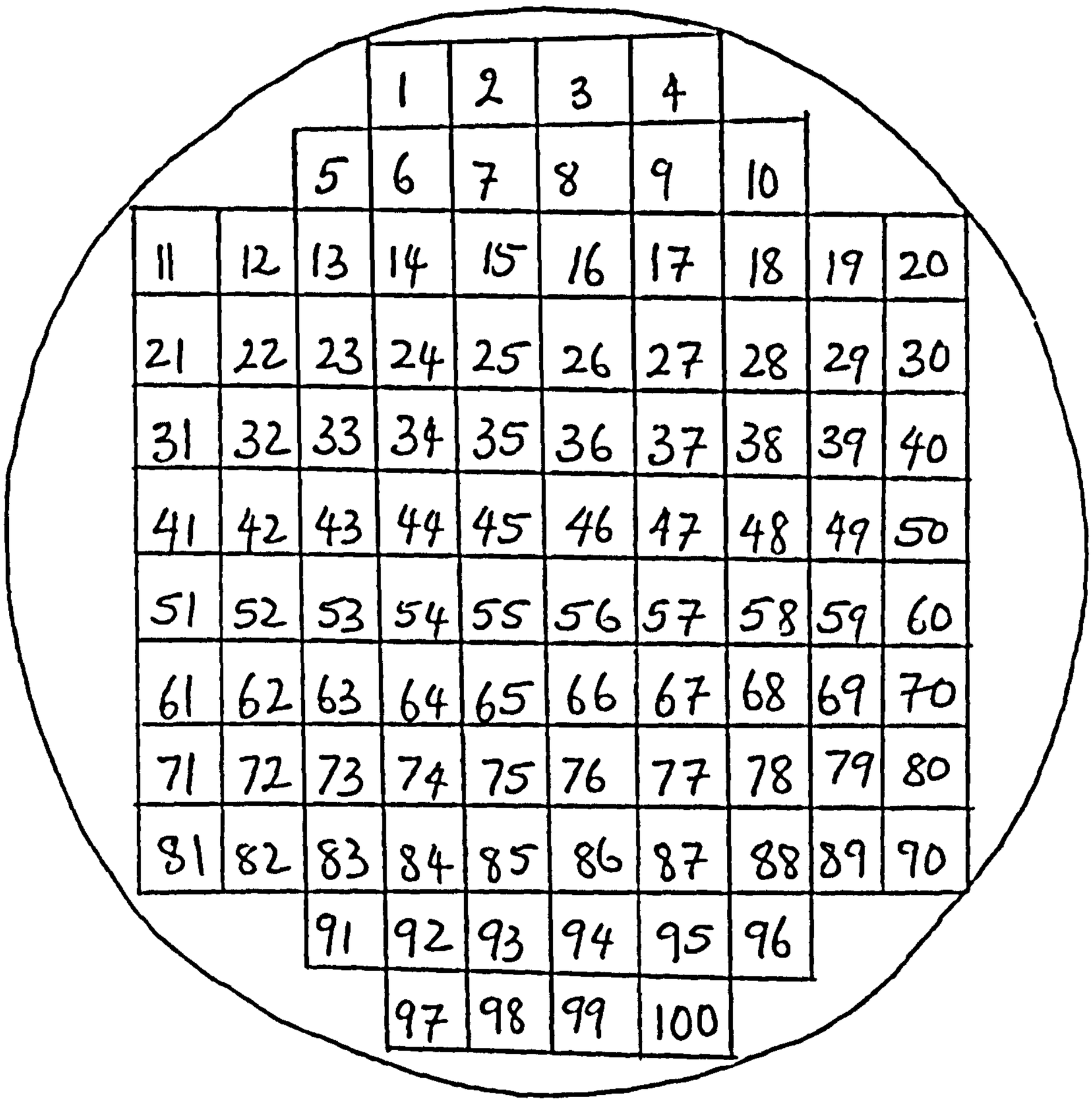


Fig. 2.1: Sample grided culture plate for isolation of recombinant bacterial colonies

2.5.3 Transfer and Lysis of Recombinant Bacterial Colonies onto Nylon Membrane Filters

Bacterial colonies were transferred from culture plates onto nylon (Hybond N, Amersham) membranes, and *in situ* lysis of the colonies done as described by Sambrook *et al.* (1989). Grided LB-plates used to generate a recombinant library (section 2.5.2) served as templates for colony lifts. After overnight incubation at 37°C, plates were chilled at 4°C for 30 - 60 min (up to 4 d) before being transferred.

Four sets of Whatman 3MM paper (4 pieces per set) were cut in circles, the same diameter as the bottom of culture plates, and each set placed inside a sterile culture plate. The plates were sequentially labelled, 1-4. The first plate was flooded with 10 % (w/v) SDS such that the filters were completely soaked but not dripping. Similarly, the second set of filters was saturated with denaturation solution, the third set with neutralisation solution, and the fourth set with 2 x SSC. Circular nylon membrane filters (Hybond-N, Amersham) were numbered with soft pencil, the numbers on filters corresponding to the plates from which colonies would be lifted.

To lift the colonies, the filter was placed numbered side down on the agar medium in contact with the bacterial colonies until it was completely wet, taking care not to move the filter once it had touched the medium and colonies, and avoiding air bubbles. The filter and underlying medium were then marked at 3 or 4 asymmetric locations, by stabbing through with a sterile 19-gauge needle. Using blunt-ended forceps, the filter was peeled off and placed colony side up on a pad of 3MM paper saturated with 10 % (w/v) SDS (first pad), and left on to soak for 3 min. This step was intended to limit diffusion of plasmid DNA during denaturation and neutralization, and help to give a sharp hybridisation signal. Cells were lysed *in situ* by transferring the filter to the denaturing solution (second pad), and soaking for 5 min, colony side up. The filter was then transferred to neutralisation solution (third pad), where it was maintained colony side up for 5 min, before being soaked, colony side up onto the wash solution (fourth pad), for 5 min. Finally, the filter was laid colony side up on a clean sheet of 3 MM paper, and allowed to air dry for at least 30 min. It was then wrapped in cling film and the released

DNA noncovalently attached to the filters by UV- cross linking on a transilluminator for 4 min.

Two lifts were made per plate, then the plates were incubated overnight at 37°C to regenerate the colonies. To avoid carry over during transfer from one solution to another, the edge of the plate was used to remove excess fluid on the bottom of the filter. Alternatively, the filter was touched briefly on dry paper towel.

2.5.4 Colony hybridizations

Recombinant colonies were screened by differential colony hybridizations with ^{32}P-labelled, gradient-enriched phytoplasma-DNA, or with total DNA from healthy coconut palm. Probe DNA was labelled with ^{32}P-dATP by using random oligoprimers according to the manufacturer's instructions (random primed DNA labelling kit, Bochringer Mannheim Biochemica).

2.5.4.1 *Labelling of probe DNA with radioactive isotope, ^{32}P (random primed labelling)*

A stock solution of Sephadex G-50 for preparation of chromatography column was prepared by addition of 10 g of G-50 medium (dry powder) to sterile distilled water. The resulting slurry (160 ml) of swollen resin was washed several times with distilled water to remove soluble dextran, then equilibrated in 1 x TE (pH 7.6), and stored at 4°C.

A labelling reaction mixture was prepared by combining together in a microfuge tube on ice:

100 ng DNA sample, denatured by heating 10 min at 100°C, and cooled on ice

2 µl reaction buffer

3 µl dNTP mixture (dCTP, dGTP, dTTP, 1 µl of each individual solution, mixed)

5 µl = 50 µCi [α^{32}P] dATP, 3000 Ci mMol^{-1}, aqueous solution

sterile distilled H_2O to make volume to 19 µl.

1 µl Klenow enzyme.

The mixture was incubated for 30 min at 37°C, and the labelling reaction stopped by addition of 2 µl of 0.2 M EDTA, pH 8.0.

Unincorporated label was removed by chromatography on a Sephadex G-50 column: A sterile 1 ml syringe was plugged with siliconised glass wool, placed inside a clean plastic centrifuge tube, and filled to the top with Sephadex beads (from stock solution). It was centrifuged 5 min at 2,500 rpm in a bench top centrifuge to compact the beads. Buffer collected in the centrifuge tube was discarded, the syringe topped up with more beads, and centrifugation repeated. The column was fully set at the end of the run, buffer in the bottom of the tube was discarded, and the labelled probe was loaded onto the column. The syringe was inserted into a microfuge tube with cap removed, and both placed in the centrifuge tube, and centrifuged 5 min at 2,500 rpm. A Geiger counter was used to check the whole column for radioactivity. The labelling reaction was considered successful, and unincorporated label removed by the spin column when counts were high in the top part of the column, low in the bottom part, and also high in the eluate that collected in the microfuge tube (the probe). The probe was either used immediately for hybridisation, or stored at -20°C and used within 2 days.

2.5.4.2 *DNA -DNA hybridizations*

Membranes containing bacterial colonies (colony lifts, section 2.5.3) were prehybridized overnight (16 h) at 68°C in a solution containing 6 x SSC, 5 x Denhardt's, 0.5 % SDS, and 1 mg ml^{-1} denatured salmon sperm DNA. This was replaced with a similar solution containing the probe DNA that had been denatured by boiling for 5 min at 100°C, and the blots hybridised to the probes overnight at 68°C. After hybridisation membranes were subjected to high stringency wash conditions of two washes in 2 x SSC, 0.1 % SDS at 25°C (15 min each), one wash in 0.2 x SSC, 0.1 % SDS at 68°C (30 min), and once again in 0.2 x SSC, 0.1 % SDS at 25°C (10 min). Membranes were then sealed in plastic wrap and exposed to X ray film (Fuji photo film Co., Japan) with an intensifier screen. One membrane of each colony lift was probed with ^{32}P-labelled gradient-enriched phytoplasma DNA, and its replica with ^{32}P- labelled healthy coconut DNA. Colonies

that hybridised to phytoplasma-DNA but produced weak or no signal with the healthy coconut DNA were selected for further screening as potential probes.

2.6 SELECTION OF POTENTIAL PROBES

2.6.1 Small Scale Preparation of Plasmid DNA (Miniprep DNA) by Alkaline Lysis Method

Colonies that hybridised to phytoplasma-DNA but produced weak or no signal with the healthy coconut DNA in colony hybridizations were subcultured into LB broth, and recombinant plasmids extracted from small scale preparations (minipreps) of each culture by the alkaline lysis method of Sambrook *et al.* (1989). Each selected bacterial colony was tooth-picked into 5 ml of LB medium with ampicillin, in a 15 ml tube whose cap was loosely fitted. The tubes were incubated overnight at 37°C with vigorous shaking on a rotary shaker at 2,500 rpm. The medium became turbid.

Two 1.5 ml aliquots of each culture were transferred into 2 ml microfuge tubes, and centrifuged for 1-2 min at 12,000 x g at 4°C in a microfuge. The medium was removed by aspiration leaving the pellets dry. Each pellet was resuspended in 100 µl of ice-cold miniprep solution 1 by vigorous shaking on a vortex (full resuspension essential for subsequent lysis), then tubes incubated on ice for 5 min. 200 µl of freshly prepared lysis solution 2 was added to each tube. Tubes were closed tightly and mixed by inverting rapidly five times (but no vortexing), then incubated on ice for 5 min. 150 µl of ice-cold 3 M sodium acetate, pH 5.2, or 5 M acidified potassium acetate was added, and the mixture vortexed slightly to mix, then incubated on ice for 30 min. Tubes were vortexed, then centrifuged at 12,000 x g for 15 min at 4°C.

The supernatant (ca 0.45 ml) was transferred to a fresh tube, and nucleic acids precipitated with 2 volumes of chilled absolute ethanol. Tubes were inverted several times to mix, left at room temperature for 2 min, then centrifuged at 12,000 x g for 30 min at 4°C to pellet nucleic acids. The supernatant was either removed with a Pasteur pipette, leaving the

pellet dry or carefully poured off, and tubes drained on tissue. Pellets were washed with 70 % (v/v) ethanol, left to stand at room temperature for 5 min, then centrifuged briefly and the ethanol carefully poured off. Pellets were either allowed to air dry or dried under vacuum. Each pellet was redissolved in 50 µl of 1 x TE, pH 8.0, containing 20 mg ml^{-1} RNAse, finger vortexed to dissolve, and left at room temperature for 1.5 h. A 5 µl aliquot was removed for restriction enzyme digestion, and the rest stored at -20°C.

2.6.2 Southern Blotting and Screening of Potential Probes

Restriction endonuclease digestion of the recombinant plasmids to release cloned DNA inserts was done overnight with *Eco* RI enzyme by the method previously described (section 2.4.3). Sizes of cloned DNA inserts were estimated after electrophoresis of 10 µl aliquots of the digests in 1 % (w/v) agarose gels (section 2.3.4), using 1 kbp Ladder DNA as standard. These plasmid DNA digests were then blotted from gels onto nylon membrane filters (Hybond N, Amersham) by the method of Southern (1975) using 4 x SSC as the transfer buffer. Gel blotting was done by either capillary transfer, or by use of a vacuum blotter.

For capillary transfer, the gel was first placed in 0.25 M HCl until the colour of the tracking dye changed to yellow, then left for another 10 min. The gel was rinsed in distilled water, covered completely with denaturation buffer and gently agitated for 30 min at room temperature, during which time the colour re-appeared. It was again rinsed in distilled water, transferred to neutralization buffer and agitated for 15 min at room temperature.

A capillary blot was set up by placing 4 pieces of 3 MM paper (larger than the gel by at least 3 cm on all sides) on top of a large piece of stretched cling film, and soaked completely with 20 x SSC. Excess liquid and air bubbles were removed by rolling a pipette over them. The gel was drained from neutralization buffer and placed well-side down over the pad of 4 wet 3 MM paper. A nylon membrane filter (Hybond N, Amersham) cut to the same size as the gel, wet in distilled water and pre-soaked in 20 x SSC was placed on top of the gel, excluding air bubbles. On top of the membrane were

placed two pieces of 3 MM, cut to the same size as the gel and pre-soaked in 20 x SSC. The cling film was then folded around the sides of the gel so that it just touched the sides of the gel to ensure that the transfer buffer would move through the gel to the filter, and not around the sides. Paper towels (about 5 cm high), were then placed on top of the 3 MM paper, followed by a piece of glass. On top, a weight of about 0.75-1 kg (1 l conical flask filled with water) was placed, and the set up left undisturbed overnight.

The weight and towels were removed next day, and the gel turned over such that it was on top of the filter and the 3 MM paper. Gel well positions were marked on the filter membrane using a soft pencil. The left corner the membrane was nicked to indicate orientation of the filter to the gel. The membrane was then washed in 6 x SSC by gentle agitation for 5 min at room temperature, blotted in between two clean sheets of 3 MM paper, then air dried at room temperature for at least 30 min. It was wrapped in cling film and DNA noncovalently attached by UV- cross linking on a transilluminator for 4 min. They were then hybridised to either ^{32}P-labelled healthy coconut DNA or labelled, gradient-enriched phytoplasma-DNA probes (section 2.5.4.2).

The vacuum blotter was used as an alternative to decrease the time of blotting, because many membranes had to be screened. The blotter was connected to the in-line liquid trap and vacuum pump, and the porous gel support screen wet with distilled water. A filter membrane, cut slightly larger than the gel (approx. 1 cm on all sides), and pre-wet in distilled water was placed in the centre of the support screen, taking care to remove air bubbles. The plastic mask was adjusted on top of the membrane, such that the window was in the centre of the membrane with the latter extending beyond the window on all sides by about a centimetre. While avoiding air bubbles, the gel was gently slid on top of the membrane so that it fit exactly over the window of the plastic mask. The set up was clamped in place, the gel flooded with depurination solution, and vacuum switched on. The vacuum gauge was set at 50 mbar.

Depurination was for 10 min, by the end of which the colour of the membrane had turned yellow. The blotter was tilted and all the liquid sucked out using a plastic pipette, then the gel flooded with about 50 ml of denaturation buffer, which was left on for 20 min, by

which time the colour changed to blue again. All the liquid was sucked out with a plastic pipette, then the gel flooded with same volume of neutralisation buffer for 30 min. This was sucked out, and the whole blotter flooded with 500 ml of 20 x SSC, which was left on for 1 h. The salt solution was decanted off and positions of the gel slots marked on the filter with a thin lead mechanical pencil, while the vacuum was still on. The scaling frame was then unclipped to release vacuum, the gel removed, and vacuum switched off. The membrane was washed in 2 x SSC for 5 min, blotted between two layers of 3 MM paper, and air dried, then UV-cross linked for 4 min on a transilluminator. Hybridisation to labelled probes was as described above.

2.7 USE OF DNA PROBES TO SCREEN FOR PHYTOPLASMA INFECTIONS

2.7.1 Dot Blot hybridization

Initially, dot blot hybridizations were used to check the effectiveness of the different methods for extraction of total DNA. For this purpose, probe DNA was prepared by labelling the DNA fragment amplified up in PCR from LD-infected tissue (Rohde's primers) with a non-radioactive plant derivative, Digoxigenin (DIG). After selection of potential DNA probes, all experiments undertaken to determine the effectiveness of these probes for detection of phytoplasma infections in plants and insects were by nonradioactive dot blot hybridisation.

For Blotting the DNA onto nylon membrane, 2 µg of total DNA extracted from LD-infected or healthy coconuts, was denatured by boiling for 10 min in 50 µl of TE buffer that contained 3 µl of 2 N NaOH. The mixture was immediately cooled on ice, and neutralised by addition of 3 µl of 2 M Tris-HCl, pH 7.0. The DNA was then diluted with an equal volume of 12 x SSC, and blotted as a series of two-fold dilutions (initial DNA dilution = 1 µg) onto nylon (Hybond N, Amersham) membranes by using a Bio-Dot manifold (Bio-Rad Laboratories). DNA extracted from insects suspected to be potential vectors were blotted as single spots only. 10 µl of insect DNA (out of the total volume of

50 µl per insect) was made to 23 µl with TE and 3 µl of 2N NaOH, and boiled as described above. Neutralisation was as described, after which the DNA was diluted with an equal volume of 12 X SSC and directly blotted as described without further dilution. The membranes were air dried, and exposed to UV-light for 4 min to cross link the DNA.

2.7.2 Purification of Cloned DNA for Labelling

Recombinant plasmid DNA was prepared from bacterial cultures by the alkaline lysis procedure (section 2.6.1), and insert DNA separated by enzyme restriction digestion with *Eco* RI (section 2.4.3). The restricted DNA fragment was then purified from the agarose gel after electrophoresis, either by the freeze squeeze method, or by use of the Quiaquick gel extraction kit (Quiagen GmbH, Germany).

2.7.2.1 *The Freeze squeeze method*

The enzyme was denatured after restriction digestion by addition of 2 µl of 0.2 M EDTA, then the DNA electrophoresed in 0.8 % agarose according to the normal procedure (section 2.3.4). The bands were visualised with a hand held lamp to avoid 'nicking' the DNA. The fluorescing bands were excised with a sharp razor or scalpel, and excess agarose chopped off. Each band was then cut into 2-3 smaller portions and transferred into sterile microfuge tubes (volume per tube not more than 150 µl). To each microfuge tube was added 0.5 ml of 0.3 M sodium acetate containing 1 mM EDTA, pH 7.0, and the tubes incubated in the dark at room temperature for 45 min, with occasional inverting.

After incubation, the gel slices were transferred into 0.5 ml microfuge tubes whose bottoms had been punched with a 19 G needle and holes plugged with siliconised glass wool. The tubes were capped and immersed in liquid nitrogen for about 1 min, until the gel slices froze. Each of these was then placed inside a 1.5 ml Eppendorf tube whose cap had been removed, and centrifuged at room temperature for 10 min at full speed. The small microfuge tubes were discarded, the eluate transferred into normal 1.5 ml Eppendorf tubes, and DNA precipitated by addition of 1/50 volume of 0.5 M $MgCl_2$, 5 % (v/v) acetic acid solution, 1 µl of glycogen carrier (from a 20 mg ml^{-1} stock solution at -20°C), and 2.5

volumes of absolute ethanol. Tubes were chilled at -70°C for 30 min, or overnight at -20°C, then centrifuged 10 min at full speed in the microfuge to pellet the DNA. The pellets were each washed with 70 % (v/v) ethanol, dried under vacuum, and dissolved in 10 µl sterile distilled water or 1 x TE, pH 8.0. A sample (1 µl) was electrophoresed in a mini-gel of 0.8 % agarose to estimate the quantity of DNA recovered before labelling.

2.7.2.2 *Quiaquick gel extraction kit*

Insert DNA was extracted from agarose gel after electrophoresis of restriction digests, according to the manufacturer's instructions. Fluorescing bands were excised, the gel slices weighed, and three volumes of buffer QX1 added (e.g., 300 µl for 100 µg). The mixture was incubated for 10 min at 50°C, with occasional inverting. Each sample was then loaded onto a Quiaquick spin column and the column placed inside a 2 ml collection tube, then centrifuged 1 min at maximum speed in a tabletop microfuge. The eluate was discarded, the column topped with 0.75 ml of buffer PE, and centrifugation repeated for 1 min to wash the column. The eluate was discarded, and the columns centrifuged for another 1 min to remove excess buffer PE.

The columns were then transferred to clean microfuge tubes, and the DNA eluted by addition of 50 µl of 10 mM Tris-HCl, pH 8.5 followed by centrifugation for 1 min at maximum speed. The DNA was precipitated from the eluate by addition of 0.1 volume of 5M NaCl, 2.5 volume cold absolute ethanol, and 1 µl glycogen carrier then chilled overnight at - 20°C. After centrifugation for 15 min at maximum speed, the pellets were washed with 70 % (v/v) ethanol, air dried, and each dissolved in 10 µl sterile distilled water, or 1 x TE, pH 8.0.

2.7.3 Labelling Insert DNA with Non-radioactive Digoxigenin-11-dUTP.

The labelling reaction was according to the manufacturer's instructions. Reagents were combined in a sterile microfuge tube on ice:

100 ng purified insert DNA

2 µl 10 x hexanucleotide mixture (random hexanucleotides, 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mM Dithioerythritol [DTE], 2 mg ml^{-1} BSA, pH 7.2).
2 µl 10 x dNTP labelling mixture (1 mM dATP, 1 mM dCTP, 1 mM d GTP, 0.65 mM dTTP, 0.35 mM DIG-11-dUTP, pH 7.5)
sterile distilled water to make up volume to 19 µl
1 µl Klenow enzyme (2 Units).

The reaction mixture was incubated overnight at 37°C. 2 µl of 0.2 M EDTA, pH 8.0 was added to stop the reaction, and the labelled DNA precipitated with 0.1 volume 4 M lithium chloride and 2.5 volume absolute ethanol (-20°C). The mixture was well mixed and incubated at -70°C for 30 min, or 2 h at -20°C. The labelled DNA was pelleted by centrifugation for 15 min at 13,000 x g at 4°C, the pellet washed in 100 µl of 70% ethanol, and dried under vacuum. It was resuspended in 50 µl of 1 x TE buffer, pH 8.0, and immediately used as probe DNA, or stored at -20°C for later use. The concentration (yield) of the labelled probe was determined by activation of the label (DIG-11-dUTP) with either the colour or chemiluminescent detection reagents, and comparison of the signal to that of a DNA standard supplied with the kit.

2.7.4 DNA -DNA Hybridization with DIG-labelled probes

Prehybridization and hybridisation with DIG-labelled probes were as described for colony hybridisation (section 2.5.4.2), except that the reagents used were those supplied with the kit (Boehringer Mannheim, Biochemica, section 2.1.1). After overnight hybridisation at 68°C, the membranes were washed twice, 5 min per wash in 2 x SSC, containing 0.1 % SDS at room temperature, followed by two washes, 15 min each in 0.1 x SSC, containing 0.1 % SDS at 68°C. These washes were to remove unbound probe, which could otherwise lead to high background. The membrane was equilibrated in buffer 1 (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 5 min at room temperature. 0.3 % Tween 20 was included in buffer 1 if a chemiluminescent detection method was to be used.

The membrane was then gently agitated in 100 ml of buffer 2 (blocking reagent stock solution diluted 1:10 in buffer 1) for 60 min at room temperature, to block non-specific sites. The membrane was then incubated in the anti-DIG-alkaline phosphatase antibody

(diluted 1:5,000 in buffer 2) for 30 min at room temperature, with gentle agitation to ensure the antibody covered the whole membrane surface. The membrane was transferred to a fresh tray, and washed twice, 15 min each in buffer 1 containing 0.3 % Tween 20. The membrane was next equilibrated 2 min in 20 ml of buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), to 'activate' the alkaline phosphatase conjugated to the antibody. While ensuring that the membrane was kept wet, the hybridization signal was then detected by either chemiluminescent or colorimetric detection method, depending on availability of reagents.

2.7.4.1 *Chemiluminescent detection method*

AMPPD (Lumigen TM PPD), diluted 1:100 in buffer 3, was applied onto the DNA side of the membrane, inside an open hybridisation bag at a ratio of approximately 0.5 ml per 100 cm^2, by scattering drops of the substrate over the whole surface with a sterile pipette. The top sheet of the bag was then gently lowered over the membrane, thereby spreading the liquid to form a continuous film over the membrane. The surface of the top sheet was gently wiped with a paper towel to remove any air bubbles and create a liquid seal over the membrane, and the membrane incubated at room temperature for 5 min in the dark. Excess liquid was removed by rolling a pipette over the top sheet, then the bag sealed, making sure not to trap air bubbles inside. The sealed bag was incubated at 37°C for 15 min to bring the alkaline phosphatase chemiluminescent reaction to steady state, then the membrane exposed to X-ray film at room temperature, with intensifying screen. The film was developed after 1 h, and where the signal was not sufficient, exposure increased to 2-3 h, as necessary.

2.7.4.2 *Colorimetric detection method*

For preparation of the colour substrate, 45 µl NBT solution and 35 µl X-Phosphate (the colour detection reagents) were mixed in 10 ml of buffer 3. The substrate solution was immediately poured over the membrane in a tray or a sealed plastic bag, and the membrane incubated at room temperature in the dark, making sure to exclude air bubbles, and not to shake the container until colour development was complete (few minutes to

12h). The membrane was washed in 50 ml of buffer 1 for 5 min to stop the reaction, after spots of desirable size had developed. Results were documented by photographing, photocopying, or dried and stored at room temperature. To revitalise the colour on dry membranes, the latter were wet in buffer 4 (100 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.8 AMPLIFICATION OF PHYTOPLASMA DNA BY THE POLYMERASE CHAIN REACTION (PCR).

2.8.1 Detection of Phytoplasma DNA in Palm Tissue by PCR

Six different 16S rRNA oligonucleotide primers were used for PCR to prime the amplification of phytoplasma 16S rDNA from LD-infected palms. They were:

1. Rohde forward primer, 5'-GAGTACTAAGTGTCGGGGCAA-3'
2. Rohde reverse primer, 5'-AAAAACTCGCGTTTCAGCTAC-3' (1 and 2 are a primer pair developed for specific amplification of LD phytoplasma rDNA by Rohde *et al.* (1993).
3. Forward primer P1, 5'-AAGAGTTTGATCCTGGCTCAGGATT-3'
4. Reverse primer P6, 5'-TGGTAGGGATACCTTGTTACGACTTA-3' (3 and 4 are the Mollicute-specific primer pair developed by Deng and Hiruki (1991a).
5. Forward primer LD 16-1, 5'-CGGAAACCTTCGGGTTTTAG-3', synthesised from LD phytoplasma sequences (Harrison, 1994)
6. Reverse primer LD SR, 5'-GGTGCCATATATATTAGATTG-3', synthesised from the 16S-23S intergenic region of LD phytoplasma (Tymon, 1995).

PCR conditions were optimised for each of the six primer combinations used, namely; Rohde's forward / Rohde's reverse; Rohde's forward / LD SR; LD 16-1 / Rohde's Rev; LD 16-1 / LD SR; P1 /P6; and P1 / LD SR.

For PCR, a 25 µl reaction mixture contained about 50 ng template DNA extracted from LD-infected tissue, 150 µM mixed deoxynucleotide triphosphates (dNTPs), 50

ng each primer, 1 Unit of Taq Polymerase (Promega Corporation), and standard PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01 % gelatin, 0.5 % (v/v) Nonidet P40, 0.5 % (v/v) Tween 20). The mixture was overlaid with 25 µl of mineral oil, and subjected to 36 cycles in an automated thermocycler (Biometra, UNO Thermoblock) using the following parameters: 1 min (2 min for the first cycle) denaturation at 94°C, 1 min 20 s of annealing at 57°C, and 2 min 10 s (5 min for last cycle) extension at 72°C. Reaction mixtures containing DNA template from healthy palms, and/or water substituted for DNA served as negative controls in each experiment. The annealing temperature varied depending on primer combination as follows: 57°C for Rohde F / Rohde Rev., 54°C for Rohde F / LD SR, 53°C for LD 16-1 / LD SR, and 52°C for P1 / LD SR, P1 /P6, and LD 16-1 / Rohde Rev., respectively.

PCR products were analysed by electrophoresis through a 1 % (w/v) agarose gel as described before (section 2.3.4), and visualised in the gel by UV transillumination after staining with ethidium bromide.

2.8.2 Screening Putative Insect Vectors for Phytoplasma DNA by PCR.

Homopteran insects suspected to be putative vectors of LD were collected from the leaves of palms showing typical LD symptoms, and DNA extracted from them as described before (section 2.3.6). DNA extracted from single insects or batches of three to four insects were screened by PCR for presence of phytoplasma DNA. DNA of 5,000 out of the 15,000 insects extracted was screened by PCR. Primer pairs used to screen insects were either Rohde's forward and reverse, or Rohde's forward and LD SR. DNA bands amplified from some of the insects during PCR reactions were digested with restriction enzymes to confirm whether the restriction fragments were similar to those found in the phytoplasma DNA amplified from LD-infected coconut tissue.

2.9 DETERMINATION OF LD-ASSOCIATED PHYTOPLASMA STRAINS

The genetic relationship of the LD-phytoplasma to phytoplasmas associated with LY in Jamaica and Florida, and LYD in Kenya, Mozambique, and Ghana, as well as 12 other non-coconut phytoplasmas was determined by restriction fragment length polymorphisms (section 2.9.1). The relationship to LYD in Ghana and Mozambique was also determined by dot blot hybridization. Dot and southern blots were used as well, to determine whether phytoplasma isolates from different regions of Tanzania showing significant differences in disease incidence are similar or different strains.

In an alternative approach to study the possible existence of strains of phytoplasma associated with LD in different regions of Tanzania, a portion of the 16S ribosomal RNA gene was amplified, cloned and sequenced. The more variable intergenic region between the 16S and 23S ribosomal RNA genes of the phytoplasma isolates, was similarly studied (sections 2.9.2 - 2.9.4).

2.9.1 Restriction Fragment Length Polymorphisms (RFLP)

5 µg of total DNA for each of the phytoplasma and spiroplasma isolates tested was digested overnight with *Eco* RI enzyme, or *Eco* RI and *Hind* III according to standard procedures (Section 2.4.3). Test isolates included LD phytoplasma isolated from four locations in Tanzania, LYD (two isolates each) from Kenya, Mozambique, and Ghana, LY isolates from Florida and Jamaica (one each), as well as the DNA of 12 other phytoplasmas and 2 spiroplasmas (section 2.2.3)). For double digestion of non-coconut phytoplasmas and spiroplasmas with *Eco* RI and *Hind* III enzymes, 1 µg of total DNA was digested. Each double digestion reaction, whether digesting 1 or 5 µg DNA, used 2 Units of each enzyme, and reaction buffer 2. Only 1 µg of the Florida LY isolate was digested in each reaction due to limited quantities available. After electrophoresis in a 1 % (w/v) agarose gel, the digests were blotted onto nylon membranes (Hybond, Amersham) by the method of Southern (section 2.6.2). The blots were UV-crosslinked, then prehybridized and hybridized to DIG-labelled probe

LD 12-66, as described before (section 2.7.4). Detection of the hybridization signal was by chemiluminescent method (section 2.7.4.1).

For preparation of dot blots, 1 µg of total DNA in a volume of 20 µl was mixed with 3 µl of 2 N NaOH and denatured by boiling for 10 min at 100^{0}C in a boiling water bath. The mixture was cooled on ice for 5 min, then neutralised by addition of 3 µl of 2 M Tris-HCl, pH 7.0. The DNA was then diluted with an equal volume of 20 X SSC, and blotted onto nylon membranes as single spots using a dot blot manifold as described before (section 2.7.1). The membranes were UV cross-linked, then prehybridised and hybridised as above. Detection was by either chemiluminescent or colourimetric method depending on availability of reagents.

2.9.2 Amplification of the 16S Ribosomal DNA (rDNA) and 16S-23S Intergenic Spacer Sequences by PCR

Oligo 4, the 16S forward oligoprimer, 5'-GAA GTC TGC AAC TCG ACT TC and Oligo 7, the 23S reverse oligoprimer, 5'-CGT CCT TCA TCG GCT CTT (Kirkpatrick *et al.*, 1994a) were used to prime the amplification of the intergenic spacer region (SR) between the 16S and 23S ribosomal RNA genes of the LD-associated phytoplasma. Three LD DNA samples were tested for each of the three locations representing particular disease incidence categories. Kifumangao was representative of the high incidence area, and Chambezi and Kigombe, the moderate and low incidence areas respectively. A 25 µl reaction mixture contained about 100 ng template DNA extracted from LD-infected tissue, 150 µM mixed deoxynucleotide triphosphates (dNTPs), 50 ng each primer, 1 Unit of Taq Polymerase and standard PCR buffer. The mixture was overlaid with 25 µl of mineral oil, and subjected to 35 PCR cycles in an automated thermocycler (Robocycler) using the following parameters: 1 min denaturation at 94°C, 2 min at 53°C, and 2 min (10 min for last cycle) extension at 72°C. Reaction mixtures without DNA template, and water substituted for DNA served as negative controls in this experiment. For amplification of 16S ribosomal DNA (rDNA), Rohde's forward and reverse primers, and the reaction conditions described before (section 2.8.1) were used.

2.9.3 Cloning of the PCR Amplification Products

PCR generated rDNA fragments were cloned directly into the pTAg vector by use of the LigATor kit (R and D Systems Europe Ltd). The vector, provided with the kit, was already pre-cut and contained T overhangs, which facilitated direct ligation of the PCR fragments that have single A overhangs at the 3' ends.

2.9.3.1 *Purification of insert DNA*

Gel electrophoresis in 1% (w/v) agarose was used to check the purity of the PCR products. For the products from the spacer regions there were three bands initially amplified (data not shown). This made it necessary to re-amplify the PCR products in order to obtain single clean bands of the product. The PCR products were then electrophoresed in 1% (w/v) agarose, and specific bands excised under longwave UV. The DNA fragments were recovered from agarose by use of the Geneclean II Kit, and dissolved in 10 µl of 1 x TE.

2.9.3.2 *Ligation of the purified fragment to the pTAg vector*

Ligation reactions were set up using only 2 µl of the PCR fragment. Each of the three samples from the three locations of Kifumangao, Kigombe and Chambezi respectively were amplified and ligated. For ligation, the sample DNA fragment was combined in a 1.5 ml microfuge on ice with:

1 µl 10 x Ligase buffer

0.5 µl 100 mM DTT

0.5 µl 10 mM ATP

1 µl 50 ng μl^{-1} pTAg vector

Nuclease-free water to make up volume to 9.5 µl.

The tube was vortexed briefly to mix contents, then centrifuged briefly to bring the contents to the bottom of the tube. 0.5 µl of T4 DNA ligase (2-3 Weiss units) were added, and the contents gently mixed with a pipette tip, then incubated overnight at 16°C. Control ligation reactions were also set up. In one reaction, a 50 bp control insert

DNA provided with the kit (has single A overhangs at each end for compatible cloning into the vector) was used, while in the other, insert DNA was replaced by nuclease free water. These controls were intended to respectively test the efficiency of the ligation reaction, and the integrity of the T overhangs of the pTAg vector, which would prevent self ligation if intact.

2.9.3.3 *Transformation of competent cells*

The transformation procedure was similar to the one described before (section 2.4.5), except that a different type of competent cells was used, and hence modification to the procedure. LigATor kit competent cells {endA1 hsdR17($rk_{12}^{-}m_{k12}^{+}$)supE44thi-1recA1 gyrA96relA1 lac[F'proA^{+}B^{+}lacI^{q}ZΔM15::Tn10(Tc^{R})]} were thawed on ice, then gently mixed by inverting, and 20 µl pipetted into a pre-cooled 1.5 ml microcentrifuge tube on ice. 1 µl of the ligation mixture was added to the cells, and the tube gently tapped to mix the contents, then incubated on ice for 30 min. The cells were then given a heat shock for exactly 40 s in a 42°C waterbath, and incubated on ice for 2 min. 80 µl of the thawed SOC medium was added to the tube, and the cells allowed to recover by shaking the tubes at 250 rpm on a rotary shaker at 37°C for 1 h. A control transformation reaction was included whereby the ligation reaction was replaced by 1 µl of a test plasmid provided with the kit. 50 µl of each transformation mixture was plated on an LB plate containing 50 µg ml^{-1} ampicillin and 15 µg ml^{-1} tetracycline. The plates were left at room temperature for 5 min for the liquid to be absorbed, then the plates inverted and incubated overnight (at least 16 h) at 37°C.

2.9.4 Screening of Recombinant Colonies

White colonies were selected for further screening for presence of insert DNA. The white colour was a basis for identifying potential recombinant colonies because insert DNA cloned into the pTAg vector would interrupt the LacZα peptide sequence. The interruption would block the vector's ability to complement the LacZ peptide synthesised in the competent cells for production of functional β-galactosidase

enzyme in presence of X-gal. The resulting absence of the enzyme would cause production of white colonies instead of the usual blue colour.

Transformants were rapidly screened for the presence or absence of DNA inserts by the colony PCR technique (Gussow and Clackson, 1989; Pampfer, 1993). A bulk PCR enzyme pre-mix (for 100 colonies) was prepared by combining in a microcentrifuge tube on ice:

100 µl 10x Taq buffer

100 µl (0.2 mM) 2mM dNTPs

0.5 nmol pTAg SEQ5' primer

0.5 nmol pTAg SEQ3' primer

5 Units Taq polymerase

Sterile distilled water to make volume to 1 ml

10 µl aliquots of the PCR pre-mix were dispensed into 0.5 ml PCR tubes, and overlaid with 30 µl mineral oil. Each colony was then touched lightly with a sterile toothpick, and the bacteria inoculated into the sequentially numbered PCR mix-containing tubes. The PCR reaction was performed for 30 cycles, as follows:

PCR Cycles	Denaturation	Annealing	Synthesis
1 x	94°C 1.5 min	50°C 30 s	72°C 3 min
9 x	94°C 30 s	50°C 30 s	72°C 3 min
10 x	94°C 30 s	50°C 30 s	72°C 4 min
10 x	94°C 30 s	50°C 30 s	72°C 5 min

At the end of the reaction, 2.5 µl of gel loading dye was added to each tube, and the samples electrophoresed in 1 % (w/v) agarose as described before (section 2.3.4). The two pTAg primers anneal at approximately 100 bp on either side of the T cloning site. Thus, non-recombinant transformants were identified as bands of 200 bp, whereas the recombinants were the larger bands of about 700 bp.

2.9.5 DNA Sequencing

The rDNA products were prepared for sequencing with Taq DNA Polymerase (Taq DyeDeoxy Terminator Cycle sequencing) according to the ABI instructions for the Catalyst robotic workstation and cycle sequencing protocols, which uses fluorescent dideoxy nucleotides. All reagents were provided in the ABI Taq DyeDeoxy™ Terminator Cycle Sequencing Kit.

The PCR templates were purified for sequencing according to the directions of the kit manufacturers. For cycle sequencing, a reaction premix of kit reagents (for 20 reactions) was prepared by mixing in a tube on ice:

80 µl	5 x TACs Buffer
20 µl	dNTP Mix
20 µl	DyeDeoxy™A Terminator
20 µl	DyeDeoxy™T Terminator
20 µl	DyeDeoxy™G Terminator
20 µl	DyeDeoxy™C Terminator
10 µl	AmpliTaq® DNA Polymerase

5 x TACS Buffer (Terminator Ammonium Cycle Sequencing buffer) = 400 mM Tris-HCl, 10 mM $MgCl_2$, 100mM $(NH_4)_2SO_4$, pH 9.0.

dNTP mix = 750 µM dITP, 150 µM dATP, 150 µM dTTP, 150 µM dCTP

The reagents were thoroughly mixed by tapping the bottom of the tube several times to ensure uniform distribution of the enzyme, and stored at 4°C. Cycling reactions were then prepared in labelled 0.6 ml double-snap-cap microcentrifuge tubes on ice by mixing:

9.5 µl	Reaction premix
7.0 µl	Purified PCR fragments
3.2 pmol	Primer for purified PCR fragments

Deionized water to make up total volume to 20 µl.

Each reaction mixture was overlaid with a drop of mineral oil approximately 40 µl.

The tubes were placed in a thermal cycler (Perkin Elmer Cetus Model 9600) preheated to 96°C, and immediately subjected to 25 cycles as follows:

- Rapid thermal ramp to 96°C
- 96°C for 15 s
- Rapid thermal ramp to 50°C
- 50°C for 1 s
- Rapid thermal ramp to 60°C
- 60°C for 4 min
- After the last cycle, rapid thermal ramp to 4°C and hold.

2.9.6 Purification of Extension Products and Separation of Bands on the Sequencing Gel

The sequencing extension products were purified on Centri-Sep™ spin columns to remove excess DyeDeoxy™ Terminators according to manufacturer's instructions. Each column to be used was gently tapped to cause the gel material to settle to the bottom of the column. The column stopper was then removed, 0.75 ml of deionized water added, and the column stoppered and inverted several times to mix. The gel was allowed to hydrate for 30 min at room temperature, and air bubbles removed by inverting the column several times and allowing the gel to settle to the bottom. The upper-end cap was then removed followed by the lower-end one, and the column allowed to drain completely by gravity. The column was then placed into the provided wash tube, and centrifuged at 1300 x g for 2 min to remove the interstitial fluid. The column was then placed into a sample collection tube, and the whole 20 µl of the reaction mixture loaded onto the column, avoiding the oil as much as possible. The column was again centrifuged for 2 min at 1300 x g. The eluate (sample) was dried carefully in a vacuum centrifuge, avoiding heat and overdrying.

5 µl of deionized formamide was mixed with 1 µl of 50 mM EDTA, pH 8.0, and 4 µl of this mixture added to the sample tube and agitated vigorously to dissolve the dry residue. The solution was centrifuged briefly to bring all liquid to the bottom of the tube. When the gel was ready for loading, all samples were heated at 90°C for 2 min

to denature the DNA, then cooled on ice, and immediately loaded onto the DNA sequencer (ABI, Model 373A, Version 1.2.1) according to the instructions in the User's manual.

Chapter 3: DEVELOPMENT OF DNA PROBES

3.1 INTRODUCTION

For any successful disease control programme, early and accurate diagnosis of the pathogen is essential to effectively manage and / or eradicate plant infections. Diagnosis of the coconut lethal disease in Tanzania, like many other diseases caused by phytoplasmas, has been most difficult to tackle. The main obstacle has been lack of a simple, sensitive and specific method for pathogen detection. Difficulty to develop a reliable detection method has been a consequence of the inability to isolate the pathogen from the host *in vitro*. Lack of a detection method for the LD pathogen has also been the main factor limiting our understanding of disease epidemiology, making it difficult to devise a sound control programme.

LD diagnosis has for a long time depended on biological characteristics such as the disease syndrome, complemented by observation of the organisms in the phloem of infected tissue with the electron microscope. However, electron microscopy has limitations. The preparation of tissues is time consuming, and the equipment is expensive to maintain, which makes the technique unviable for routine use. Thus, LD infections have been diagnosed routinely by staining razor-cut sections from LD-infected tissue with a fluorochrome, DAPI (4'-6'-diamidino 2-phenylindole 2HCl). Adoption of this technique is based on the association of fluorescence in LD-infected tissues with presence of phytoplasmas by electron microscopy (Deutsch and Nienhaus, 1983). However, this technique is not specific, because DAPI stains all DNA-containing organisms, and cannot differentiate between different phytoplasmas.

The development of new detection techniques which target only the genome or coat protein of the pathogen has overcome problems formerly hindering correct diagnosis of phytoplasma infections (Lee and Davis, 1988). Nucleic acid hybridization probes are one of those techniques. Their production and utilisation has been facilitated by

the recombinant DNA technology. This technology has made it possible to clone 'random' fragments of phytoplasma DNA from infected plants or insect vectors, and use them as molecular probes for specific pathogen detection and disease diagnosis (Davis *et al.*, 1988a; Kirkpatrick *et al.*, 1987; Kollar *et al.*, 1990; Lee *et al.*, 1990).

The technology of cloning (producing recombinant DNA) provides a convenient means for purifying and propagating specific segments of DNA (Grierson and Covey, 1984). For production of a phytoplasma DNA probe, phytoplasma DNA is first digested with a restriction enzyme to produce random fragments. These are inserted into a similarly cut cloning vector that is capable of replicating in bacteria (*Escherichia coli*) by recombination *in vitro*. The chimaeric molecules containing the vector DNA and insert phytoplasma DNA are then introduced into individual bacterial cells, and the latter separately multiplied. The multiple copies of inserted phytoplasma DNA can subsequently be recovered by purifying the hybrid vector from bacterial cells and separating out the vector DNA with an enzyme. Selected phytoplasma DNA thus multiplied is then labelled and used as a probe in nucleic acid hybridizations to detect the presence of complementary sequences in infected tissue.

Purification of phytoplasma DNA has been one of the limitations to the production of specific phytoplasma DNA probes, because of the relatively low titres in plant hosts. Production of specific probes to phytoplasmas has been possible when the organisms could be propagated in herbaceous hosts (Sears *et al.*, 1989; Davis *et al.*, 1990; Harrison *et al.*, 1991), or insect vectors (Kirkpatrick *et al.*, 1987; Davis *et al.*, 1988a), in which the organisms readily multiply to high titres and can be purified. This is because the phytoplasmas do not generally multiply to high titre in their hosts, they are phloem-restricted, and separation of the pathogen from host tissue during DNA extraction is very difficult. The difficulties in extraction often leads to production of probes which anneal to host DNA during hybridization reactions, and thus reduce the sensitivity of pathogen detection by the respective DNA probes.

The approach of extracting DNA from herbaceous hosts or insect vectors in order to avoid background hybridization to host DNA, has not been utilised for coconut

infecting phytoplasmas, because it has not been possible to transmit these organisms to herbaceous hosts. Production of probes to these diseases has been complicated further by the fact that the concentration of phytoplasmas in coconut tissue is generally very low (Deutsch and Nienhaus, 1983; Thomas, 1979). Vectors are another limitation, since the vector for LD is still unknown. However, the vector for LY is known, and the disease infects other palm species in which the concentration of phytoplasmas have been found to be reasonably high for DNA extraction (Harrison *et al.*, 1992). Disease specific probes have been produced using DNA extracted from the apical meristems of LY-infected manila (*Veitchia merrillii*) palm. This has helped to overcome the problem of contamination with host DNA. Similarly, DNA for screening purposes has been successfully extracted from the apical meristems of LY infected coconut palms.

The report of Harrison *et al.* (1992), and our experiences on diagnosing LD infections by the DAPI technique (Schuiling and Mpunami, 1990), were the basis for the decision to attempt in this study, to produce DNA probes to the LD phytoplasma by using DNA from infected coconut tissue. Despite anticipated difficulties in extraction, it was considered feasible that careful selection of LD infected palms would provide coconut tissues of sufficient phytoplasma concentration to produce reasonable DNA yields for production of probes. The probes might facilitate pathogen detection, and assist in identification of the insect vector(s).

3.2 PURIFICATION OF DNA FROM PALM TISSUE

3.2.1 Efficiency of Total DNA Extraction Procedures

All the procedures tested for extraction of total DNA from coconut tissue were effective in isolating DNA that was clean enough for use in various assays, including hybridizations and PCR. This was reflected in the $A_{260/280}$ ratios (Table 3.1) of the different preparations. However, preparations extracted by the phytoplasma-enrichment procedures of Harrison *et al.* (1992), and Ahrens and Seemuller (1992) respectively, were better.

Table 3.1 **Quantity and optical density ratios ($A_{260/280}$) of total DNA obtained from LD-infected coconut tissue by different extraction procedures**

Total DNA (µg gm^{-1} Fwt), and corresponding $A_{260/280}$ ratios (in parenthesis)

Location / Palm No.	Harrison *et al.* (1992)	Dellaporta *et al.* (1983)	Ahrens and Seemuller (1992)	Doyle and Doyle (1990)	Rohde *et al.* (1993)
Kenya, No.1	20 (1.8)	100 (1.9)	10 (1.9)	90 (1.9)	90 (1.9)
Kenya, No.2	10 (1.7)	90 (1.9)	10 (1.9)	180 (1.8)	200 (1.9)
Kenya, No.3	10 (1.7)	130 (1.9)	20 (1.9)	210 (1.8)	270 (1.9)

This is because they contained smaller quantities of contaminating substances with peak absorbency at 210-220 nm as seen on sample print-outs of the spectrophotometer absorbency readings (Fig. 3.1). The amount of these substances was higher in DNA extracted by the other procedures (Fig. 3.1). A possible explanation for these differences is that the former methods use differential centrifugation to eliminate chloroplasts, starch, and carbohydrates, before the cell nuclei are lysed with SDS or CTAB to release DNA. This step also reduces polysacharrides and other carbohydrate-associated particulate material that often adhere to DNA following centrifugation and become co-extracted with it. Some of these substances have been reported to be the main source of contamination to plant DNA (Jofuku and Goldberg, 1989). Chloroplast DNA is very similar to prokaryote DNA (Grierson and Covey, 1984), and so it would be unlikely to contribute to that type of peak. This was verified by DNA obtained by the procedure of Rohde *et al.* (1993). Although Triton X-100, which causes complete lysis of the chloroplasts was included in the extraction buffer, the DNA still contained the contaminants. According to Jofuku and Goldberg (1989), leaf tissue does not contain starch. Since DNA was extracted from young leaves, either carbohydrates, polysacharrides, carbohydrate-associated particulate material, phenolic compounds or their combination may have contributed to the observed absorbancy at short wavelengths.

Total DNA yields varied with the extraction method. Yields were lowest for the procedures of Harrison *et al.* (1992), and Ahrens and Seemuller (1992) that enrich for phytoplasmas and mitochondria early in the extraction process (Table 3.1). The reason for this is that by differential centrifugation at the beginning of extraction, these procedures enrich for phytoplasmas and mitochondria, and eliminate chloroplasts and plant chromosomal nuclei that would otherwise be a source of additional (undesirable) DNA (Sears *et al.*, 1989). Thus, the DNA obtained by these methods, was more enriched for phytoplasma than that from other procedures. This was demonstrated in the number of repeated density gradient centrifugation steps which were necessary to produce a visible band of phytoplasma DNA. When DNA produced by the procedure of Harrison *et al.* (1992) was loaded onto CsCl-bisbenzimide density gradients and centrifuged, it was possible in one experiment to

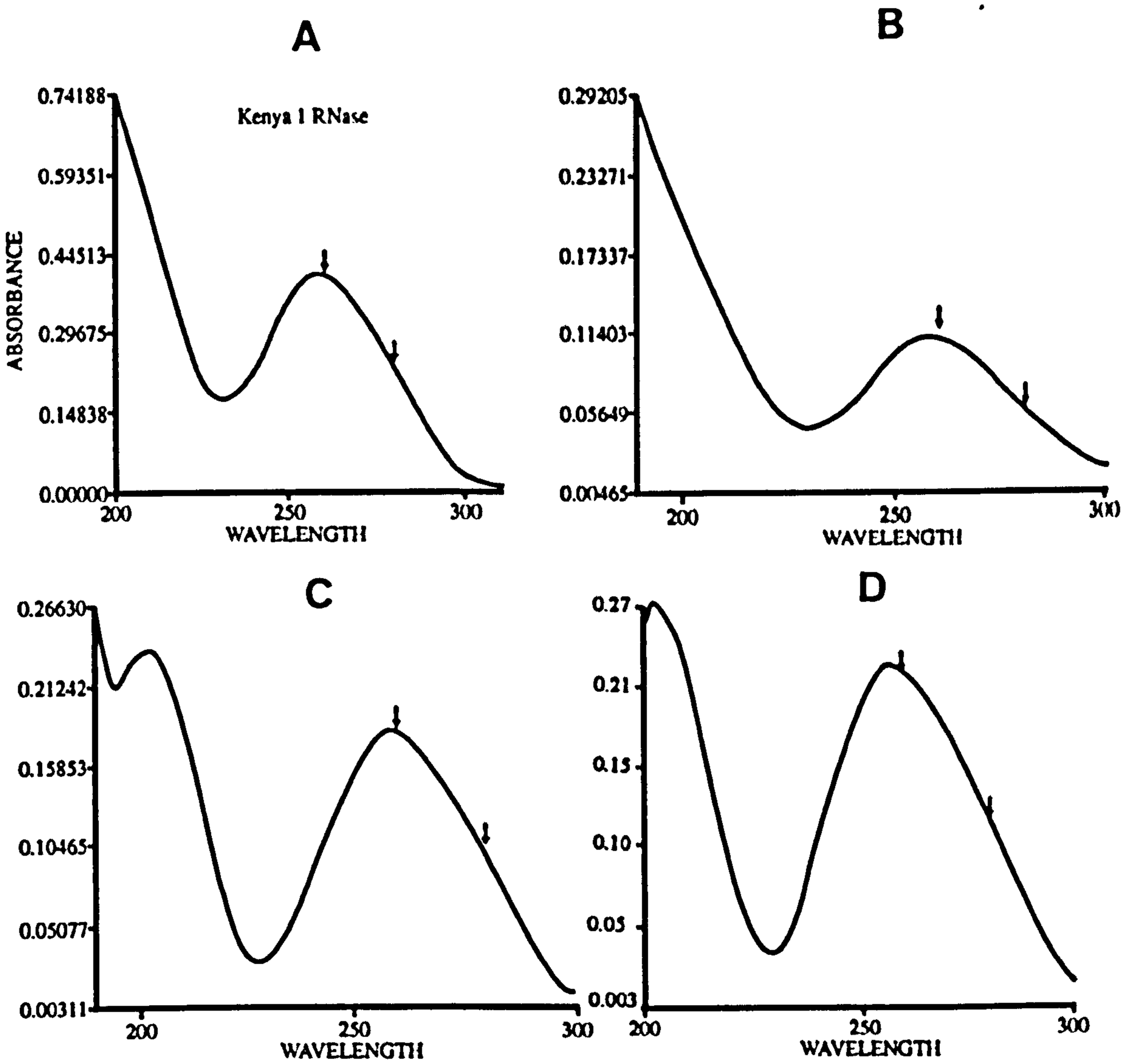


Fig. 3.1: Spectrophotometer absorbency readings for DNA samples extracted by four different procedures from the meristematic tissue of one LD-infected coconut palm.

(A) Phytoplasma-enrichment procedure of Harrison *et al.*, 1992.

(B) Phytoplasma-enrichment procedure of Ahrens and Seemuller, 1992.

(C) CTAB extraction procedure of Doyle and Doyle (1990)

(D) Rapid extraction procedure of Rohde *et al.*, 1993.

Arrows indicate wavelengths (260 nm and 280 nm) and the corresponding light absorbency values attributed respectively to nucleic acids and proteins in each sample.

visualise a weak phytoplasma-band after three rounds of pooling and re-centrifugation of the top band (i.e. combining fractions from 6 gradients). However, in gradient separations where DNA prepared by other methods was loaded onto the gradients, at least five rounds of centrifugation (i.e. combining fractions from at least 10 gradients) were necessary to produce a visible phytoplasma-DNA band. Therefore, the higher yields obtained by the procedures which do not enrich for phytoplasmas (Table 3.1) did not reflect the amount of phytoplasma DNA obtained, but rather a mixture with DNA from chloroplast, mitochondria and chromosomal coconut DNA, in addition to other contaminants observed above.

3.2.2 Determination of Total DNA Quality

The quality of DNA obtained by different extraction procedures was ascertained by blotting and hybridization with DIG-labelled phytoplasma ribosomal DNA (rDNA) that had been amplified from LD-infected coconut tissue using LD-specific primers (Rohde *et al.*, 1993) (Plate 3.1). Phytoplasma DNA was detected in all extracts, including that from an apparently healthy coconut palm. Detection was in as little as 7.8 ng of blotted total DNA. However, there were no clear differences in the hybridization signal to the ribosomal DNA by the DNA from different extraction procedures. The signal was almost equally strong (spot size) for the DNA extracted by the phytoplasma enrichment procedures of Harrison *et al.* (1992), and Ahrens and Seemuller (1992) respectively, and the other three methods (Plate 3.1).

The sensitivity demonstrated by the DIG probes during this initial test was the basis for selecting DIG labelling as a nonradioactive method for use in field screening tests. Since phytoplasmas could be detected in total DNA extracted by all of the tested procedures, it was considered safe to adopt for field screening extraction methods which are simple, quick and do not require sophisticated equipment and chemicals. Hence, the procedure of Doyle and Doyle (1990), was modified for routine DNA extraction in field experiments by eliminating the need to use liquid nitrogen.

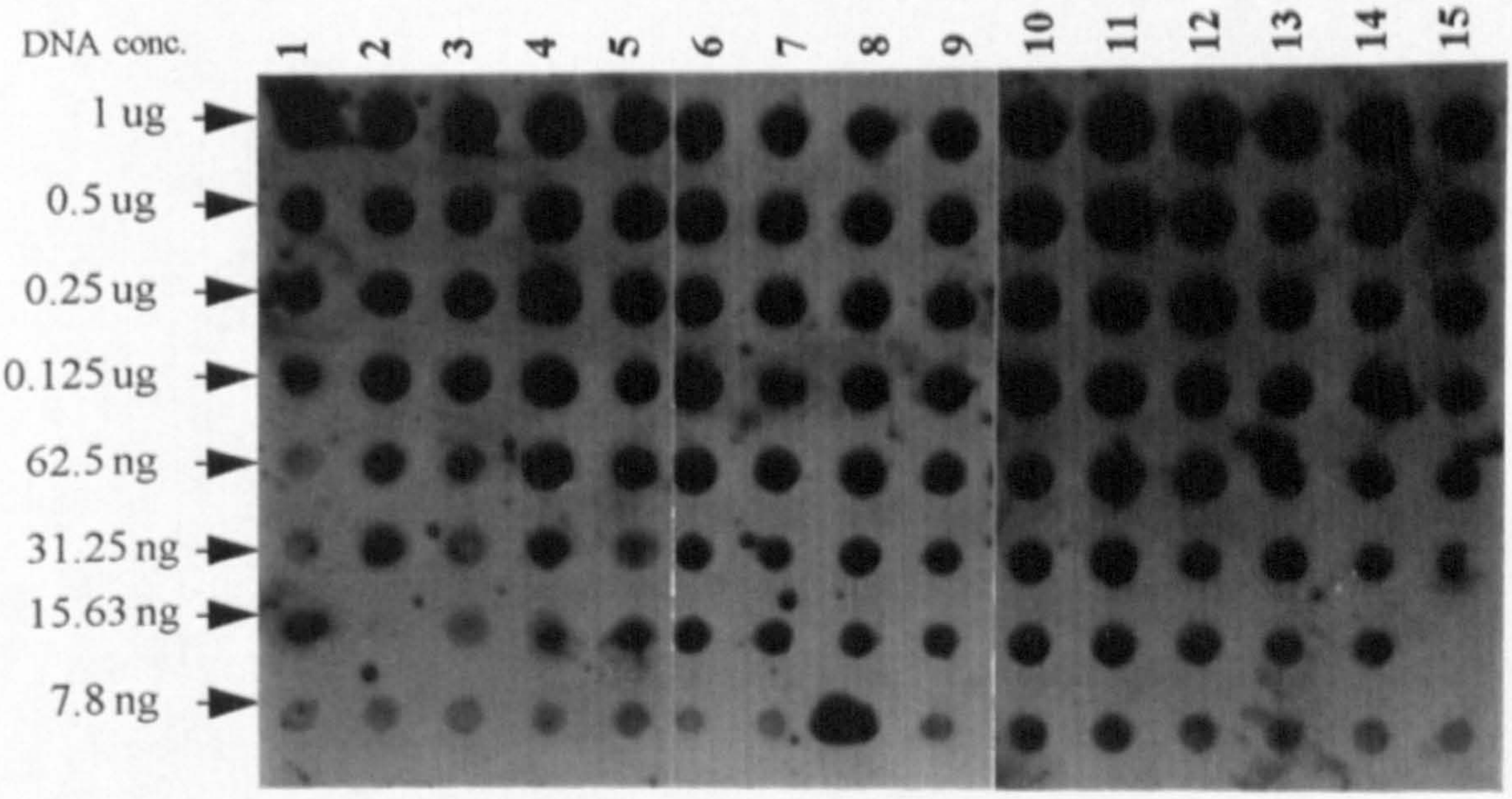


Plate 3.1: Dot blot of total DNA extracted by different procedures from LD infected coconut hybridized to a DIG-labelled LD rDNA probe. Probe DNA was amplified from LD infected coconut DNA using Rohde's forward and reverse primers.

The extraction procedures were: Phytoplasma enrichment procedures of Ahrens and Seemuller (1992, lane 1-3), and Harrison *et al.*(1992, lane 4-6). Others were Dellaporta's miniprep method (lanes 7-9), CTAB method of Doyle and Doyle (lane10-12) and Rohde's rapid extraction method (Rohde *et al.*, 1993)(lane 13-15).

3.2.3 Separation of Phytoplasma DNA by CsCl-Density Gradient Ultracentrifugation

The first round of density gradient centrifugation with DNA from LD-infected coconut resulted in separation of one major band and two minor ones below it. The major band had a buoyant density of 1.658 g cm^{-3}. The two minor bands had buoyant densities of 1.678 and 1.720 g cm^{-3} respectively (Plate 3.2). A 1 ml fraction immediately above the main band in each gradient was withdrawn from gradients, pooled, and centrifuged again. After repeating this procedure 3-5 times, a faint band was evident approximately 1 mm above the much diminished major band (Plate 3.2). This unique band with a buoyant density of 1.619 g cm^{-3}, was considered to be enriched for phytoplasma-DNA and was carefully recovered for molecular cloning.

Approximately 2.5 μg of this enriched phytoplasma-DNA was recovered after pooling fractions from the first six gradients (which were concentrated over three rounds of centrifugation). This amount represents 0.10 % of the total DNA loaded onto the initial gradients.

The tissue from an apparently healthy coconut palm which was included in the purification procedure as a healthy control, turned out to be already infected by phytoplasma when tested by PCR. Thus, there was no healthy control for comparison among the gradients. For screening purposes therefore, healthy coconut DNA was extracted from tissue that was previously obtained from Chambezi (during 1991), and had been kept frozen at -20°C at Rothamsted.

3.3 CLONING LD PHYTOPLASMA DNA

3.3.1 The LD Recombinant Plasmid Library

Recombinant bacterial colonies (those containing plasmids with insert DNA) were identified by the white colour, different from the blue colour of normal bacteria. A total of 3,118 recombinant colonies were obtained in the cloning of enriched LD phytoplasma

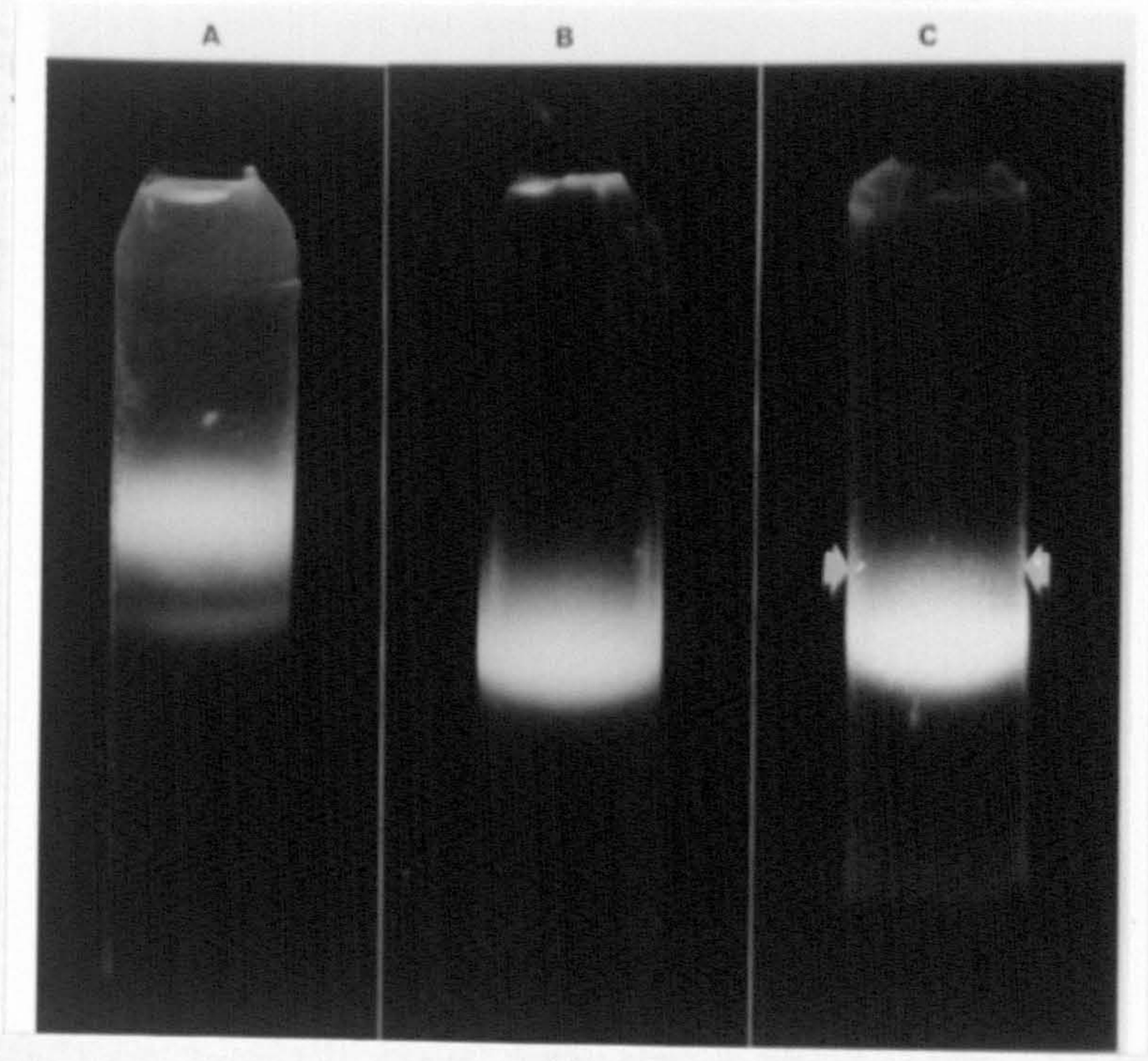


Plate 3.2: Separation of lethal disease phytoplasma DNA from palm DNA. Bisbenzimide/$CsCl_2$ gradients loaded with total DNA from LD-infected coconut after first (A), after second (B) and after third (C) round of ultracentrifugation. The phytoplasma fraction (density, 1.619g cm^{-3}) is marked with arrows. Buoyant density of the main DNA band (coconut) was 1.658g cm^{-3}.

DNA from Kifumangao. Transformations in which a 1:1 ratio of vector to insert was used produced the highest number of recombinant colonies, while the 3:1 ratio produced the lowest number. Whereas transformations using a 1:1 vector to insert ratio produced 2,120 recombinant colonies, 615 colonies were obtained from transformations using the 1:3 ratio, and only 383 colonies were obtained from transformations using the 3:1 ratio. Each of the 3,118 colonies was individually multiplied, and stored in LB broth at - 70^{0}C, as part of the LD recombinant plasmid library.

3.3.2 Differentiation of Recombinant Colonies

Differential colony hybridization with ^{32}P-labelled total DNA from healthy coconut palm and ^{32}P-labelled gradient-enriched phytoplasma-DNA was used to identify potentially useful colonies. 434 colonies hybridized strongly to the phytoplasma-DNA probe, but only weakly or not at all to the healthy coconut DNA (Plate 3.3). These clones, which made up 13.9 % of the total recombinants in the cloning experiment were selected for further screening.

High stringency washing conditions were used in all colony hybridizations during the initial screening tests. This had the advantage of reducing numbers by allowing selection of only the clones homologous to phytoplasma. During further screening, however, this approach was of no advantage because some of the clones give a high signal due to repetitive sequences in the DNA fragment. Selection based on high stringency conditions alone could have led to the discarding of some good probes. Therefore, both moderate and high stringent washing conditions were used when examining the performance of individual DNA inserts as probes.

3.4 SCREENING FOR POTENTIAL PROBES

3.4.1 Southern Blot Hybridizations and Selection of Probes

Seventy of the 434 selected clones which showed stronger hybridization signals to the phytoplasma DNA in differential colony hybridizations were subcultured.

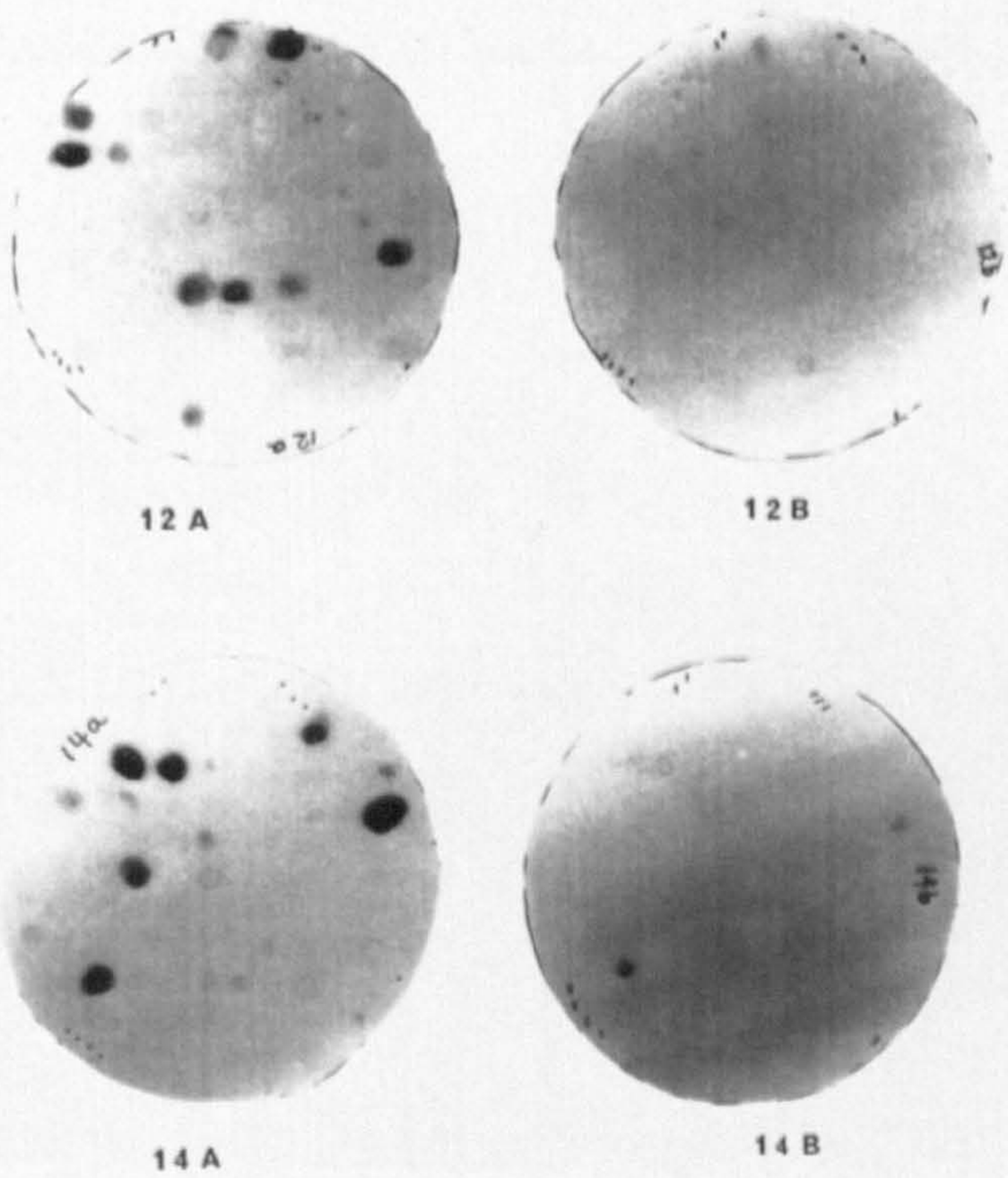


Plate 3.3: Screening of cloned DNA for phytoplasma. Representative filter lifts of transformed colonies probed with (A) ^{32}P-labelled phytoplasma DNA and (B) ^{32}P-labelled healthy coconut DNA. Potentially good clones hybridize to the phytoplasma probe only.

Insert DNA was separated from vector DNA by electrophoresis on 1 % agarose gels, after restriction endonuclease digestion of the recombinant plasmids with *Eco* RI enzyme. Southern blots of the gels were screened using both ^{32}P-labelled gradient-enriched phytoplasma- DNA and ^{32}P-labelled healthy coconut DNA (Plate 3.4). Although 38 clones hybridized strongly to the phytoplasma DNA, only 22 of these hybridized weakly or not at all to healthy coconut DNA (Table 3.2). These 22 were considered potentially good for further screening as probes, but selection of good probes involved other criteria.

To determine the size of insert DNA, the DNA size marker (Lambda DNA digested with *Hind* III and *Eco* RI enzymes) was labelled with ^{32}P and mixed with the probe DNA during hybridization. This made the bands of the marker DNA visible on the autoradiographs. The distance migrated by the size marker bands on the agarose gel was therefore directly proportional to that on the corresponding autoradiograph. The size of the marker DNA bands was plotted against the distance migrated for each radiograph. From a standard curve (Fig. 3.2) so generated for each gel, the distance migrated by insert DNA for all the clones on the blot were estimated by comparing the distance they migrated to that migrated by the marker DNA. For the screened clones, insert sizes ranged between 0.45 kbp and 3.30 kbp (Table 3.2).

From the 22 clones that did not hybridize at all or hybridized weakly to healthy coconut DNA, the best six which contained inserts larger than 1 kilobase pair, and showed a very strong hybridization signal to phytoplasma DNA were selected as potentially good probes. They include (insert size in parenthesis) clones LD 12-1 (1.05 kbp and 1.70 kbp), LD 12-20 (1.05 kbp and 1.70 kbp), LD 12-30 (2.20 kbp), LD 12-66 (2.65 kbp and 0.45 kbp), LD 19-11 (2.65 kbp), and LD 19-87 (1.40 kbp and 1.95 kbp) (Table 3.2). Clones LD 12-30, LD 12-66 and LD19-87 were selected as good probes despite weak hybridization to healthy coconut DNA because of the comparatively larger size of DNA inserts and stronger hybridization signal to phytoplasma DNA. All blots were stripped after hybridization to the phytoplasma DNA probe, and hybridized with healthy periwinkle DNA, in order to identify clones which were not LD specific, but had not been detected by healthy coconut DNA.

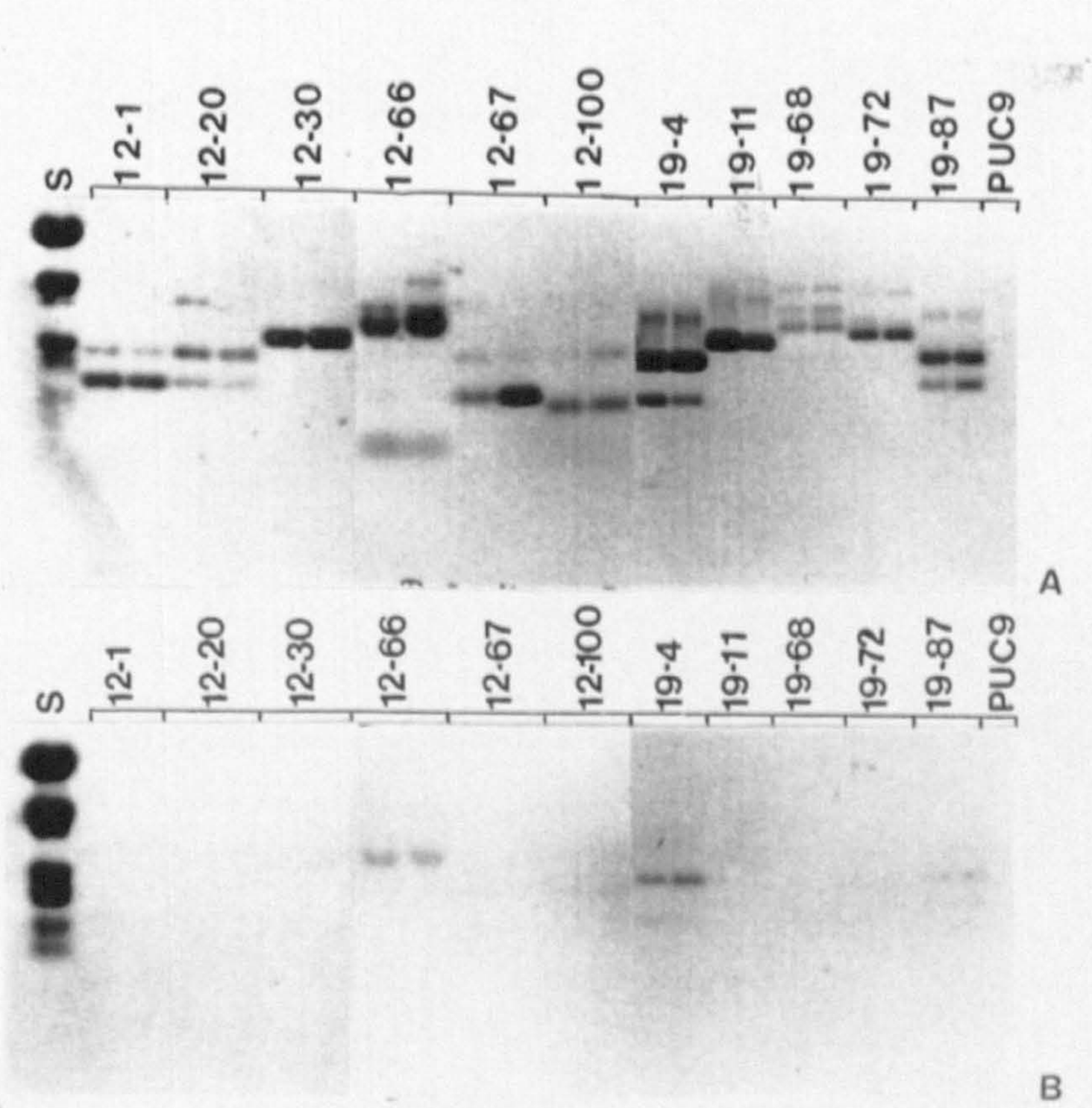


Plate 3.4: Screening for potential LD-specific probes. Representative Southern blot of cloned phytoplasma DNA after hybridization with (A) ^{32}P-labelled phytoplasma DNA and (B) ^{32}P-labelled healthy coconut DNA. Potential probes on this blot were clones 12-1, 12-20, 12-30, 12-66, 12-67, 12-100, 19-11, 19-68, 19-72, and 19-87 which hybridized strongly to phytoplasma DNA but weakly or not at all to healthy coconut DNA. S, Lambda DNA digested with *Hind* III and *Eco* RI enzymes.

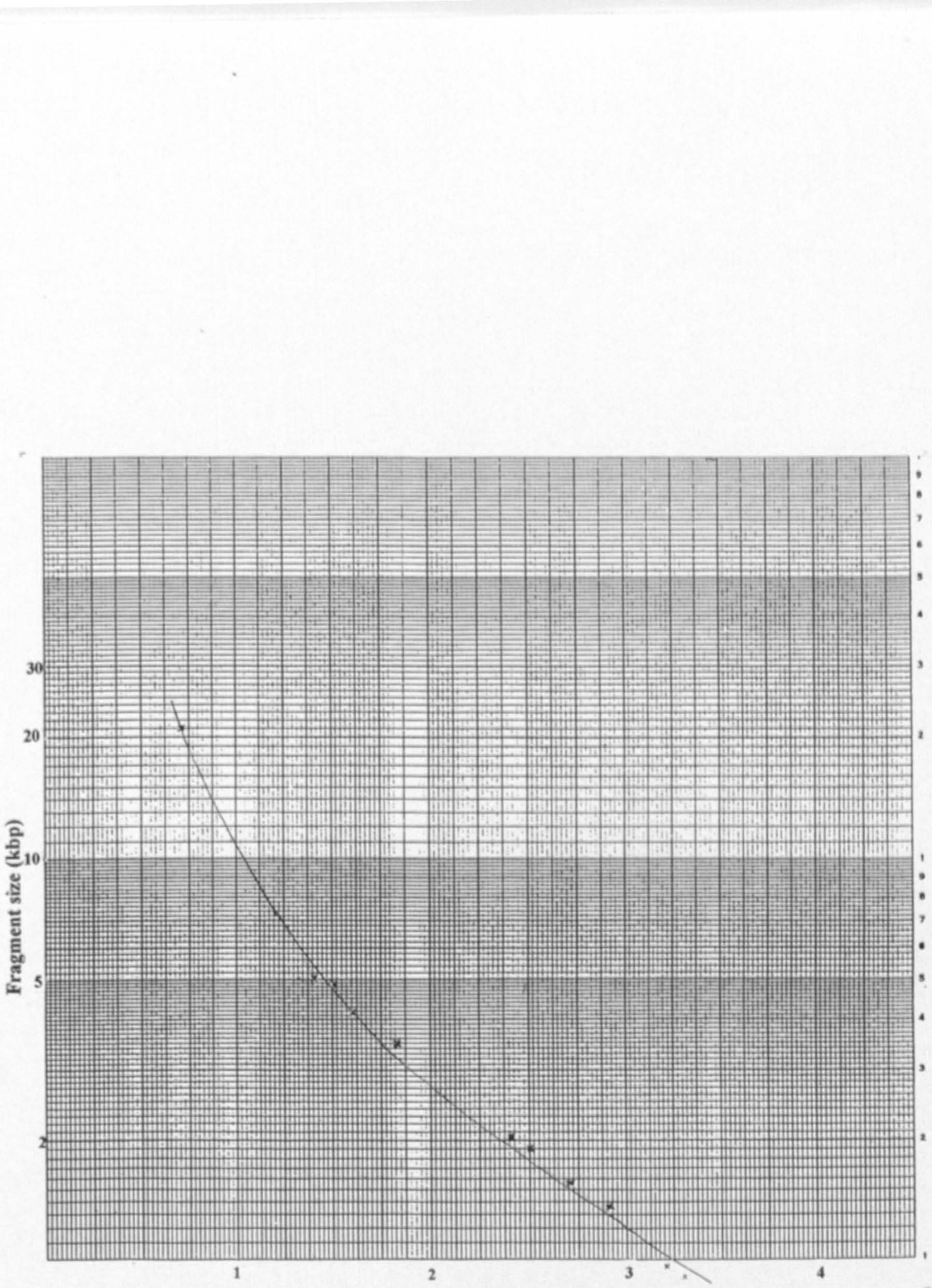


Fig. 3.2: A sample curve showing the electrophoretic mobility of Lambda DNA digested with *Hind* III and *Eco* RI enzymes (DNA size marker) on 1% agarose gel. Such standard curves were used for estimating the sizes of insert DNA in clones electrophoresed on the same gel.

Table 3.2 Size and hybridization signal of insert DNA screened as potential DNA probes

Clone No. and DNA inserts		Intensity of hybridization signal to probe		
Clone No.	No. and size of inserts	Phytoplasma-DNA	Healthy coconut DNA	Periwinkle DNA
LD 10-56	3.30 kbp	+++	+++	0
	0.70 kbp	++	++	0
LD 11-12	1.70 kbp	++	+++	0
	1.00 kbp	++	+++	0
LD 11-23	1.40 kbp	++	+++	0
LD 11-24	1.70 kbp	+++	++++	0
	1.0 kbp	+++	++++	0
LD 11-54	2.00 kbp	0	++	0
	1.70 kbp	+ (very weak)	++	0
LD 12-1	1.70 kbp	++	0	0
	1.05 kbp	+++	0	0
LD 12-3	1.90 kbp	+++	++	0
	1.20 kbp	+++	++	0
LD 12-20	1.70 kbp	++	0	0
	1.05 kbp	++	0	0
LD 12-30	2.20 kbp	+++	+ (very weak)	0
LD 12-51	1.00 kbp	+++	0	0
LD 12-64	2.40 kbp	++	+	0
LD 12-66	2.65 kbp	++++	+ (weak)	0
	0.45 kbp	++	0	0
LD 12-67	0.80 kbp	++	0	0
LD 12-100	0.70 kbp	++	0	0
LD 13-17	1.95 kbp	++	+	NT
	1.30 kbp	+	0	NT
LD 13-77	2.70 kbp	+++	+	NT

LD 14-17	**1.20 kbp**	**+++**	**0**	**0**
LD 14-18	**0.80 kbp**	**+**	**0**	**0**
LD 14-21	**1.20 kbp**	**+**	**0**	**0**
	0.70 kbp	**+ (very weak)**	**0**	**0**
LD 14-48	**0.90 kbp**	**++**	**0**	**0**
LD 14-79	**1.50 kbp**	**+ (very weak)**	**0**	**0**
LD 16-20	**1.10 kbp**	**+**	**++**	**0**
	0.80 kbp	**+**	**0**	**0**
LD 16-28	**1.20 kbp**	**+**	**+**	**0**
LD 19-4	**1.90 kbp**	**+++**	**+**	**NT**
	1.10 kbp	**++**	**+ (very weak)**	**NT**
LD 19-11	**2.65 kbp**	**+++**	**0**	**NT**
LD 19-68	**3.20 kbp**	**++**	**0**	**NT**
LD 19-72	**2.65 kbp**	**++**	**0**	**NT**
LD 19-87	**1.95 kbp**	**+++**	**+ (very weak)**	**NT**
	1.40 kbp	**++**	**0**	**NT**
LD 22-6	**1.00 kbp**	**++**	**+**	**0**
LD 22-8	**0.90 kbp**	**+**	**0**	**0**
LD 22-36	**1.35 kbp**	**++**	**0**	**0**
LD 25-2	**1.00 kbp**	**+**	**0**	**0**
LD 25-28	**150 bp**	**+ (very weak)**	**0**	**0**
LD 34-4	**0.85 kbp**	**++++**	**++**	**0**
LD 34-33	**1.30 kbp**	**+**	**+ (very weak)**	**0**
LD 34-34	**0.95 kbp**	**++++**	**+++**	**+ (very weak)**
LD 34-66	**0.80 kbp**	**++**	**0**	**0**

(+, weak; ++, strong; +++, very strong; NT, Not tested)

Clones highlighted in bold were the best selections

3.4.2 Characterisation of probes

Insert DNA from clone LD 12-66 was labelled with DIG-dUTP and used to probe a Southern blot of *Eco* RI-digested total DNA from four LD-infected palms from Tanzania, two LYD-infected palms each from Kenya, Mozambique and Ghana, an isolate of LY from coconut in Jamaica and 12 other non-coconut phytoplasmas and two spiroplasmas (section 2.2.3). A similar blot contained the same DNA (excluding the LYD isolates from Ghana and Mozambique) doubly digested with *Hind* III and *Eco* RI enzymes. On this second blot, the Jamaican LY isolate was replaced by an LY isolate from *V. merrillii* in Florida. It was also probed with DIG-labelled clone LD 12-66. Whereas the first blot contained 5 µg digested DNA per sample, the second one contained only 1 µg of digested DNA per sample.

The probe hybridized to LD and all other LYD phytoplasma DNA, but not to healthy coconut DNA or any of the non-coconut infecting phytoplasmas and spiroplasmas, both after moderate and high stringency washes. When the blot containing a higher concentration of *Eco* RI-digested total DNA was probed, the probe hybridized generally to all size fragments of LYD DNA between 1 kbp and 21 kbp, but the strongest signal was at approximately 1 kbp (Plate 3.5). However, when hybridized to the blot containing 1 µg total DNA doubly digested with *Eco* RI and *Hind* III, the probe hybridized only to the LD isolates, and the Kenyan LYD isolate at four discrete positions, and one of these bands was the same size as the probe (2.65 kbp) (data not shown). Even then, the hybridization signal was faint despite prolonged autoradiography.

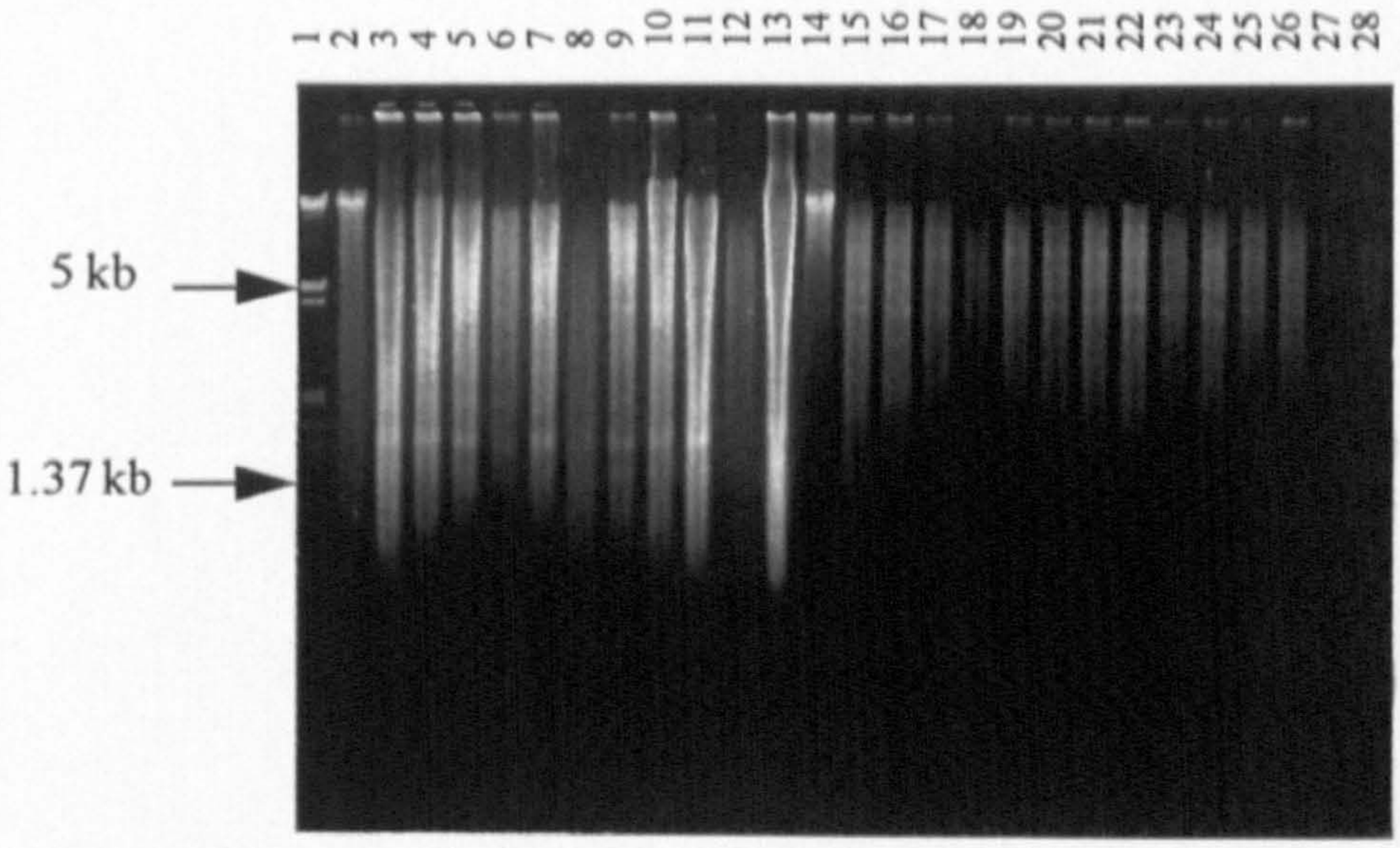


Plate 3.5: Ethidium bromide stained agarose gel of phytoplasma- infected coconut and periwinkle total DNA digested with *Eco*-RI enzyme (A) and autoradiogram of the same gel after Southern blotting and hybridization to DIG-labelled probe LD12-66 (B).

Lane 1, Size marker; 2 and 14, healthy coconut and periwinkle DNA; 3-6, LD isolates - Tanzania; 7-8, LYD-Mozambique; 9-10, LYD-Kenya; 11-12, LYD-Ghana; 13, LY (Jamaica); 15-27, non-coconut infecting phytoplasmas; 28, corn stunt spiroplasma.

The probe hybridized strongly to all the LD isolates from Tanzania. The hybridization signal was also very strong to the LY isolate from Jamaica (Plate 3.5), but it did not hybridize to the isolate from Florida (data not shown). This was probably due to the low titre of phytoplasma in the latter because only 1 µg of that sample was digested compared to 5 µg for the former. Although the probe hybridized to all LYD isolates, there were differences in the intensity of the hybridization signal. For instance, one isolate from Mozambique and one from Ghana hybridized very weakly to the probe, while their counterparts hybridized strongly. This may also be due to low phytoplasma concentrations in the DNA preparations, rather than strain differences.

The LD 12-66 insert DNA was also labelled with DIG and used to probe a dot blot of the DNA samples mentioned above (excluding LY). After moderate stringency washes, the probe hybridised to the LD phytoplasma DNA, as well as the LYD-infected DNA from Kenya and CSPW DNA from Ghana, though less intensely (Plate 3.6). The probe also hybridized weakly to healthy coconut DNA, but not to periwinkle DNA or any of the other non-coconut phytoplasmas. Results of hybridization of probe LD 12-66 to different phytoplasma isolates are summarised in Table 3.3.

The DNA of other non-coconut infecting phytoplasmas was available in limited amounts. Consequently, the other five selected probes could not be characterised in the same way as clone LD 12-66. They were only partially characterised by hybridization to dot blots of several isolates of LD and LYD affecting coconut palms in East and West Africa. For this, insert DNA from each of the selected recombinant plasmid (potential probes) was labelled nonradioactively with digoxigenin (DIG-dUTP) and used to detect phytoplasma DNA in healthy and LYD-infected coconut palms on the blots. Each dot blot contained serially diluted DNA of five LD isolates, three isolates of LYD from Kenya, and one isolate of Cape St Paul wilt from Ghana. All the clones hybridized to infected DNA after high stringency washes, but also produced weak hybridization signals to healthy coconut DNA (Table 3.4; Plate 3.7 for example of probe LD 19-87).

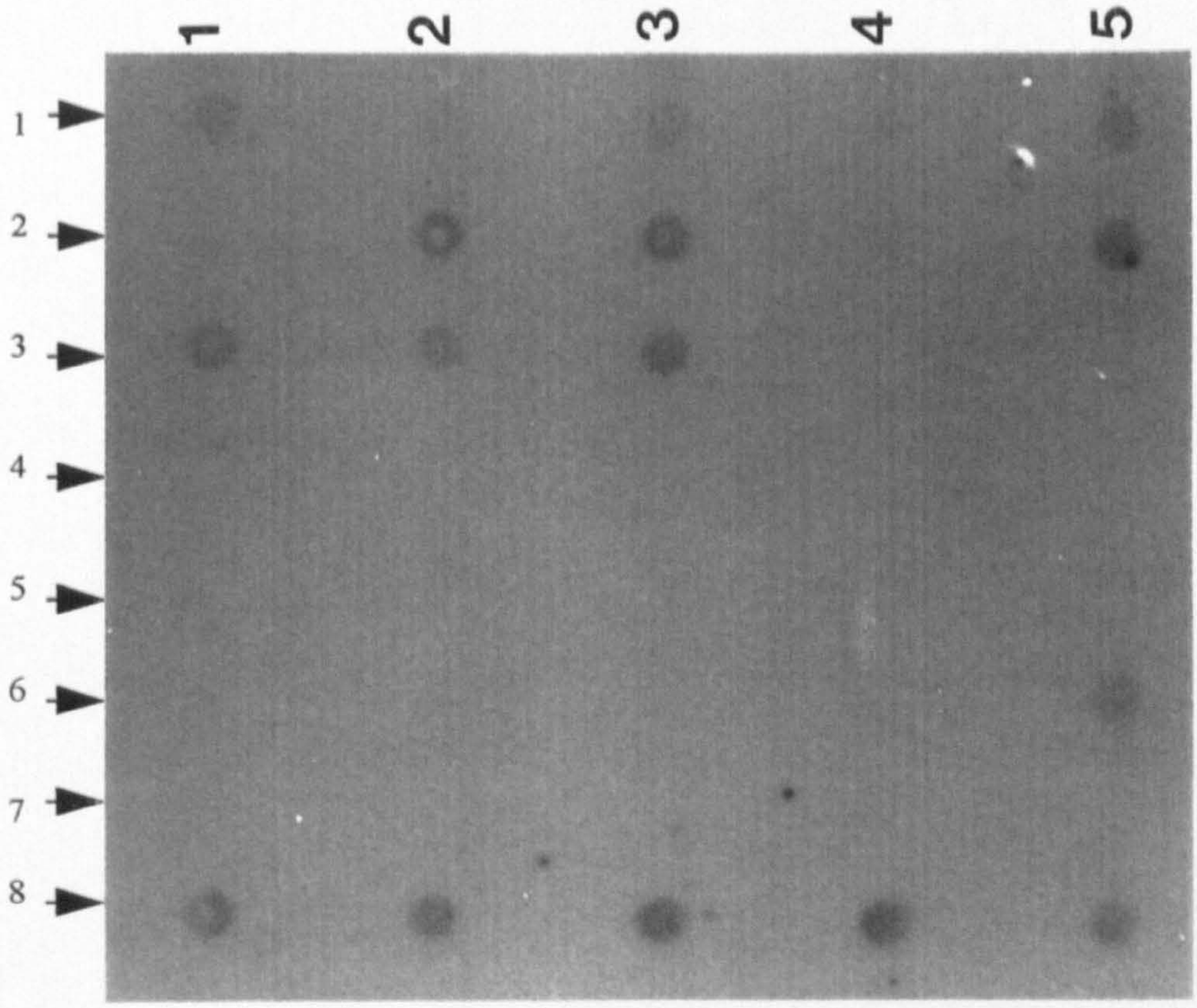


Plate 3.6: Dot blot of different phytoplasma DNA hybridized to DIG-labelled probe LD12-66. Phytoplasma DNA blotted as single spots down the columns are: Column 1: healthy coconut, Ghana LYD, Kenya LYD, PLP, GVX, AEY, healthy periwinkle, LD (Kifuma.).

Column 2: healthy coconut, LYD-Ghana, LYD-Kenya, AYS, FBP, STOL, healthy periwinkle, LD (Chambezi).

Column 3: healthy coconut, LYD-Ghana, LYD-Kenya, EEY, VAC, SC, healthy periwinkle and LD (Tanga).

Column 4: healthy coconut, LD (Kigamboni), APF, CST, ACL, CAV, healthy periwinkle and LD (Mpiji).

Column 5: healthy coconut, LYD-Kenya, WX, CP, LD (Tanga), LD (Sotele), healthy periwinkle and LD Kifumangao.

Table 3.3 Dot blot hybridization of phytoplasmas and Spiroplasmas to DNA probe LD 12-66

	Hybridization signal
Phytoplasmas	
LD - Tanzania (all five isolates)	+
LYD- Kenya	+
LYD- Mozambique	+
LYD- Ghana (Cape St Paul Wilt)	+
Green valley X	-
Apple Proliferation (AP)	-
American Elm Yellows (AEY)	-
European Elm Yellows (EEY)	-
Plum Leptonecrosis (PLP)	-
Apple chlorotic leaf roll (ACLR)	-
Faba bean phyllody (FBP)	-
Stolbur of pepper (STOL)	-
Catharanthus virescence (CAV)	-
Aster Yellows (AY)	-
Vaccinium witches broom (VAC)	-
Clover phyllody (CP)	-
Spiroplasmas	
Corn stunt (*S. kunkeli*)	-
Spiroplasma citri	-

+ The isolate hybridized to the probe

- No hybridization signal

Hybridization to blots with isolates of LYD from Mozambique were conducted separately, and each of these probes hybridized to the phytoplasma associated with LYD in Mozambique (data not shown).

All DIG-labelled probes hybridized to healthy coconut DNA on dot blots. For probe LD 19-87, with two inserts of sizes 1.40 kbp and 1.95 kbp, weak hybridization was from the larger insert. For probes LD 12-66 and LD 19-11, which each contain inserts of 2.65 kbp, complete separation of the insert DNA from the vector sequences was impossible. This vector contamination may have contributed to non-specific hybridization and increased the signal to healthy coconut DNA which was previously not detected for ^{32}P-labelled LD 19-11. The hybridization of probes LD 12-1 and LD 12-20 to healthy DNA was unexpected as neither probe hybridized before when labelled with radioactive isotope. No explanation could be found for this unexpected positive hybridization. The cause of non-specific hybridization signals on dot blots with DIG probes has yet to be established.

After restriction digestion of the plasmid DNA, the insert DNA in clones LD 12-66 and LD 19-11 comigrated in agarose gels to about the same position as the vector DNA, and were visualised as one thick double band. To confirm that these clones did in fact contain inserts which are very difficult to separate away from the vector, miniprep DNA of these two clones was digested with enzymes *Cla* I and *Sca* I. *Sca* I enzyme has only one recognition site in the vector sequences (within the ampicillin resistance gene), whereas *Cla* I has none. Both enzymes have six base recognition sequences, thus, are infrequent DNA cutters. Plasmid DNA was digested overnight at 37 ^{0}C, the restriction products analysed by electophoresis in a 1% agarose gel and bands visualised on the UV transilliminator after staining with ethidium bromide.

The enzyme *Cla* I produced one predominant band for each of the clones LD 12-66 and LD 19-11, of approximate size 7.0 kbp and 7.5 kbp respectively. However, *Sca* I digested both clones more than once, generating bands of different sizes for each as estimated and shown in table 3.5 below.

Table 3.4 Relative sensitivity of selected DNA probes for detection of the LYD phytoplasmas in coconut palms by dot blot hybridization.

Probe	Insert size	Dot blot hybridization to DNA of LYD isolate from:										
		Tanzania						Kenya			Gha	Mo
		HL	KL	KF	SO	CB	KG	KY 1	KY 2	KY 3	CSPW	MOCM
LD 12-1	1.05 and 1.70 Kb	+	++	++	++	+++	++	++	++	++	++	NT
LD12-20	1.05 and 1.70 Kb	+	++	++	+	+++	+++	+	++	++	+	++
LD12-30	2.20 Kb	+	++	++	++	+++	+++	+	++	++	+	+++
LD12-66	2.65 Kb	+	++	++	++	++	++	+	+	++	+	NT
LD19-11	2.65 Kb	+	++	+++	+++	+++	+++	++	++	++	++	+++
LD19-87	1.40 and 1.95 Kb	+	+++	+++	++	++	+++	+	++	++	++	+++

Gha, Ghana; Mo, Mozambique

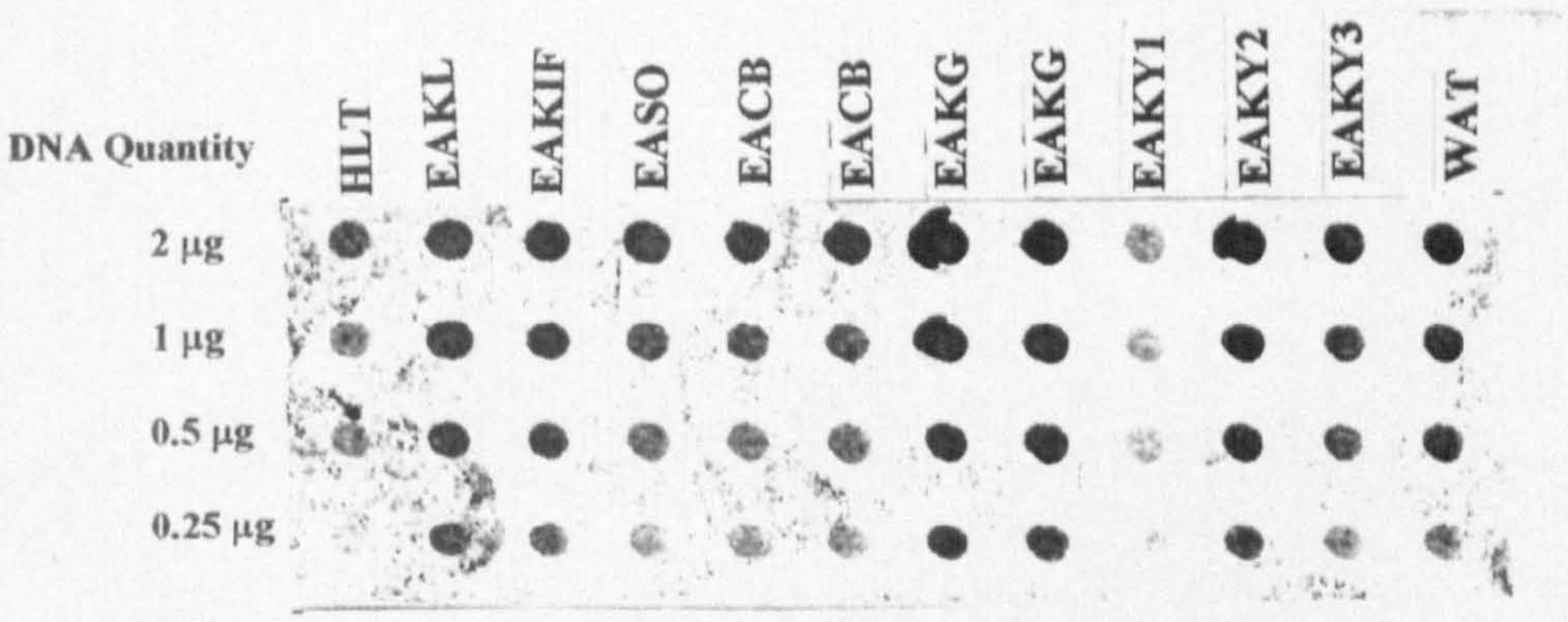
HL, Healthy coconut palm; KL, Kilwa, KF, Kifumangao; SO, Sotele; CB, Chambezi; KG, Kigombe; KY, Kenya; CSPW, Cape St. Paul wilt (Ghana); MOCM, Mocimboa da Praia (Mozambique).

+, weak; ++, strong; +++, very strong hybridization signal; NT, Not tested

Table 3.5 **Estimated sizes of restriction fragments generated by digesting LD 12-66 and 19-11 plasmid DNA with enzyme *Sca* I**

	Clone LD 12-66		Clone LD 19-11
Fragment size	1.	0.70 kbp	0.85 kbp
	2.	1.40 kbp	3.05 kbp
	3.	1.60 kbp	3.20 kbp
	4.	3.50 kbp	
Total length		7.20 kbp	7.10 kbp

The total length produced by digestion of each plasmid by either enzyme was more than 5.40 kbp, which is the expected length if each clone had contained two joined vector molecules. Therefore, these results confirmed that each of these clones contain double DNA inserts. Double digestion of the plasmid DNA by use of either one of these enzymes in combination with an enzyme that cuts in the vector cloning site, may provide more information about the nature of these inserts.



HLT	Healthy coconut
EAKL	Tall from Kilwa
EAKIF	Kifumangao
EASO	Sotele
EACB	Chambezi
EAKG	Kigombe (Tanga)
EAKY	Kenya
WAT	West African Tall from Ghana infected with CSPW disease

Plate 3.7: Detection of LD in infected coconuts by non-radioactive (DIG-labelled) probes. Dot blot of coconut DNA infected with LD or other LYD after probing with clone LD 19-87 and high stringency wash conditions.

3.5 DISCUSSION

Total DNA for use in production of probes in this study was extracted from coconut meristematic tissue using the procedure which enriches for phytoplasma and mitochondria DNA by differential centrifugation. Buoyant density centrifugation in CsCl (Sears *et al.*, 1989; Kollar *et al.*, 1990) was then used to separate phytoplasma DNA from mixtures with plant DNA. This technique was preferred because it has been used to obtain sufficient DNA for cloning from hosts with low pathogen titre (Harrison *et al.*, 1992).

Phytoplasmas represent a minute proportion of the total components of their hosts, but because they have not been cultured *in vitro*, infected hosts must serve as sources of DNA for molecular cloning (Lee and Davis, 1988). This makes it necessary to selectively concentrate phytoplasma DNA from host DNA during isolation. Strategies for extracting DNA for use in production of probes, have evolved over time, from techniques that involve concentrating host extracts to enrich for intact phytoplasmas and mitochondria (Davis *et al.*, 1988b; Lee and Davis, 1988), to those employing buoyant density centrifugations to separate phytoplasma DNA from crude extracts of host DNA (Kollar *et al.*, 1990). Concentration of phytoplasma from host extracts has been achieved either by differential centrifugation (Davis *et al.*, 1988b), or by solubilization of the vascular tissue with enzyme and separation of sieve elements (Lee and Davis, 1988). Differential centrifugation was preferentially selected over solubilization in this study because it is faster and economical.

The concentration of phytoplasmas in tissues of coconut palms affected by LD and LY has been reported to be very low, and the distribution uneven (Deutsch and Nienhaus; 1983, Thomas, 1979). Results obtained in this study support those reports, since it was not possible to resolve a phytoplasma DNA band after initial CsCl-bisbenzimide density gradient centrifugation of total DNA from large quantities of meristematic tissue. Three, and up to five rounds of pooling and ultracentrifugation were necessary to resolve a phytoplasma DNA band (Plate 3.2).

Furthermore, the relative amount of enriched LD phytoplasma DNA (0.1%) recovered from coconut DNA, was much lower than the proportion of LY phytoplasma DNA (0.4%) recovered from LY infected manilla palm DNA by Harrison *et al.* (1992). By use of LY-specific probes in dot hybridization analysis, Harrison (1996) estimated the titre of LY phytoplasma in coconut palms to be 10- to 12-fold lower than in other LY-susceptible palm species such as cliff date palm (*Phoenix rupicola*), spindle palm (*Hyophorbe verschaffeltii*) and footstool palm (*Livistona rotundifolia*). The low percentage recovery of the LD phytoplasma from coconut tissue in this study provides additional evidence of similarity to LY, whereby both pathogens are able to infect coconut palms (their major host) at very low concentrations. This feature differs from some phytoplasmas which infect other perennial hosts. For instance, the apple proliferation phytoplasma was found to constitute 2 % of total DNA extracted from phloem tissue of apple trees (Kollar *et al.*, 1990).

During extraction of the DNA of several phytoplasmas from periwinkle, Kollar *et al.* (1990) reported that the proportion of phytoplasma DNA recovered from total DNA was significantly influenced by the plant part used for DNA extraction. Phytoplasma DNA constituted 0.1-1 % of total DNA when stems and leaves were used for extraction, compared to 0.2-2 % when stems alone were the DNA source, or 0.3-3 % when roots were the source. Harrison *et al.* (1991) reported that pigeon pea witches'- broom phytoplasma DNA formed 0.67% of total DNA extracted from periwinkle stems. By pointing out the differences in the distribution of phytoplasmas in host tissue, these reports suggest that it is possible for higher concentrations of phytoplasmas to be obtained from coconut root tips. Since these tissues have been demonstrated to contain phytoplasmas (Schuiling and Mpunami, 1990) but they were not tested as a source of total DNA, this possibility will be investigated in future studies.

All the procedures tested for extraction of total DNA from coconut tissue produced DNA that was of sufficient quantity and quality for various uses, including PCR and DNA-DNA hybridizations (Table 3.1, Plate 3.1). This demonstrated that extraction

procedures which are simple, quick, economical, and do not require sophisticated equipment could be safely adopted for field screening without compromising useful information. Consequently, the miniprep extraction procedure of Doyle and Doyle (1990) was modified to avoid use of liquid nitrogen during extraction in order to adopt it for routine use in field screening experiments.

Based on the number of recombinant colonies obtained, it can be deduced that a reasonable level of transformation frequency was achieved in cloning. However, a large proportion of the transformants contained host DNA inserts, or mixtures with host DNA (Table 3.2). This indicates incomplete separation of phytoplasma DNA from host DNA. It further points to the fact that the concentration of LD phytoplasma in coconut tissue is very low, because recovery of pure phytoplasma DNA was not achieved despite the large amount of tissue used, and the tedious extraction process. Only 22 out of 454 (5%) transformants which hybridized to phytoplasma DNA did not hybridize at all, or hybridized weakly to healthy coconut DNA. This proportion is very low compared to 14% obtained when the LY pathogen was cloned using DNA isolated from manila palm (Harrison *et al.*, 1992). Periwinkle DNA which is comparatively A + T rich, proved very useful as an additional probe to cross-check the sensitivity of cloned phytoplasma DNA in hybridizing to homologous sequences. The screening mechanism of periwinkle DNA is two fold (Dr. Harrison, personal communication). First, it detects the hybridization signal shown by clones to phytoplasma DNA that is due to nonspecific (background) hybridization resulting from homology between phytoplasma genomic DNA away from the insert (such as the conserved prokaryotic rRNA genes) and cloning vector and / or residual *E. coli* chromosomal sequences. Secondly, it helps to identify chimeric clones, which contain more than one piece of DNA insert.

Hybridization analyses with probe LD 12-66 indicated that it specifically detected the phytoplasma associated with LD of coconut palm, although it did hybridise weakly to healthy coconut DNA in dot blots. Use of genomic RFLP analysis to detect differences in hybridization to DNA of different phytoplasmas showed that the probe hybridizes only to LYD (lethal yellowing-like diseases) DNA, but not to non-coconut

phytoplasmas or spiroplasmas (Plate 3.5). Hybridization to the LYD isolates by the probe was very similar, and no polymorphisms were detected. However, on a blot containing a low concentration of genomic DNA, the probe hybridized at only four discrete positions to each of the LD phytoplasma isolates, and to one isolate from Kenya, but not to other phytoplasmas (data not shown). This suggests that by adjusting the amount of digested DNA transfered on Southern blots, it may be possible to detect polymorphisms among the LYD isolates using this probe.

The probe hybridized strongly to the LY isolate from Jamaica, but not to the LY isolate from Florida. This result is rather unexpected, because the LD phytoplasma is genetically related to LY, and was previously found to cross-hybridize to the LY-specific DNA probe during dot blot hybridizations (Harrison *et al.*, 1994a). The only possible explanation for lack of hybridization would be a low phytoplasma titre because small quantities of DNA (1 µg) were digested compared to all other isolates.

During dot blot hybridizations, the probe did hybridize to LYD-infected palms from Kenya, and Ghana (Plate 3.6), as well as Mozambique (data not shown). However, it did not hybridize to any of the 12 different non-coconut phytoplasmas and two spiroplasmas (Plate 3.6). The LY phytoplasma DNA was not included on the dot blot, because it was not available at the time of the experiment. It may be necessary, therefore, to repeat the hybridization test to the LY DNA to confirm the presence or absence of cross-hybridization. The level of specificity shown by probe LD 12-66 for detection of LD is quite promising, and ensures that it could be useful in the search for potential insect vectors and other plant hosts of the LD phytoplasma. Although this probe was not specific to LD phytoplasma alone, as demonstrated by cross hybridization to other LYD phytoplasmas, its usefulness lies in the fact that it does not hybridize to non-coconut infecting phytoplasmas and spiroplasmas. This ensures that it could be utilised in future studies to determine the level of genetic relatedness between LD and other LYD.

Selection of the best probes was based on the intensity of their hybridization signal to phytoplasma DNA and the size of insert DNA. The insert size of 2.65 kbp for probe

LD 12-66 was particularly favourable. When hybridized to a low concentration of doubly digested, LD-infected total DNA, this probe hybridized at four discrete positions, one of which was the same size as the cloned insert (data not shown). This implies that this probe contains reiterative sequences, and this accounts for the strong hybridization signal. Presence of reiterative sequences was backed by results of restriction digestion of the recombinant plasmid with enzymes *Cla* I and *Sca* I which showed that the clone in fact contains two fragments (Table 3.5). The fragments may have similar sequences, or may be different. A difference in the sequences of these fragments would explain why the probe had high affinity to phytoplasma DNA, while at the same time there was background hybridization to healthy coconut DNA. It is possible one of the fragments is a portion of coconut DNA. Further investigations will be neccesary to establish the exact nature of these inserts.

Each of the six probes was able to detect LYD in infected palms from Tanzania, Kenya, Ghana and Mozambique on dot blots, but the levels of detection were not uniform. Among the factors which could influence their action are the concentration of phytoplasmas in the sample extracts, probe size, the copy number of cloned sequences, and the extent of base sequence homology between probe DNA and phytoplasma DNA. The weaker hybridization signal to Ghanian CSPW infected coconut DNA by some of the probes suggest that variability does exist between the phytoplasmas causing diseases in coconuts in Tanzania and Ghana. This is in agreement with a report by Harrison *et al.* (1994a) that phytoplasmas associated with the LYDs of the Carribean, East and West Africa are similar but not genetically identical.

Differences in specificity of detection were observed depending on whether probes were labelled with a radioactive isotope or non-radioactive Digoxigenin (DIG). Some DIG probes were found to hybridize to healthy coconut DNA when, previously, no hybridization was observed when radioactively labelled, i.e. LD 12-1, LD 12-20 and LD 19-11. These apparent false positives may be due to phosphatase activity in the coconut tissue. Randles *et al.* (1992) while using DIG-labelled cDNA probes for the detection of coconut foliar decay virus (CFDV) in coconut palms, reported the

presence of endogenous enzyme activity in palm tissue which tended to interfere with the DIG detection procedure. They found that this interference could be eliminated by incubating leaf extracts in 0.5 NaOH for two hours at 37^{0}C before blotting for hybridization. This approach will be tested in future.

Chapter 4 THE USE OF DNA PROBES FOR DETECTION OF PHYTOPLASMA IN LD-INFECTED PALMS

4.1 INTRODUCTION

LD occurs throughout the coastal belt of mainland Tanzania, except for the islands of Zanzibar and Pemba. There are, however, big differences in disease incidence among the affected regions. Whereas the disease is widespread in the south and has killed about 56% of the palms since 1965, only 8.5% are affected in the northern districts (Schuiling *et al.*, 1992a). These epidemiological differences have been difficult to reconcile because environmental conditions including moisture, soils, flora and insect fauna on palms in all the regions appear to be similar. Among postulated causes for the discrepancy are genotypic differences within the local coconut populations, existence of different strains of the pathogen, and possibly different insect vectors.

The differences in disease incidence observed in Tanzania, extend across its borders into neighbouring Kenya to the north, and into Mozambique to the south in a similar pattern. In both these countries, lethal yellowing-like diseases (LYD) have been reported, but the magnitude of damage they cause to palms differs. In Kenya, the damage is generally minor (Schuiling *et al.*, 1991, unpublished report), while in Mozambique, the one locality found affected was totally devastated (Mpunami and Seguni, 1996).

Despite attempts to study it, the epidemiology of LD has remained a mystery, mainly due to lack of a rapid and sensitive method of disease detection under field conditions. For the same reason, the relationship between LD and similar diseases in the neighbouring countries has remained unestablished. The insect vector has also remained unidentified, and previous attempts to transmit the disease using suspected insects caught from leaves of LD-infected palms have been unsuccessful (Anonymous, 1987).

For most phytoplasma incited diseases, the problems of pathogen detection and

disease diagnosis have been overcome by application of the recombinant DNA technology. Cloned, random fragments of phytoplasma DNA extracted from infected plants have been used in DNA hybridization analyses to reliably and specifically detect the pathogens in their hosts (Bertaccini *et al.*, 1990a; Daire *et al.*, 1992; Harrison *et al.*, 1992; Nakashima *et al.*, 1992; Ko and Lin, 1994). Similarly, it has been possible to detect phytoplasmas in insect vectors by the same technique (Kirkpatrick *et al.*, 1987; Davis *et al.*, 1988b; Rahardija *et al.*, 1992). In addition to providing a reliable means for disease diagnosis, DNA recombinant technology has also facilitated taxonomic studies (Lee and Davis, 1992), and epidemiological studies including those on the ecology and distribution of the respective diseases (Kirkpatrick *et al.*, 1990).

Thus, by developing DNA probes to the LD phytoplasma (section 3.4) alternative new prospects for improvement in techniques for disease diagnosis were opened up. This also provided an avenue from which epidemiological aspects of this disease could be studied. The homologous DNA probes containing fragments of LD phytoplasma DNA were used in dot hybridization analyses to detect LD infections in palms, and in restriction fragment length polymorphisms (RFLP) to investigate possible existence of different strains of the pathogen.

4.2 DETECTION OF PHYTOPLASMAS IN DISEASE-INFECTED PALMS BY DNA PROBES

4.2.1 Identification of Suitable Tissues to Sample

In order to determine which part of the affected palm was suitable to sample for disease detection, it became necessary to establish where in the palm the phytoplasmas accumulate, and which tissues have the highest phytoplasma concentration. The best part to sample would be the one with the highest phytoplasma concentration. To determine where phytoplasmas accumulate, insert DNA for probe LD 12-66 was labelled nonradioactively with digoxigenin (DIG-dUTP), and hybridized to a dot blot of DNA collected from different meristematic tissues of LD-affected palms. The DNA was extracted by either the miniprep extraction procedure of Doyle and Doyle (1990), or the

miniprep procedure of Dellaporta *et al.* (1983) (section 2.3.5). The DNA was serially diluted to only 125 µg for each sample. This was sufficient to show differences in the hybridization signal to identify which tissues had the highest DNA concentration, and which extraction procedure yielded more phytoplasma DNA.

Phytoplasma DNA was detected in all meristematic tissues sampled, including the petioles of very young leaves, the area below the growing point, root tips, inflorescences, and the spear leaf (Plate 4.1). The highest concentration was in the petioles of young unopened leaves and the area below the growing point (Plate 4.1). The probe also detected phytoplasma DNA extracted from the spear leaves of two apparently healthy palms. These palms were closely observed, and one month later, they developed LD symptoms (data not shown). There was a stronger hybridization signal to DNA extracted by the CTAB procedure of Doyle and Doyle than those extracted by the miniprep method of Dellaporta (Plate 4.1), indicating higher recovery by the former method.

In addition to probe LD 12-66, insert DNA from each of the other selected potential probes (section 3.4.1) was labelled with DIG and used to detect phytoplasma DNA in healthy and LD-infected coconut palms by dot blot hybridization as described (section 3.4.2). They all hybridized strongly to phytoplasma DNA, but also weakly hybridized to healthy coconut DNA (data not shown). Although they all were good probes, and were occasionally used for detection of phytoplasma DNA, preference was given to probes LD 12-66 and LD 19-87 which produced a stronger hybridization signal in most experiments.

4.2.2 Studies on the Distribution of Phytoplasmas in LD-Infected Tissue

For studying the distribution of phytoplasmas in the meristematic tissues of LD-infected coconut palms, probes LD 12-66 and LD 19-87 with large size DNA inserts were used. They produced a much stronger hybridization signal to LD phytoplasma DNA than other probes. These probes were each labelled non-radioactively with DIG and hybridized to serially diluted DNA of LD-infected coconut tissue on dot blots. The individually blotted DNA samples were taken from all meristematic tissues of LD-infected palms. Palms from

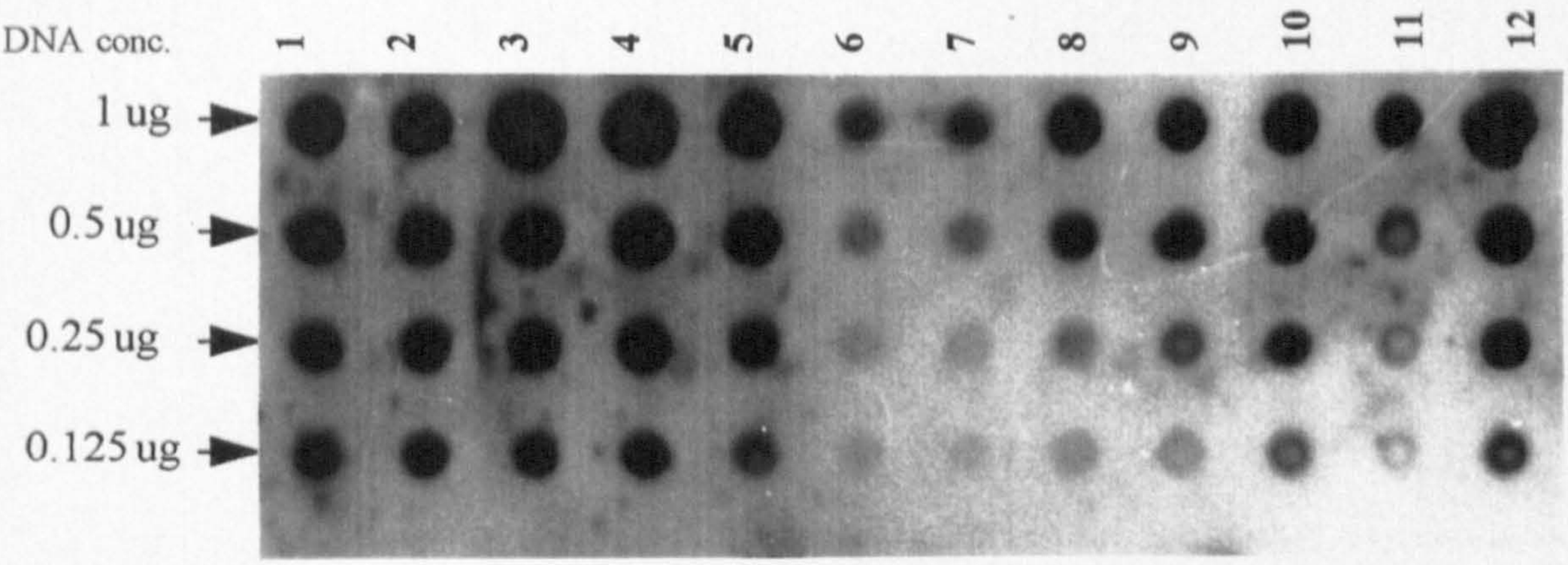


Plate 4.1A: Detection of phytoplasmas in the meristematic tissues of LD- infected coconut DNA by dot blot hybridization to probe LD 12-66. The DNA was extracted by either CTAB procedure(lane 1-5) or the miniprep method of Dellaporta (6-12). The different tissues are: below the growing point (lane 1 & 10) and leaf petioles of: opened leaf (6); first unopened (2, 7, 11 and 12); second unopened (3 & 8); and third unopened (4 & 9)

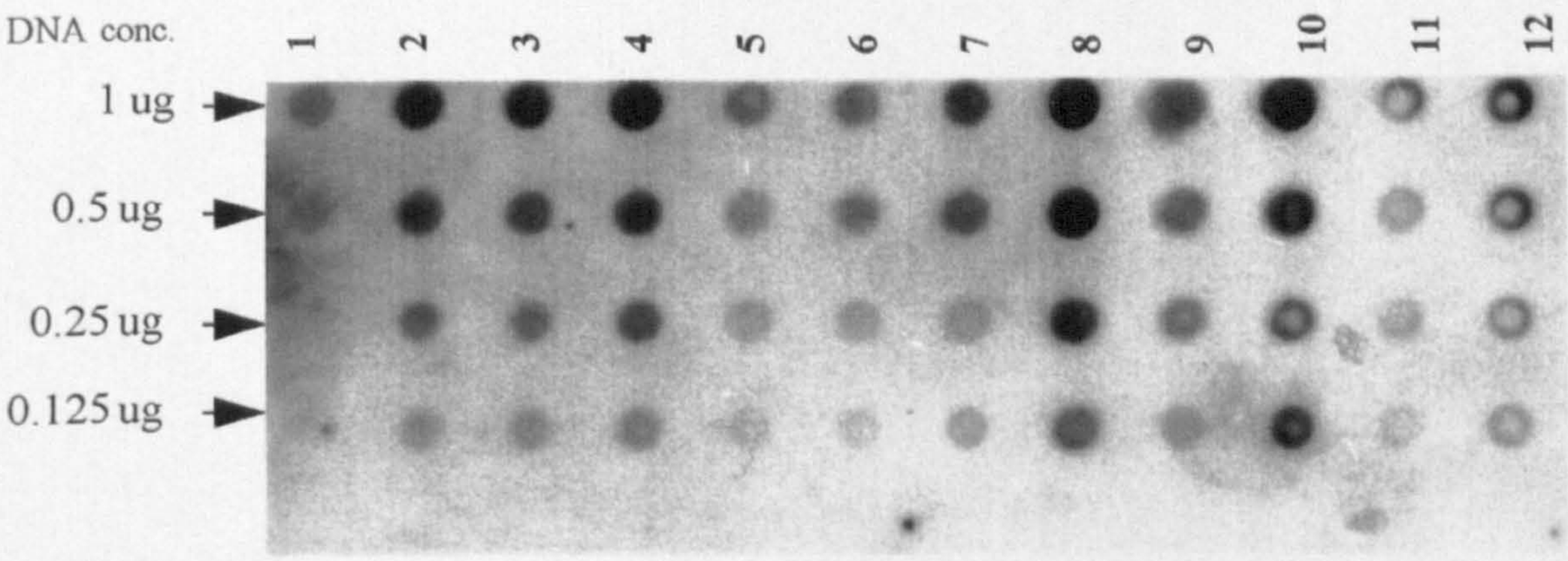


Plate 4.1B: Detection of phytoplasma in different meristematic tissues of LD-infected coconut palms on dot blots by use of DIG-labelled probe LD 12-66. The DNA was extracted by either CTAB procedure(lane 1-6); or according to Dellaporta (lane 7-12). The tissues are: Unopen inflorescences (lane 1 & 8); roots (7); below the growing point (6 & 12); and leaf petioles of first open (2), first unopened (3 & 9); second unopened (4 & 10); and third unopened (5 & 11)

which the samples were taken were specially selected to include palms at different stages of disease progression, in order to determine the stage at which phytoplasma concentration in the palm was highest. The stage of disease progression was determined by assessing the foliar symptoms, and determining the proportion which was still green relative to the rest of the already bronzing or browning canopy. For example, a four year-old palm with 4 dry brown leaves, 4 leaves with advanced bronzing symptoms, 2 leaves with slight bronzing, 5 green leaves, and slight necrosis in the spear leaf would be graded as follows:

Symptoms	No of leaves	Disease score	Stage of disease
Dry brown	4	4 x 3	
Advanced bronze	4	4 x 2	(23 x 10) /
Early bronzing	2	2 x 1	(16 x 3) = 4.8
Green	5	5 x 0	
Spear, slight necrosis	1	1 x 1	
Total	16	23	

The symptoms were assessed on a scale of 0-3, (0, healthy; 3, totally dead) for each leaf on the palm. Meanwhile, the stage of disease was evaluated on a scale of 0-10, where 0 refer to a healthy palm, and 10 refer to a completely dead palm (Schuiling, personal communication). The disease score based on the foliar symptoms was then expressed as a proportion of a possible maximum points when the palm was completely dead (i.e. 23 x 10) relative to the maximum possible disease score when all leaves on the palm were dead (i.e. 16 x 3), which is equal to 4.8.

To select palms at the different stages of disease, the affected palms were then categorised into four classes based on disease score as follows:

1.0 - 2.5 Early stage

2.6 - 4.5 Moderate

4.6 - 6.5 Advanced

6.6 - 9.9 Very advanced

The advanced stage coincided with the stage when the disease symptoms were very

apparent, and it was also re-categorised into two sub-stages; the early advanced, moderately advanced. Representative palms were selected from each of these categories and samples taken from them for DNA blotting and hybridization.

As an alternative way to describe the stage of disease, if the 5 leaves still green were expressed as a proportion of the total number of leaves, then the palm canopy was described as about 38 % green.

Plate 4.2 shows the results of screening using probe LD 19-87. Overall, phytoplasmas were detected in all the meristematic tissues, including the root tips, petioles of young opened and unopened leaves, young inflorescences, area below the growing point, and in the spear leaflets. The highest concentrations were found in the petioles of young unopened leaves (specifically, -1 to -3, where -3 refers to the youngest unopened), the root tips, and the area below the growing point. The least amount of phytoplasmas were detected in the spear leaflets (Plate 4.2).

With regard to palms at different stages of disease progression, the concentration of phytoplasmas was much less for palms at relatively earlier advanced stages of disease (palm 1, Plate 4.2). The concentration reached a peak when disease symptoms were moderately advanced (palms 2 and 3, Plate 4.2), and tended to decrease when disease symptoms were very advanced (palm 4, Plate 4.2). However, the concentration remained high in the petiole of the first unopened leaf even in the palm at a very advanced stage, with detection levels down to 7.8 ng of total DNA.

In tissues with a high phytoplasma concentration, detection was possible in 7.8 ng total DNA (Plate 4.2). The main problem in all hybridizations, however, was the high background hybridization to healthy coconut DNA, with detection in 62.5 ng of total DNA in some preparations (Plate 4.2). The background hybridization to healthy coconut DNA could be reduced by cross-absorbing the probe DNA with blots of healthy coconut DNA, before hybridization to the test blot as shown in Plate 4.3.

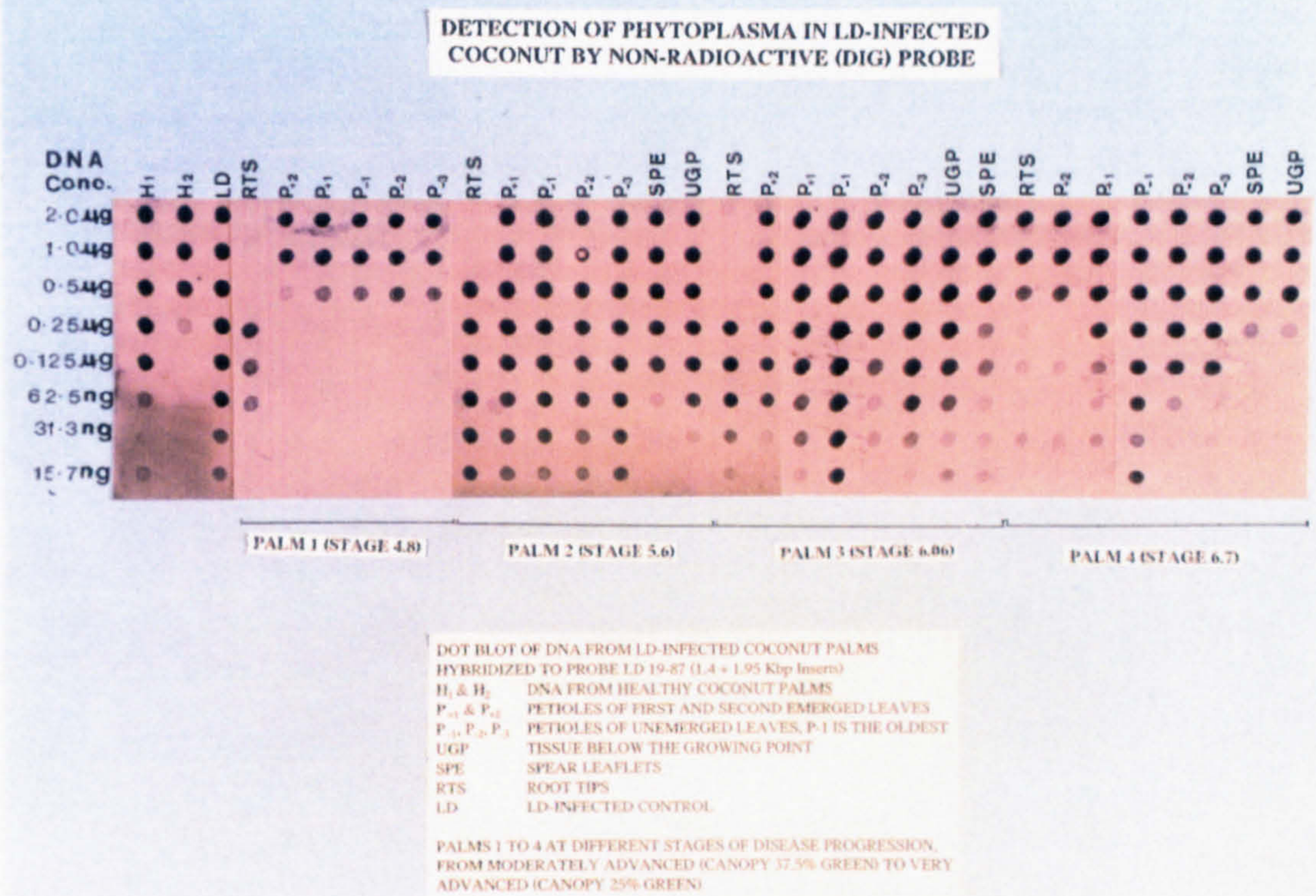


Plate 4.2: Detection of phytoplasma in palms at different stages of disease progression using DIG-labelled probe LD 19-87

4.3 SAMPLING FOR DETECTION OF INCUBATING INFECTIONS USING DNA PROBES

4.3.1 Sampling and Screening of Symptomless Palms

The symptomatological similarity of LD to LY (Schuiling *et al.*, 1981), has been the basis for assuming that LD is also associated with a prolonged latent (incubation) period. The incubation period for LY has been estimated to range between 114 - 262 days (Dabek, 1975). To confirm this assumption, selected, apparently healthy palms were screened for phytoplasma using the more sensitive DNA probe (LD 12-66) in order to detect incubating infections. For purposes of screening, spear leaves were sampled at monthly intervals for one year from 180 palms. Thirty palms (15 bearing and 15 non-bearing) were selected at each of the six representative trial sites in the disease affected regions. Two sites were in the high incidence, two in moderate incidence, and two in low incidence areas. DNA extracted from these samples was individually blotted onto nylon membranes and hybridized to the DIG-labelled probe, in order to determine the earliest time before appearance of disease symptoms at which the phytoplasma could be detected. When quantities of probe LD 12-66 for hybridization became limiting, probe LD 12-30 was used on three blots.

Due to the large volume of samples being handled, blotting and hybridization was carried out only on samples collected during the first six months, after which the experiment had to be terminated. During that period, 16 of the experimental palms were infected by LD, and developed disease symptoms. Table 4.1 shows the results of phytoplasma detection in those palms, using DNA probe LD 12-66.

Phytoplasma DNA was weakly detected in two symptomless palms (12%) as early as three months before appearance of disease symptoms. However, the background hybridization to healthy coconut DNA on corresponding blots was so high that the results were considered ambiguous / nonspecific. Similarly, in five palms (31%), phytoplasma DNA was detected two months before appearance of disease symptoms, but the reaction was masked by the background hybridization to healthy coconut DNA.

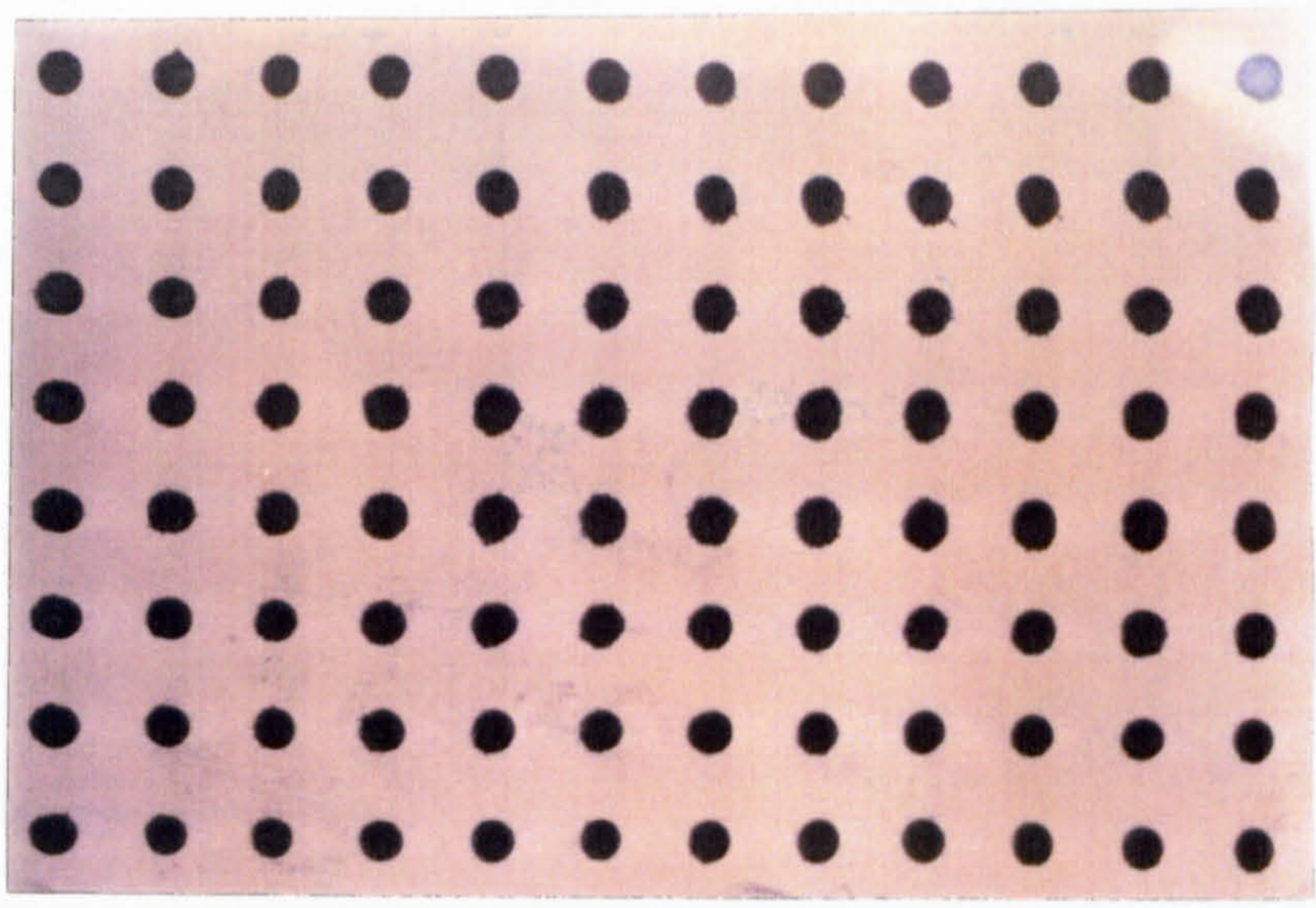
Table 4.1 Detection of phytoplasma DNA in symptomless, LD- infected palms by use of DNA probes.

Palm No. and Location	Coconut cultivar	LD Symptoms observed	Detection relative to symptoms / Intensity of hybridization signal
# 5 Chambezi	EAT-Chamb.	Jan 1995	1 mo earlier, strong
#14 Chambezi	Karkar Tall	April 1995	3 mo earlier, background to healthy
#24 Chambezi	EAT-S'Mnara	Dec 1994	1 mo later, background to healthy
#25 Chambezi	EAT-Tumaini	Mar 1995	2 mo earlier, background to healthy
#19 Kifumangao	EAT-S'Mnara	Mar 1995	same month, strong
#22 Kifumangao	EAT-Lamu	Mar 1995	2 mo earlier, background to healthy
#29 Kifumangao	Tacunan Dwf	Feb 1995	1 mo earlier, background to healthy
#01 Kigamboni	MYDxWAT	July 1995	2 mo earlier, background to healthy
#18 Kigamboni	MYDxWAT	Jan 1995	1 mo earlier, weak
#21 Kigamboni	MYDxWAT	May 1995	Undetected
#03 Kigombe	MYDxWAT	Feb 1995	2 mo earlier, background to healthy
#04 Kigombe	MYDxWAT	Dec 1994	same mo, strong
#16 Kigombe	CRDxWAT	June 1995	2 mo earlier, background to healthy
#25 Kigombe	CRDxRLT	Jan 1995	1 mo earlier, weak
# 26 Kigombe	CRDxRLT	Mar 1995	1 mo later, strong
#29 Kigombe	MYDxWAT	July 1995	3 mo earlier, background to healthy
#10 Sotele	EAT-Boma	May 1995	Undetected

However, for the 3 palms (19%) in which phytoplasma was detected one month before the appearance of disease symptoms, the hybridization signal was strong enough to overshadow the background hybridization to healthy coconut DNA.

In order to confirm whether the probes were able to detect phytoplasma DNA in infected palms three months before the appearance of disease symptoms, each of the probes LD 19-87 and LD 19-11 were labelled with DIG and cross-absorbed with healthy coconut DNA by hybridizing overnight to blots containing only healthy coconut DNA. The cross-absorbed probes were then transfered to tubes containing blots of total DNA from sample experimental palms which had developed disease symptoms, and hybridized overnight as usual. The DNA was arranged on the blots according to the sampling sequence, and was blotted in two-fold dilutions. The results showed that cross-absorption of the probe before hybridization to the test samples reduced the background hybridization signal and considerably improved the sensitivity of detection. Under those conditions, detection of phytoplasma in palms as early as three months before development of disease symptoms was confirmed, as shown in Plate 4.3b. Thus the presence of coconut DNA sequences in the probes was affecting the sensitivity of detecting incubating infections.

It was also observed that the sensitivity of phytoplasma detection was influenced by the interaction between the pathogen, the host and environmental conditions at the time of symptom development. The calender year has two rainy seasons, with heavier rain falling during the long rainy season (March - June) than the short rainy season (Nov - Dec). In between them are two dry seasons. All except one of the seven palms (86%) in which phytoplasma was detected 2-3 months before disease expression, developed symptoms during the long rainy season or within a month after the rains (Table 4.1). Furthermore, all except one (86 %) were the ecotypes that are very susceptible to LD, i.e. 4 hybrid coconuts (MYD X WAT and CRD X WAT), and two susceptible talls (Karkar Tall, EAT-ex Tumaini). The only exception, an EAT collection from Lamu, which has so far been tolerant in field trials, developed disease symptoms during the long rainy season. It can be deduced that for the phytoplasmas to reach detectable levels long before the onset of disease, host susceptibility and moisture availability facilitated rapid multiplication.



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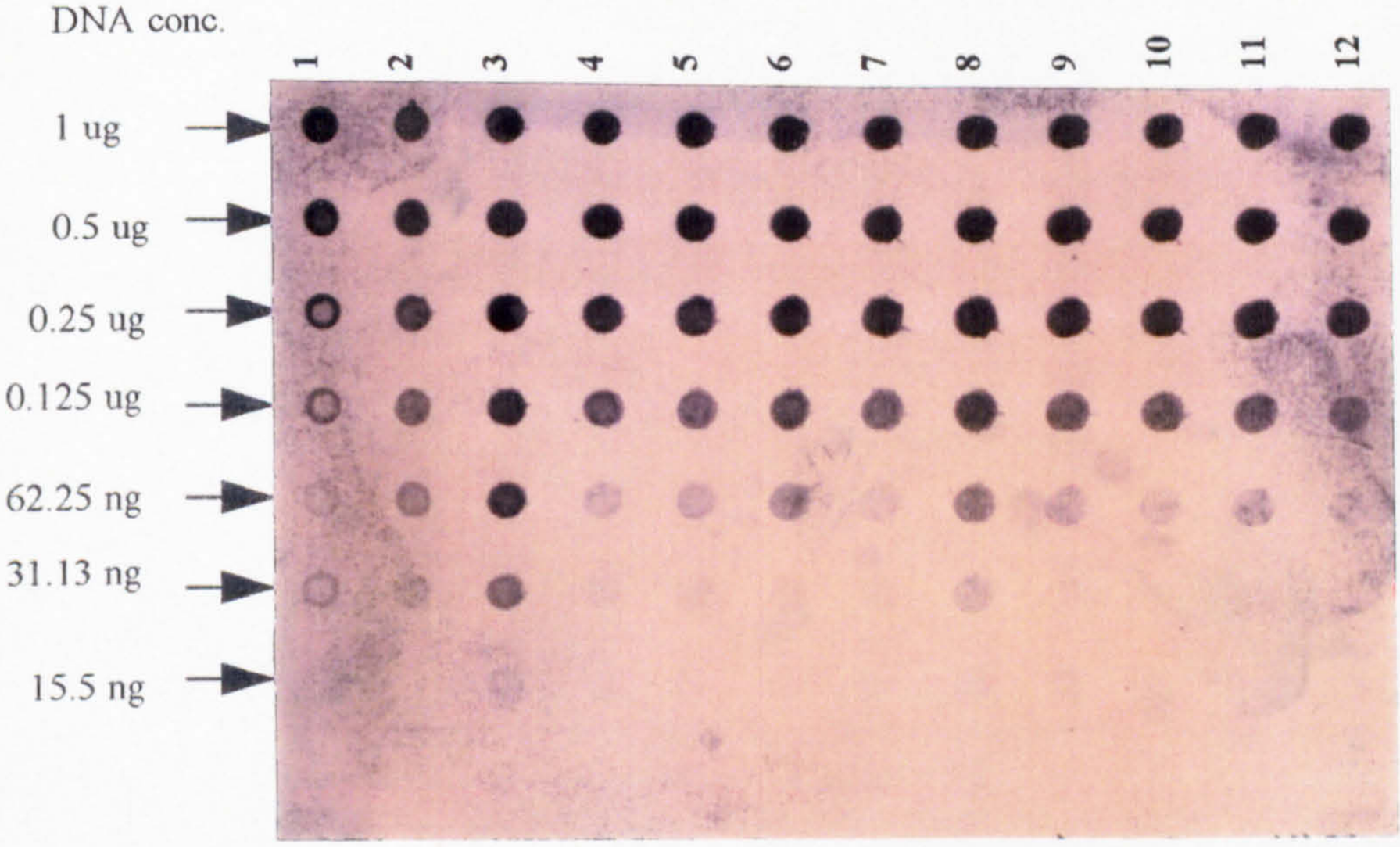


B

Plate 4.3: Detection of phytoplasma DNA in symptomless palms using the cross-absorbed probe LD 19-87 (B). Cross-absorption of probe DNA was by hybridization overnight to healthy coconut DNA (A). Samples on blot B are: healthy coconut DNA extracted by two different procedures (lane 1 & 2), LD infected control (lane 3); palm #25 Kigombe at onset of symptoms and 1 mo later (lanes 4-5); palm #26 Kigombe two months before disease symtoms (lane 6); palm # 29 Kigombe 1 mo before disease, at onset of disease and 1- 2 mo after symptom appearance (lanes 7-10); and # 14 chambezi 1 mo before disease and at onset of disease (11-12).

4.3.2 Screening Embryos and Seedlings Raised from Nuts Harvested from LD-Infected Palms

To investigate whether the LD phytoplasma can be transmitted through seed, embryos were excised from ten mature nuts (seed) collected from four palms showing moderately advanced disease symptoms at Kigombe, in Tanga region. Embryos excised from nuts collected from each palm were pooled and DNA extracted. Where germinating embryos were found, they were extracted separately from those not yet germinating. Two non-germinating embryos were obtained from the first palm, and three from the second and third palms respectively. Two germinating embryos were collected, each from a separate palm. Embryos from the fourth palm could not be extracted because they had started to disintegrate.

DNA was also extracted from individual spear leaves sampled from eight seedlings raised from nuts which were harvested from LD-infected coconut palms at Kifumangao. The DNA from both groups of tissues was blotted as single spots and hybridized to DIG-labelled probe LD 12-66. No phytoplasma DNA was detected in the DNA extracted from the 8 non-germinated embryos. However, a weak hybridization signal to the probe was observed in both embryos which were germinating at the time of collection. Similarly, DNA from both embryos which had produced a young shoot and haustorium, and DNA of four out of the eight seedlings that were screened produced hybridization signals. Since the positive hybridization signals could not be confirmed when these DNA samples were screened by the PCR technique, they were considered non-specific hybridizations.

4.3.3 Screening Other Unrelated Plant Species for Phytoplasma by Use of Probes

Other plant species growing in coconut fields, and showing symptoms which are typical of phytoplasma infections, such as chlorosis, phyllody, and / or virescence were sampled and screened for presence of phytoplasma DNA. Young leaves and twigs were plucked from such plants, and DNA extracted from them. The DNA was then blotted as single spots, and hybridised to DIG labelled probe LD12-66. Samples were collected from three shrubs at Chambezi, two from Kilwa, and three from Kifumangao. In addition,

periwinkle plants growing in the field at Kifumangao which developed severe phyllody and leaf chlorosis, were also sampled, blotted, and analysed. None of this DNA hybridized to the probe.

4.4 USE OF PROBES TO SCREEN FOR PRESENCE OF STRAINS OF THE LD PHYTOPLASMA

The incidence of LD in different regions of Tanzania where the disease occurs varies extensively. It has been postulated that one possible cause of these differences would be existence of pathogenic strains of the LD phytoplasma. To test the validity of this hypothesis, a dot blot of DNA extracted from palms sampled at three representative locations in two-fold dilutions (starting with 1 µg) was hybridized to DIG labelled probe LD 12-66. Kifumangao represented a high incidence area, while Chambezi and Kigombe represented the moderate and low incidence areas respectively. Three palms were sampled per site, and petioles of unopened leaves which had been found to contain a higher concentration of phytoplasma were used as a source of DNA. DNA extracted from leaf petioles (mixture) of three palms sampled from the northern Kenya coast was also blotted alongside for comparison.

Plate 4.4 shows the results of the hybridization reaction after high stringency wash conditions. Although the probe did hybridize to all isolates, the blot showed differences in phytoplasma titres among palms of different locations. The hybridization signal was more intense to the DNA isolates from Chambezi, and lowest to the Kenyan isolates. The probe could detect phytoplasma DNA in 62.5 ng of total DNA for the Kifumangao and Chambezi isolates respectively, and 125 ng for the Kigombe and Kenyan isolates (Plate 4.4). There was also a significant hybridization to healthy coconut DNA.

For detection of possible restriction fragment length polymorphisms between the different LD isolates and the other LYD isolates, 5 µg DNA of one isolate each from Kifumangao, Kilwa, Kigombe and Chambezi from Tanzania, as well as two isolates each from Kenya, Mozambique and Ghana were doubly digested to completion (16 hr at 37^0C) with restriction enzymes *Eco* RI and *Hind* III.

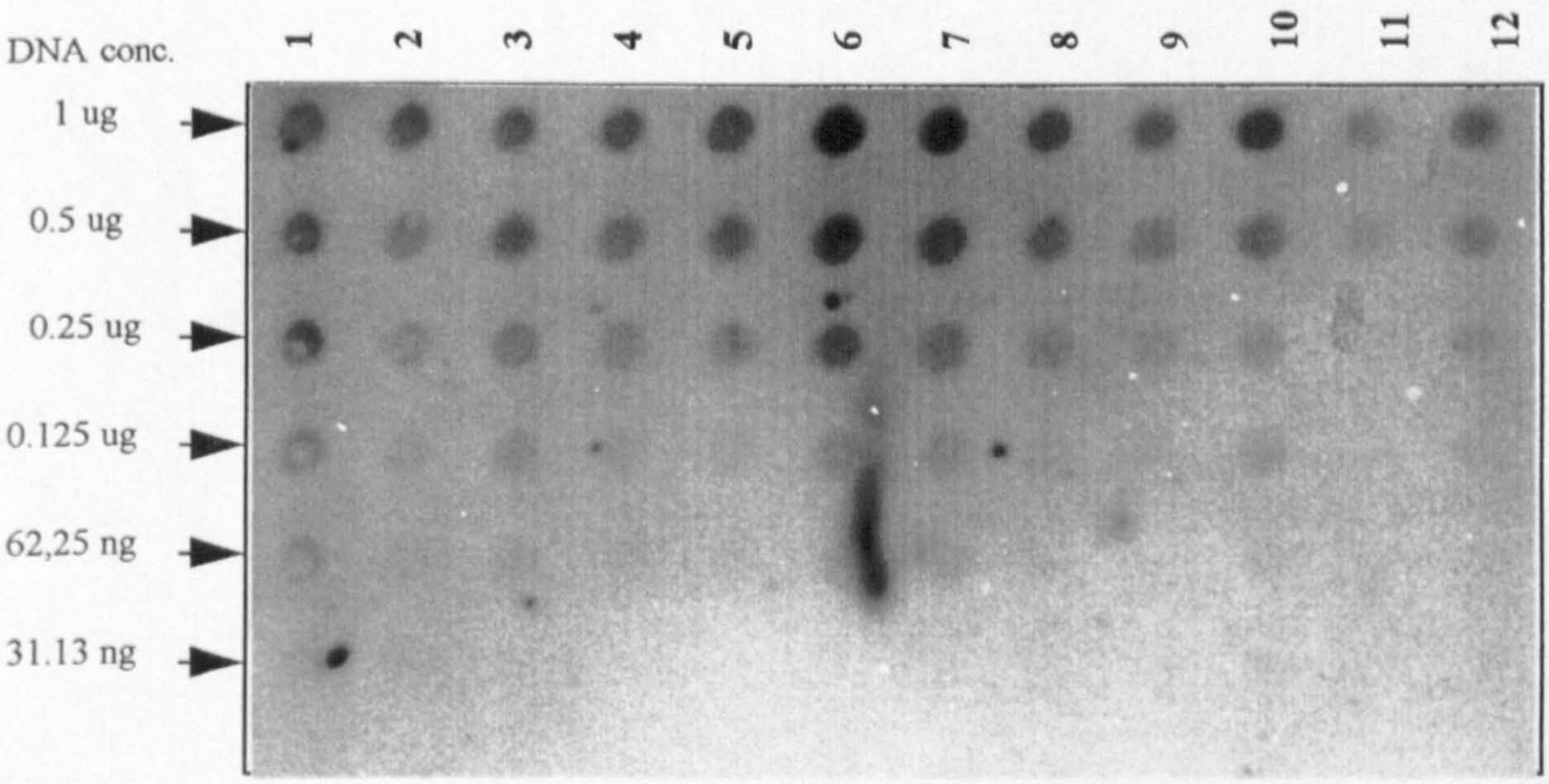


Plate 4.4: Detection of phytoplasmas in palms infected by LD in regions which show differences in disease incidence by use of probe LD12-66.

Lane 1, healthy coconut DNA, 2-4, isolates from Kifumangao; 5-7 isolates from Chambezi; 8-10, Tanga isolates, and 11-12, isolates from Kenya.

The three locations in Tanzania represent regions of different incidences of disease as mentioned above. Kilwa is also representative of the high incidence area. The isolates from Mozambique were both from Mocimboa da Praia in the northern province, near the border with Tanzania. Additional samples were an LY isolate from Jamaica, and a Spiroplasma isolate (*S. citri*). The restriction products were analysed by electrophoresis on a 1% agarose gel and visualised by UV-transillumination, after staining with ethidium bromide, then the gel was blotted by the method of Southern (section 2.6.2). The blot was hybridized to DIG labelled probe LD 12-66.

The probe hybridized to all the LD and LYD isolates after high stringency washes, but not to healthy coconut DNA, or *Spiroplasma citri* (Plate 4.5). Similar to the hybridization pattern observed to *Eco* RI-digested total DNA (section 3.4.2, Plate 3.5), the probe hybridized to fragments of all sizes between 1 kbp and 21 kbp, but the strongest signal was at approximately 1 kbp (Plate 4.5). The probe hybridized very strongly to the LY isolate from Jamaica, but there were differences in the hybridization signal to the other LYD isolates, with some isolates hybridizing very weakly. However, no polymorphisms were evident. The differences in hybridization signal are attributed to a low concentration of phytoplasma in the respective DNA samples.

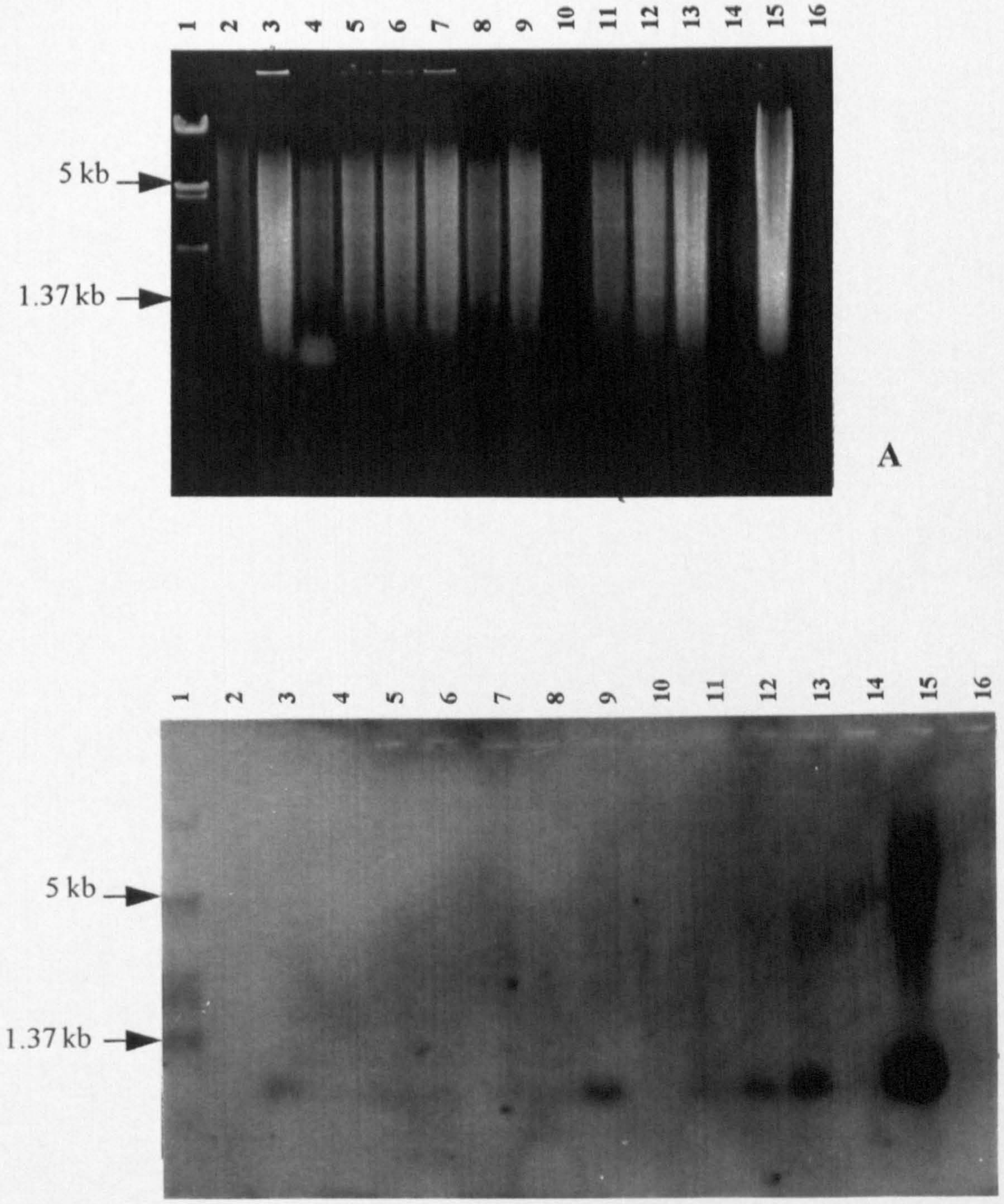


Plate 4.5: Ethidium bromide stained agarose gel of phytoplasma- infected coconut total DNA digested with *Eco*-RI and *Hind* III enzymes (A) and autoradiogram of the same gel after Southern blotting and hybridization to DIG-labelled probe LD12-66 (B).

1, Lambda DNA; 2, healthy coconut DNA; the phytoplasma isolates are:3-8, LD- Tanzania; 9-10, LYD-Mozambique; 11-12, LYD-Kenya; 13-14, LYD-Ghana; 15, LY (Jamaica); 16, *Spiroplasma citri*.

4.5 DISCUSSION

The DNA probes developed in this study were useful for detecting lethal disease in infected palms, despite difficulties experienced with background hybridization to healthy coconut DNA. Probe LD 12-66 in particular, which was extensively used provided basic information on the types of tissues where the phytoplasmas accumulate in infected palms. Results confirmed those obtained by electron microscopy studies (Deutsch and Neinhaus, 1983) that the LD micro-organisms accumulate in the sink areas (meristematic tissues), and that their distribution is uneven. These results helped to determine which parts of the palm were best to sample for disease detection.

The relative sensitivity and specificity of the probes was the main concern during probe development. By screening different palms from different localities, and at different stages of disease progression, probes LD 12-66 and LD LD 19-87 were consistently able to detect the LD phytoplasma in 7.8 ng of total DNA by dot hybridization. Hybridization by both probes to all isolates from areas with different disease incidences is an indication that the isolates share a high level of sequence homology. Variability in detection sensitivity for tissues from different sources could reflect differences in phytoplasma titres in the tissues of respective plants. The sensitivity of phytoplasma detection by use of DNA probes was reduced because of background hybridization to healthy controls. This result is explained by the difficulties experienced in separating phytoplasma DNA from coconut DNA (section 2.3.3), and is largely a consequence of low phytoplasma concentration in LD infected coconut tissues (Deutsch and Nienhaus, 1983). Researchers on the LY phytoplasma (which infects other palm species) could overcome this by producing probes using DNA from other host species, such as *Trachycarpus fortunei* and *Veitchia merrilli* (Harrison *et al.*, 1992; 1995). No alternative host for LD has yet been found.

Probe LD 12-66 which was investigated thoroughly showed a reasonable level of specificity for LD detection. However, this probe could not differentiate the LD phytoplasma from other related coconut infecting phytoplasmas in Southern blot hybridization analyses. The differences observed with a lower concentration of genomic DNA were also not conclusive. This would be due to the fact that this probe contains

reiterative sequences, which limits its value as an LD-specific probe. Difficulty in separating the cloned insert from vector sequences may also be contributing significantly to the background hybridization to healthy coconut DNA on dot blots, because this was not observed on Southern blots. Hybridization to healthy coconut DNA on dot blots could be reduced considerably by treating the DNA with proteinase K during extraction according to the procedure of Davis *et al.* (1993), as seen on plate 4.2 (lane 2). Cross-absorbing the probe DNA before use in the hybridization tests also had similar effects (Plate 4.3b). The disadvantage of these approaches, however, is that they are expensive, labour intensive and time consuming. Since these studies have confirmed the double nature of insert DNA, it may be useful to double digest the plasmid DNA and separate these inserts, and test each separately. The insert with high affinity to phytoplasma alone could then be sub-cloned for specific use. All the other potentially good probes should also be extensively screened, with the aim of determining LD-specific probes, because specific probes are more useful for disease detection and for determination of genetic relatedness among pathogens.

DNA probes proved valuable in studies on the distribution of phytoplasmas in tissues of the infected palm. Harrison *et al.* (1995) previously reported that the concentration of phytoplasmas in the tissues of LY-infected coconut palms is very low and the distribution uneven. The results of this study support their conclusions, and suggest that the petioles of young, unemerged leaves, the area below the growing point, and roots tips with higher concentrations of phytoplasmas may be more reliable tissues to sample if affected palms are to be cut down. However, sampling the petioles of young leaves and the tissue below the growing point is destructive and inappropriate for testing palms prior to the onset of disease. The concentration of phytoplasma in the spear leaves of LD-infected palms seem to be low, especially when disease symptoms have not developed in the host palm. Thus, for non-destructive sampling, and when probes are the diagnostic technique in use, the roots may prove very useful, and whenever possible should be sampled together with spear leaves to avoid incidental false negatives.

Chapter 5 DETECTION OF PHYTOPLASMAS IN INFECTED PALMS BY THE POLYMERASE CHAIN REACTION (PCR)

5.1 INTRODUCTION

The polymerase chain reaction (PCR), a procedure for primer-directed, enzymatic amplification of target DNA sequences *in vitro* (Saiki *et al.*, 1988), is the most recent and most sensitive approach to the detection of phytoplasmas (Ahrens and Seemuller, 1992; Deng and Hiruki, 1991a; Harrison *et al.*, 1994b). The specificity of PCR amplification, and hence pathogen detection is based on two synthetic oligonucleotide primers which flank the target DNA sequences to be amplified and hybridize to opposite strands (Saiki *et al.*, 1988; Erlich *et al.*, 1988). The procedure involves repeated cycles of heat denaturation of the DNA, annealing of primers to their complementary sequences on the denatured strands, and extension of the annealed primers with thermostable DNA polymerase. The primers base pair to and define each end of the target sequence on opposite strands, so that DNA synthesis by the enzyme proceeds across the region between them, ensuring simultaneous copying of both strands of the selected fragment. The extension product of one primer can serve as template for the other primer. This results in doubling of the targeted sequences at every successive cyle and causes exponential accumulation of the DNA fragment. By use of a thermostable DNA polymerase, the technique is capable of selective enrichment of the target sequences by a factor of 10^6 or more within 25 to 30 cycles (Saiki *et al.*, 1988). The amplified target DNA is detected by agarose or polyacrylamide gel electrophoresis and ethidium bromide staining.

The PCR technique is quick, very sensitive, and has been very useful for detection of pathogens which are scarce, difficult to culture, or difficult to identify once cultured. This is because it can detect the presence of minute quantities of target sequences, and those hampered by the presence of extraneous material (Saiki *et al.*, 1988; Henson and French, 1993). The specificity of primers for the target sequences can however be affected by several factors including, primer length, annealing temperature, magnesium concentration, and secondary structure of the target and

primer sequences. The effect of magnesium ion concentration on primer specificity is related to enzyme activity. These ions have been shown to be an absolute requirement for Taq polymerase activity and the optimal concentration is 1.5 mM (Chien *et al.*, 1976). Primer annealing temperature and extension time are other important factors. Saiki *et al.* (1988) reported that increasing the primer annealing temperature increases the specificity of the PCR reaction. Lower annealing temperatures result in primers annealing to mismatched target sequences, and cause nonspecific amplification. Longer extension times were also reported to affect the specificity of detection by increasing the amount of non-specific DNA amplified. Furthermore, large amounts of enzyme were noted to increase the amount of nonspecific amplification. Primer length is also important for specificity of detection. Good primers are at least 16 nucleotides long, and between 20-24 nucleotides, as longer primers could form stable hybrids at the polymerization temperature of 72^{0}C and result in lower DNA yields (Sambrook *et al.*, 1989).

For sensitive detection of phytoplasmas, primers based on *Mollicute* 16S ribosomal RNA genes have been used to selectively amplify phytoplasma DNA from mixtures with host DNA (Ahrens and Seemuller, 1992; Deng and Hiruki, 1991a; Lee *et al.*, 1993a; Namba *et al.*, 1993a). The 16S rRNA gene is a universal characteristic in prokaryotes, and has both conserved and variable regions (Razin, 1985). About 73-78% homology in rRNA sequences between phytoplasmas and the other Gram-negative prokaryotes have been detected by sequencing the 16S rRNA gene of the western X-disease phytoplasma and comparing it to the other sequences (Kirkpatrick and Fraser, 1989a). However, Kuske and Kirkpatrick (1992) have demonstrated by sequencing and comparing the 16S rRNA gene of the western aster yellows phytoplasma, that this gene contains variable regions which are unique to phytoplasmas, and which make them more closely related to each other than to other prokaryotes. These unique sequences have been utilised for the production of phytoplasma-specific or "universal" primers which are useful for detection of a broad array of phytoplasmas from infected plants, and for purposes of differentiation and classification of phytoplasmas (Deng and Hiruki, 1991a; Lee *et al.*, 1993a; Schneider *et al.*, 1993; Namba *et al.*, 1993b).

The 16S rRNA gene of plant chloroplasts is known to be very similar to that of prokaryotes (Grierson and Covey, 1984). However, it has been shown that there is sufficient variability in this gene to ensure that PCR primers based on the 16S rRNA sequences do not amplify rDNA from plant chloroplasts. By comparing the 16S rRNA sequences of the X-disease phytoplasma, plant chloroplasts and culturable mycoplasmas, Kirkpatrick and Fraser (1989b) have detected an 18-bp sequence in the 5' region of this gene which is highly conserved in phytoplasmas, but is absent in chloroplasts. This difference has been utilised in designing primers which amplify only the rDNA from phytoplasmas in PCR assays. Optimizing the primer annealing temperatures ensure that only the targeted sequences are amplified. Alternatively, restriction enzymes can be used to differentiate phytoplasma DNA from plant DNA after PCR. By use of restriction enzymes to determine restriction fragment length polymorphisms of PCR products, the band amplified from plant extracts using primers based on the conserved 16S rRNA sequences have been confirmed to be phytoplasma rDNA (Ahrens and Seemuller, 1992).

The variable regions on the 16S rRNA genes have been used to produce primers for specific pathogen detection (Namba *et al.*, 1993a; Rohde *et al.*, 1993). Similarly, pathogen-specific primers for detection of phytoplasma diseases have been developed based on the sequences of well-characterized cloned fragments of the respective phytoplasmas (Schaff *et al.*, 1992; Deng and Hiruki, 1991b; Lee *et al.*, 1993b; Harrison *et al.*, 1994b). The sensitivity of detection obtained using pathogen-specific primers based on the cloned phytoplasma sequences have been reported to substantially exceed the lower limits of detection by hybridization with the respective DNA probes (Schaff *et al.*, 1992; Harrison *et al.*, 1994b,c).

The intergenic spacer region between ribosomal 16S and 23S RNA genes have also been utilised to produce primers which specifically detect phytoplasmas. This region has been sequenced using phytoplasma-specific primers developed from the 16S rRNA sequences, and shown to contain a highly conserved transfer RNA ($tRNA^{Ile}$) gene (Kirpatrick *et al.*, 1994b). Phytoplasma-specific primers based on these sequences amplify the conserved gene as a single PCR product from phytoplasmas,

while other prokaryotes are amplified as multiple bands due to sequence variations (Smart *et al.*, 1994). The sequences flanking the highly conserved $tRNA^{Ile}$ in phytoplasmas are highly variable and have been used to produce strain-specific PCR reverse primers (Kirpatrick *et al.*, 1994a).

5.2 PRIMERS AND OPTIMIZATION OF REACTION CONDITIONS

5.2.1 Source of Primers

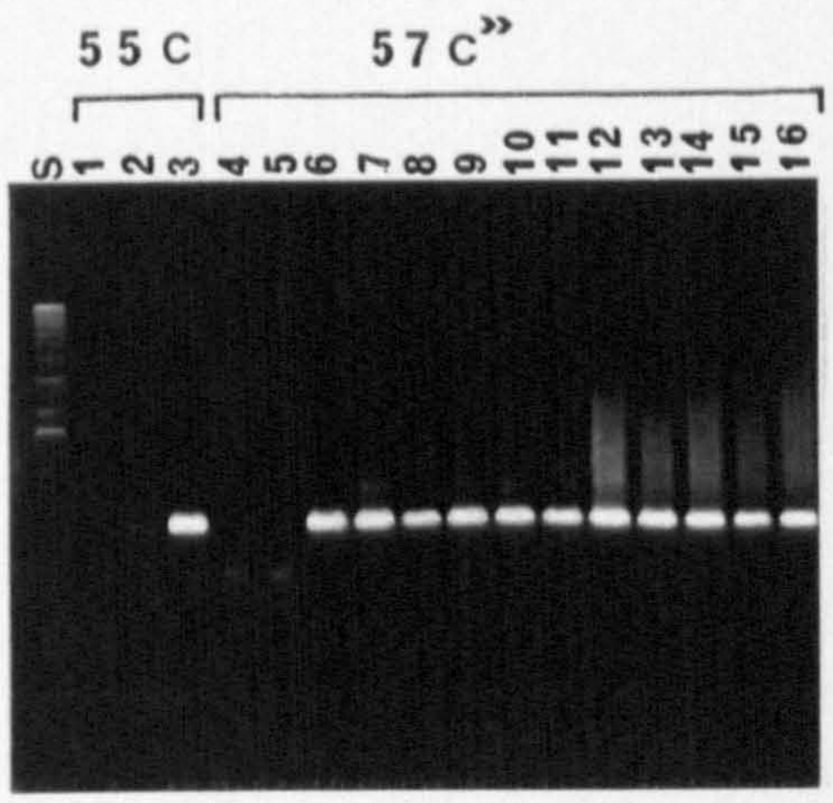
Ribosomal DNA (rDNA) sequences were amplified from reaction mixtures containing LD infected palm DNA by use of several primers. They include:

i) The Mollicute- specific 16S rRNA primer pair (Deng and Hiruki, 1991a),
P1, 5'-AAGAGTTTGATCCTGGCTCAGGATT-3' (forward), and
P6, 5'-TGGTAGGGATACCTTGTTACGACTTA-3' (reverse).

ii) Primers based on ribosomal RNA sequences of the LD phytoplasma:
LD 16-1, 5'-CGGAAACCTTCGGGTTTTAG-3' (forward) (Harrison, 1994)
Rohde's forward, 5'-GAGTACTAAGTGTCGGGGCAA-3', and
Rohde's reverse, 5'-AAAAACTCGCGTTTCAGCTAC-3, (Rohde *et al.*, 1993)

iii) A reverse primer based on the 16S / 23S intergenic RNA sequences of the LD phytoplasma:
LD SR, 5'- GGTGCCATATATATTAGATTG-3', (Tymon, 1995)

Thirty six cycles of PCR were sufficient to detect phytoplasma in LD infected tissue for all the primers used. A prominent 560 base pair DNA band was resolved by agarose gel electrophoresis from all reaction mixtures containing template DNA from LD-infected palms and Rohde's forward and reverse primers. No such band was amplified from healthy coconuts (Plate 5.1).



S	Size marker (1 KB Ladder)
1	Healthy East African Tall DNA, Chambezi
2	Symptomless Cambodia Tall,Kifumangao
3	LD-infected Tagnanan Tall, Chambezi
4	Healthy East African Tall, Chambezi
5	Symptomless Cambodia Tall, Kifumangao
6	LD-infected Tagnanan Tall, Chambezi
7	Gradient enriched LD-DNA, Kifumangao
8	Gradient-enriched LD-DNA, Kifumangao
9	Gradient-enriched LD-DNA, Kenya
10	Gradient-enriched LD-DNA, Sotele
11	Gradient-enriched LD-DNA, Chambezi
12	LD-infected brown dwarf, Kifumangao
13	Symptomless Cambodia Tall, Kifumangao (gradient ennriched DNA)
14-16	LD-infected East African Tall DNA, Kenya
>>	Annealing Temperature

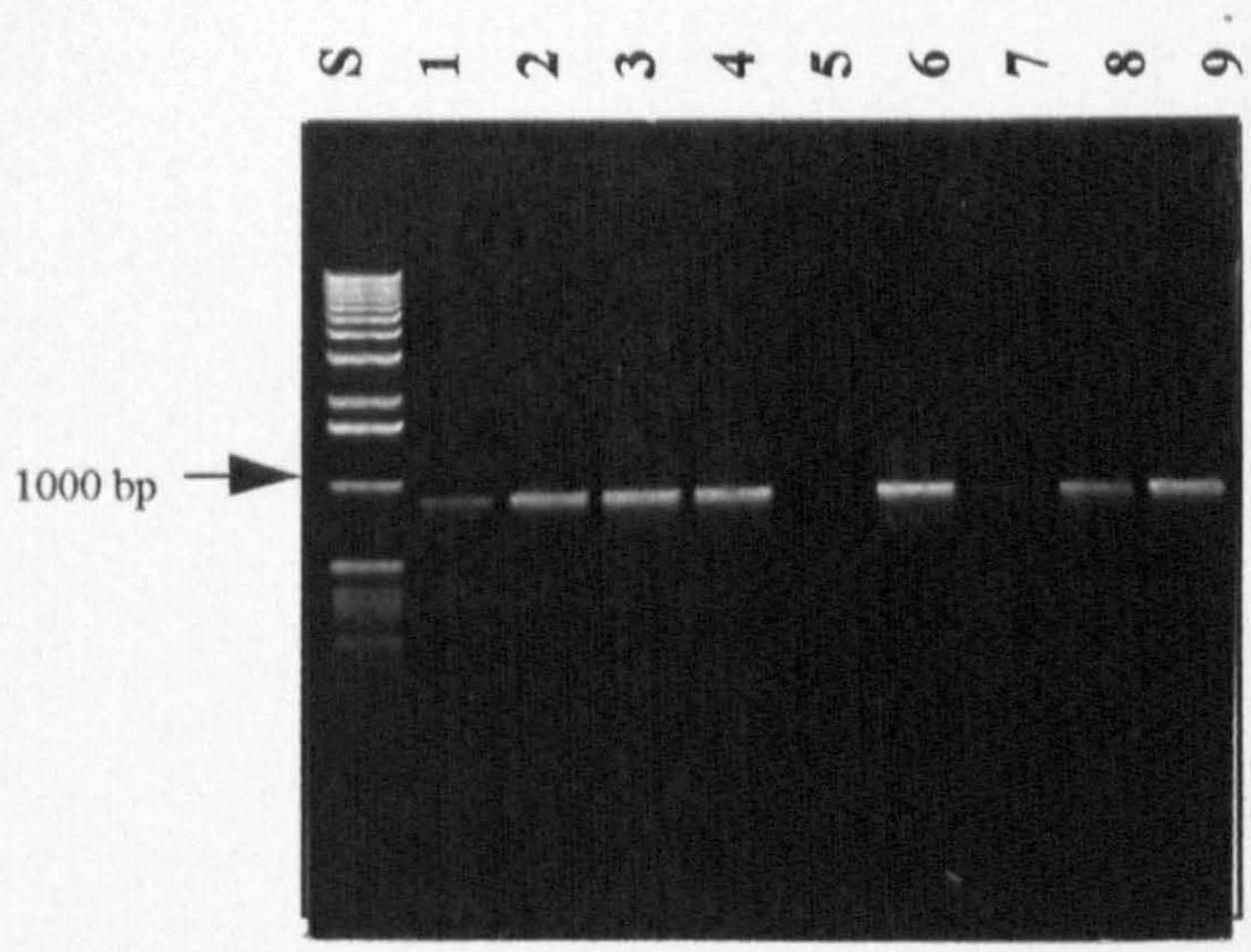
Plate 5.1: Detection of LD phytoplasma by PCR in coconut palms using primers Rohde forward and Rohde reverse. A ribosomal DNA band of approximately 500 bp was amplified from infected tissue. The optimum annealing temperature for this primer pair was 57^0C. False bands were amplified from healthy coconut tissue (lanes (1-2) when the annealing temperature was set at 55^0C which is sub-optimal. LD could be detected in a symptomless palm (lane 13).

Similarly, DNA bands of approximately 1.65 kbp were resolved from mixtures containing template DNA from LD-infected palms and either P1/ LD SR or LD 16-1/ LD SR primer combinations respectively. No DNA band was amplified from healthy coconut DNA in either case (Plate 5.2). A smaller band of approximately 1 kbp was amplified from mixtures of LD-infected DNA and either Rohde forward /LD SR or LD 16-1/ Rohde reverse primers, but not from healthy coconut DNA (Plate 5.2). Primers P1 and P6 similarly amplified a ribosomal DNA band of about 1.5 kbp from LD-infected tissue, but not from healthy tissue (Plate 5.6).

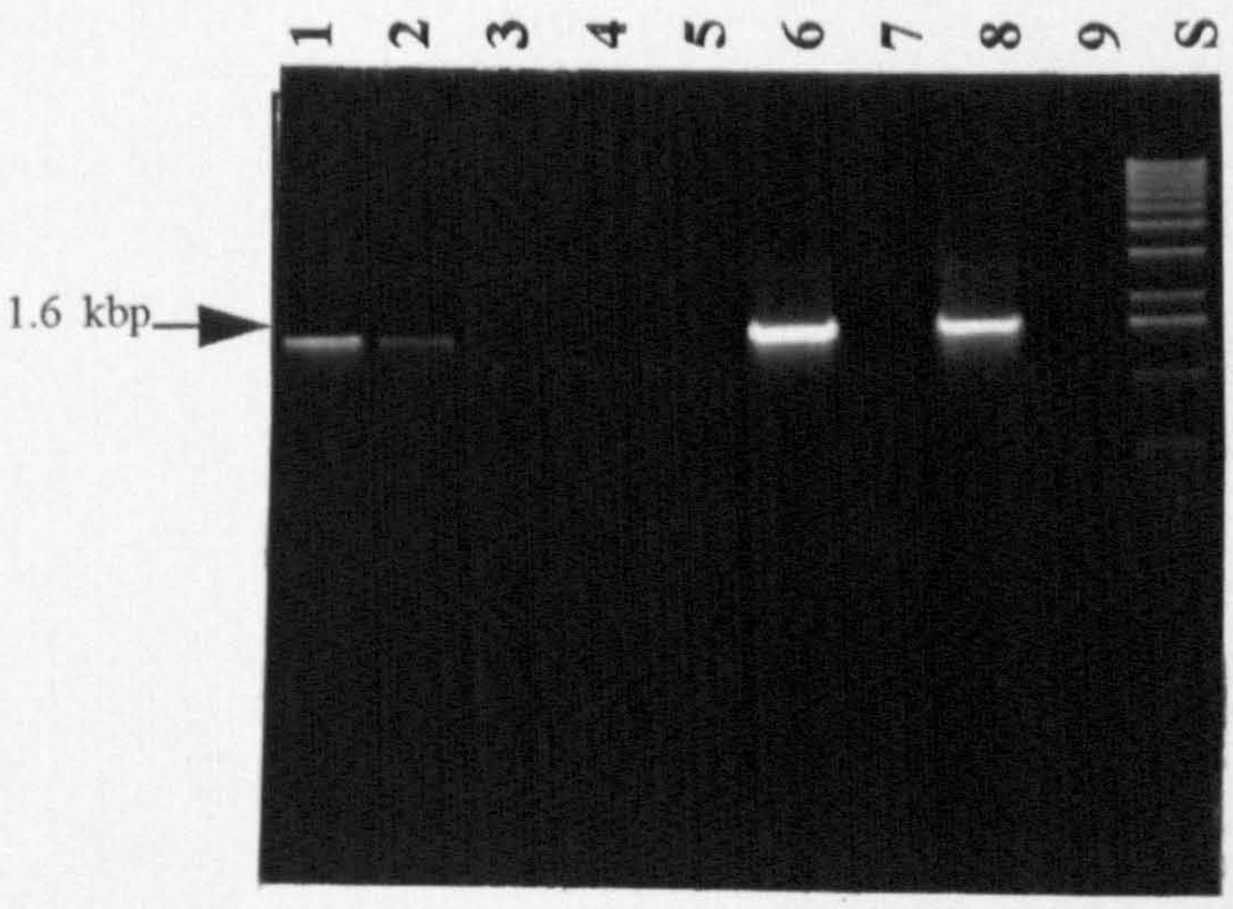
5.2.2 Determination of Optimum PCR Conditions

For each primer pair, PCR products were monitored at different temperatures, and different concentrations of magnesium ions and dNTPs, in order to determine the optimum reaction conditions. To determine the optimum annealing temperature for a primer pair, the melting temperature, Tm of each primer was first calculated from its nucleotide sequences. This was done by assigning 4^0C to every C and G, and 2^0C to every A and T nucleotides. The annealing temperature of each primer was then calculated by subtracting 5^0C from its melting temperature. A range of temperatures between the annealing temperature and melting temperature of the primer with a lower annealing temperature, was then selected and tested. The temperature at which the best PCR products were produced, was selected as the optimum for regular use. A worked example of determining the optimum annealing temperature for several primers is shown in the appendix.

The optimum annealing temperature for Rohde's primer pair was at 57^0C. At the sub-optimal annealing temperature of 55^0C, this primer pair amplified a false rDNA band from healthy coconut palms (Plate 5.1). Similarly, the optimum annealing temperatures were determined to be 52^0C for primer pairs P1 / LD SR, P1 / P6, and LD 16-1 / Rohde Reverse respectively, 53^0C for LD 16-1 / LD SR, and 54^0C for Rohde forward / LD SR (data not shown).



A



B

Plate 5.2: Amplification of LD rDNA from coconut palms using other primer combinations. (A) Rohde forward / LD SR; (B) LD16-1 / LD SR. rDNA fragments of approximately 1.0 kbp and 1.65 kbp were amplified from infected palms by these primer pairs, but no product was amplified from healthy DNA.

S, 1 kbp DNA; Gel A: LD DNA used at a concentration of 12.5, 25, 50, and 100 ng (lanes 1-4); healthy coconut (5); LD control (6); second LD DNA sample at a conc. of 12.5, 25, and 50 ng (7-9). Gel B:LD DNA (lane 1-2); insect DNA (3-5, 7); insect DNA spiked with LD DNA (6 & 8); healthy coconut DNA (9).

A prominent rDNA band was amplified from LD-infected DNA in reaction mixtures containing 1.5 mM of magnesium chloride, irrespective of primer pair. Higher concentrations of Mg++ ions in the reaction mixture resulted in amplification of multiple non-specific bands as shown using Rohde's primers (Plate 5.3). Similarly, the amount and quality of the rDNA band was better when the reaction mixtures contained deoxynucleotides (dNTPs) at a concentration of 150 µM. At higher concentrations of the dNTPs (>200 µM), multiple bands, which were smaller were amplified, even from healthy coconut tissue (Plate 5.4).

The effect of amount of enzyme on the reaction mixture was also assessed. Good amplification products were readily obtained with 1 Unit of enzyme per reaction. A combination of high enzyme concentration (2.5 Units per reaction), with high dNTPs produced multiple small bands as shown in Plate 5.4, and these bands were also amplified from healthy coconut DNA. After assessment of the ramping time for the thermocycler (Biometra thermoblock), the reaction conditions were set at denaturation for 1 min at 94^0C (2 min first cycle), 1 min 20 sec for primer annealing, and 2 min 10 sec for primer extension. The extension time in the last cycle was increased to 5 min. When the primer annealing or extension time was reduced, no amplification product was obtained from LD-infected tissues (data not shown).

5.3 DETECTION OF PHYTOPLASMAS IN PALMS INFECTED BY LD AND OTHER LETHAL YELLOWING TYPE DISEASES (LYD)

5.3.1 Detection of Phytoplasma in LD- Infected Palms

DNA was extracted from meristematic tissues of LD-infected palms and screened for phytoplasmas by PCR using all the primer pairs described above. In all reaction mixtures containing LD-infected palm DNA, ribosomal DNA (rDNA) sequences were amplified (Plates 5.1 and 5.2). Thirty six cycles of PCR were sufficient to detect the presence of phytoplasma in LD-infected tissue. No band was amplified from healthy coconut DNA. There were also differences among the primer pairs used for DNA amplification.

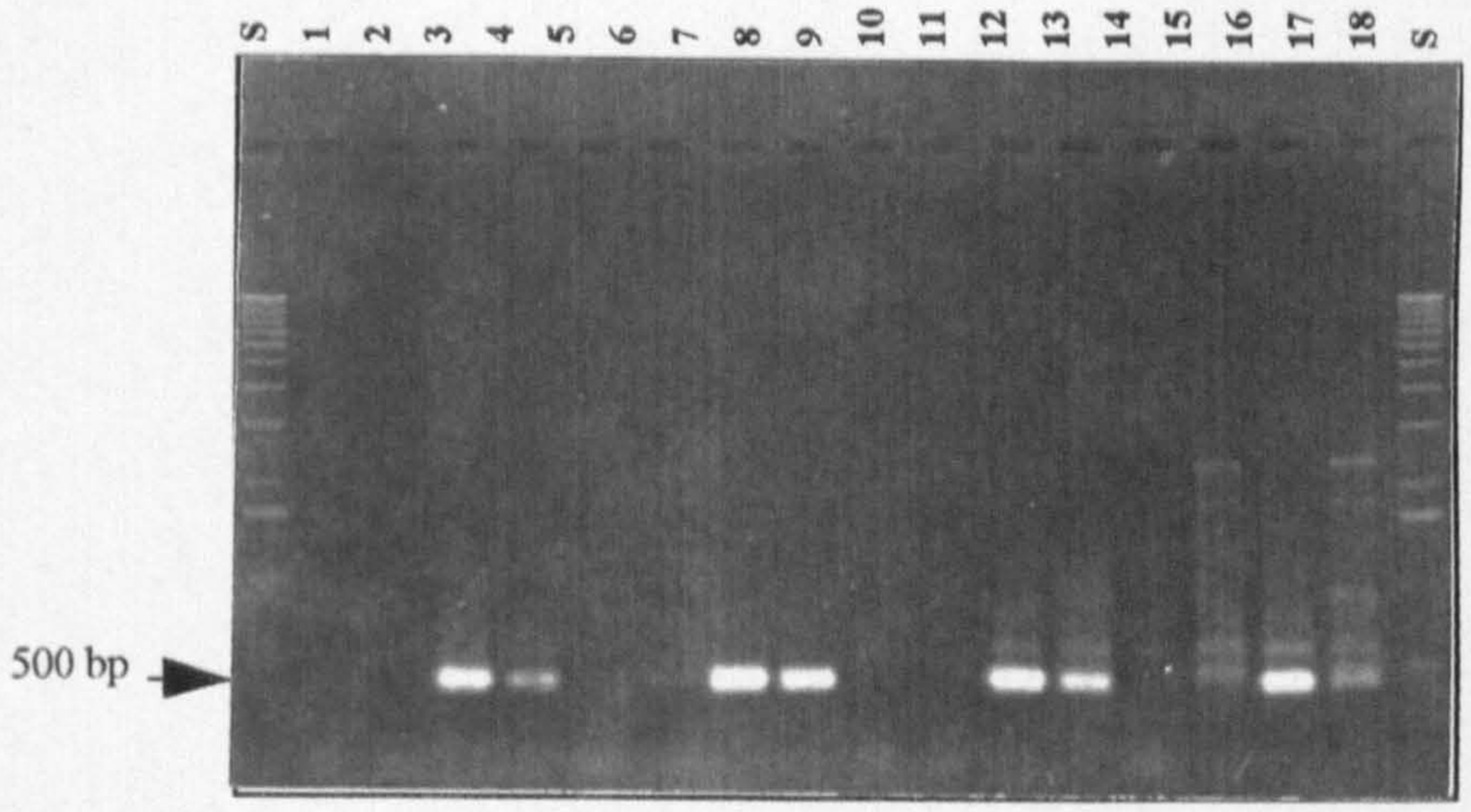


Plate 5.3: Effect of Magnesium ions on amplification of rDNA from phytoplasma- infected palmDNA by PCR. Concentrations of more than 1.5mM caused amplification of non-specific bands (lanes 12-17). S, 1 Kb DNA; lane 2-5, Std PCR buffer; lane 6-9, optimiser buffer + 1.5 mM Mg++; lane 10-13, optimiser buffer + 2.0 mM Mg++; lane 14-17, optimiser buffer + 2.5 Mg++. The first two palms in each group were healthy, and the other two LD-infected.

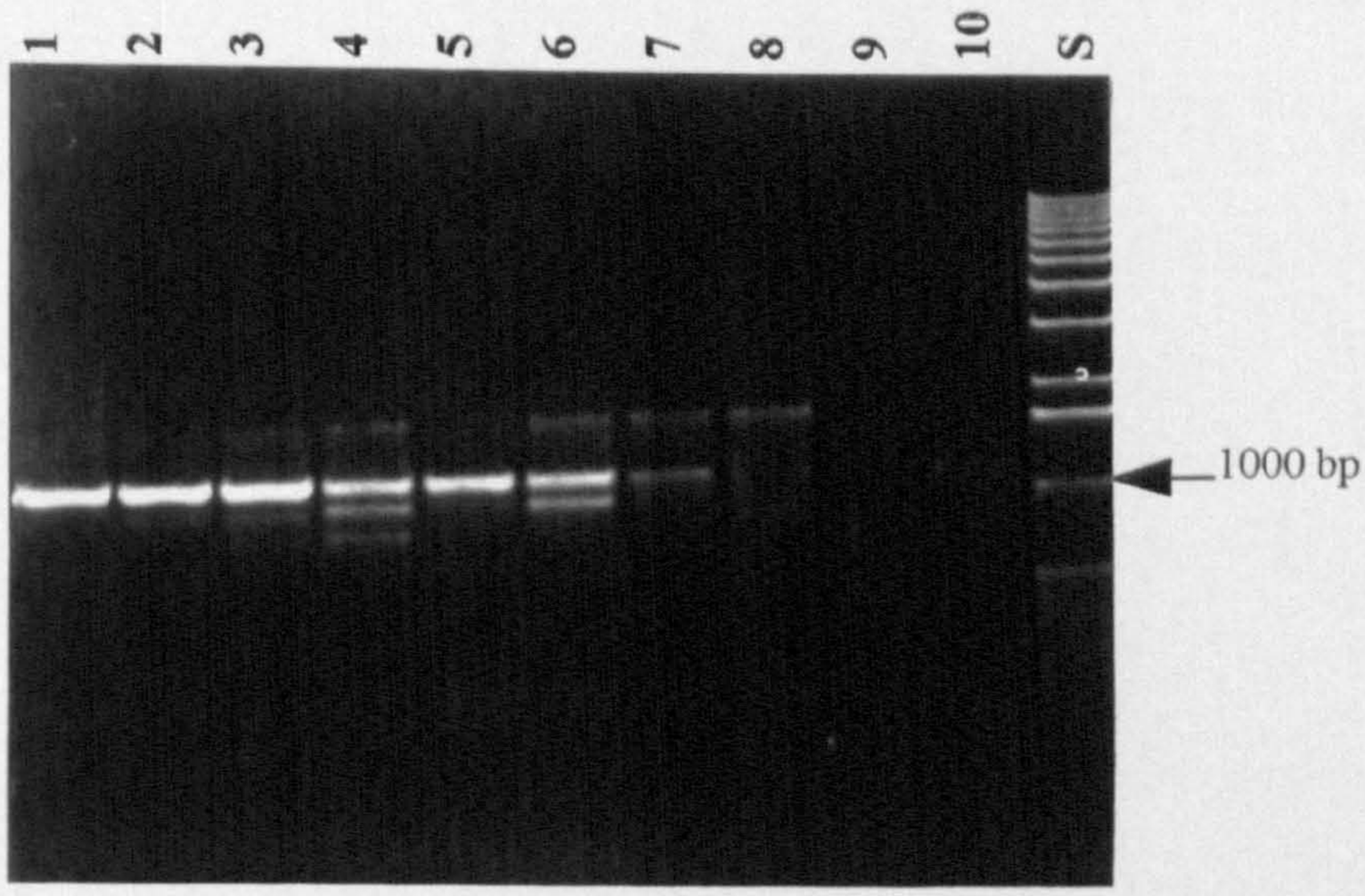


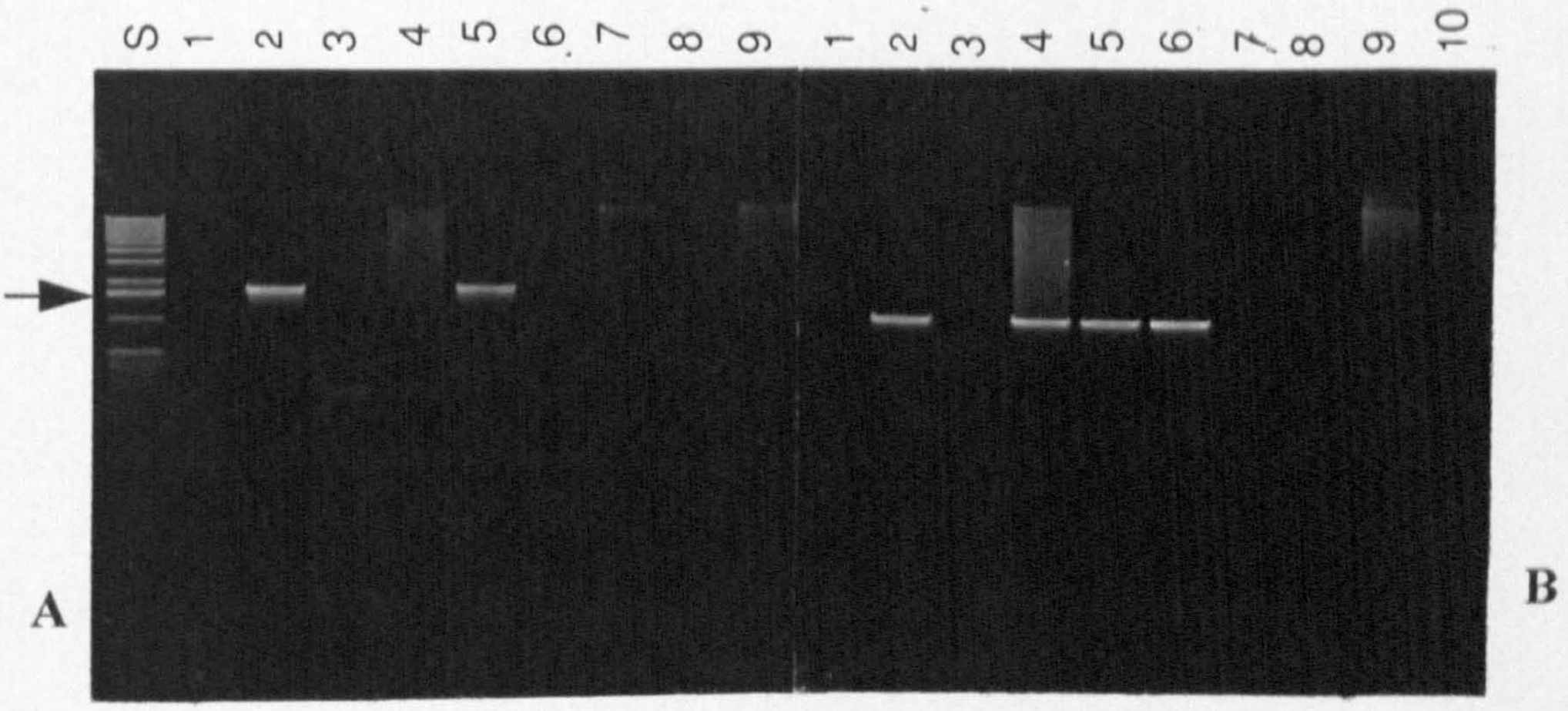
Plate 5.4: Effect of high enzyme and dNTPs concentration on PCR amplification of ribosomal DNA from LD- infected tissue. dNTPs at a concentration of 400 uM, and 2.5 U enzyme caused non-specific bands to be amplified from both healthy and LD-infected coconut DNA. Lane 1-6, DNA from different LD-infected coconut palms, 7-8 and 10, healthy coconut palms; 9, water control; S, 1 Kb DNA.

Rohde's primer pair was the most sensitive, and could detect phytoplasmas in tissues of palms at very early stages of infection when other primer pairs failed to amplify a band (Plate 5.5). Rohde forward/ LD SR was another sensitive pair, though not at the same level as the former. These primers were routinely used to screen DNA from tissues likely to contain small quantities of phytoplasmas, like the spear leaves.

5.3.2 Studies on the Effect of Sampling Technique on the Sensitivity of PCR for Phytoplasma Detection

Similar to the studies with DNA probes (section 4.2.2), DNA was extracted from all the meristematic tissues of LD-infected palms at different stages of disease progression, and screened for the presence of phytoplasma DNA. The objective was to determine in which parts of the affected palm the organisms accumulate, to assist in sampling for disease detection. The phytoplasmas were detected in all meristematic tissues, including the petioles of young opened and unopened leaves, root tips, the area below the growing point, inflorescences and the spear leaf. However, there were instances when the pathogen could not be detected in palms with clear disease symptoms, for no apparent reason. Since PCR assays were routinely done using 0.5 µl of DNA extract as template, it was considered possible that variations in the quantities of phytoplasma in the extracts, whereby some samples contained too much or too little could be causing the discrepancies. This would be indirectly determined by the type of tissue from which the DNA was extracted.

To verify whether the tissue used for DNA extraction could affect the sensitivity of disease detection by PCR, an experiment was set up using DNA from four palms at different stages of disease progression sampled at Chambezi. The stages of disease were 4.8, 5.6, 6.06 and 6.7 (Section 4.2.2). It was the same DNA used to study the distribution of phytoplasmas in tissue by use of DNA probes. DNA from each tissue was used at different concentrations (ranging between 0.5 ng and 100 ng) as template in PCR assays. The Rohde forward/LD SR primer pair was used for amplification. The results of this experiment are shown in Table 5.1.



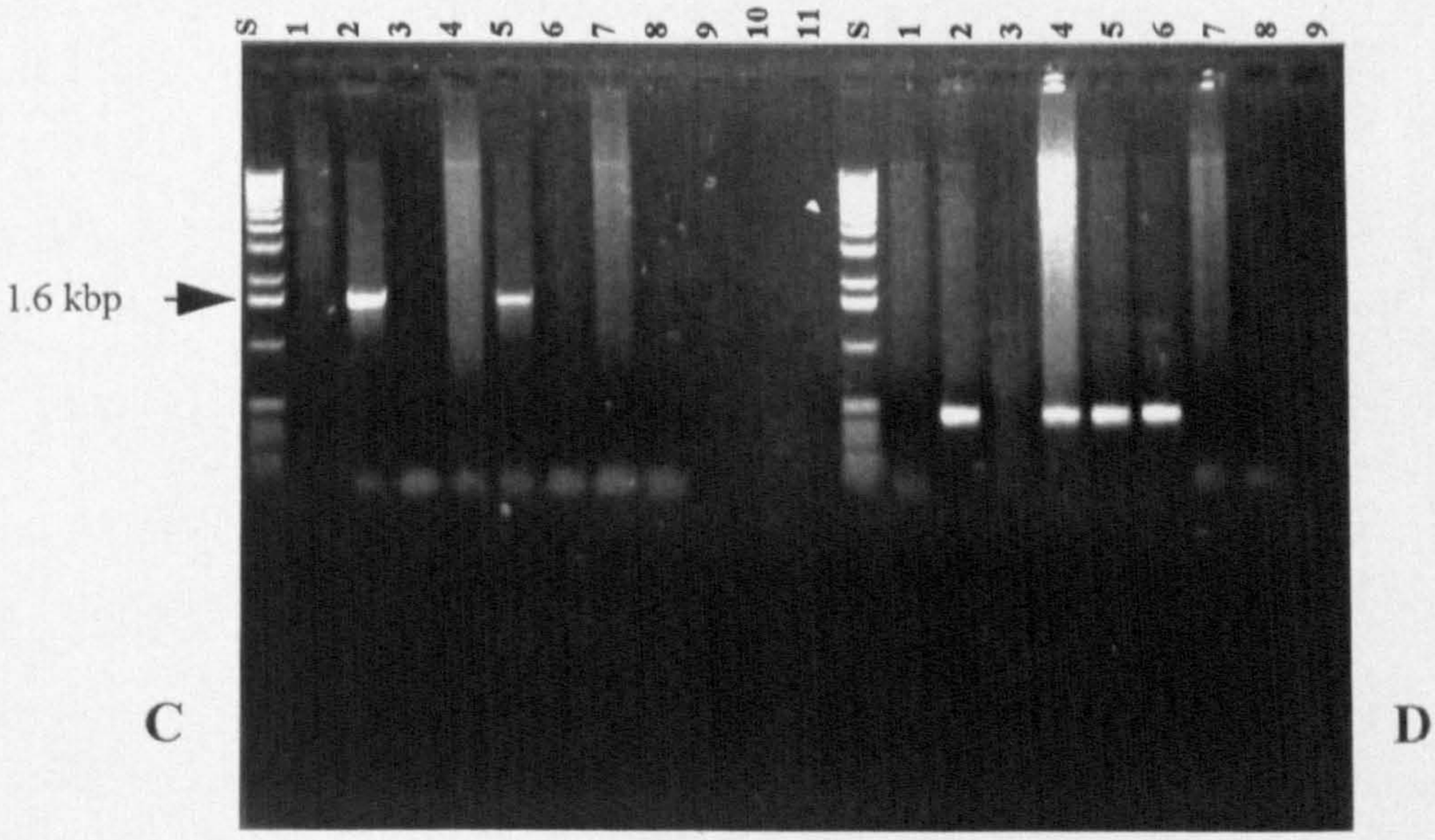


Plate 5.5: Sensitivity of different primer pairs for amplification of LD rDNA from infected palms by PCR. The primer pairs, P1 / P6 (A); Rohde forward / LD SR (B); LD 16-1 / LD SR (C) and Rohde forward / reverse (D) were used to amplify rDNA from five palms suspected to be infected by LD.

Rohde forward / reverse primer pair and Rohde forward / LD SR detected infections before disease symptoms (palm no. 4&6). All pairs detected LD when disease symptoms were fully expressed (5), but did not detect phytoplasma in palms 3&7 which were sampled 1 mo before onset of disease. S, 1 kbp DNA; 1, healthy coconut DNA; 2, LD-infected DNA; 3-7, DNA from spear leaves of palms suspected to be LD infected.

There were clear differences between tissues in each palm, and between palms at different stages of disease. For example, in palms 2, 3, and 4, the pathogen was readily detected in the leaf petioles, but not in the spear leaves. The leaf petioles, therefore would be the best source of DNA for pathogen detection at all stages of disease. In a palm at an early stage of disease progression, however, phytoplasmas were readily detected in the tissue below the growing point rather than in the leaf petioles. At this stage, there were differences even among the petioles. A ribosomal DNA band was amplified from DNA of the leaf petioles in very young unopened leaves, but not in the DNA from the petiole of the opened leaves (Palm 1 Table 5.1).

DNA extracted from the root tips also provided a good template for amplification of phytoplasma rDNA (Table 5.1). However, no rDNA band was amplified from the DNA extracted from spear leaves for all the palms assessed (Table 5.1). This indicated that this primer pair was not sensitive enough to detect phytoplasma in tissues with very low concentration of the pathogen.

Although PCR may not detect differences in pathogen concentration in tissues due to its high sensitivity, it was possible in this experiment to identify which tissues were best sources of DNA for pathogen detection by using template DNA at very low concentrations. The results are in agreement with DNA probe studies on the distribution of phytoplasmas in LD-infected coconut palms. Tissues which were shown to contain the highest concentrations of phytoplasmas by probes, were the same in which detection by PCR was highly sensitive (Table 5.1, Plate 4.2).

Table 5.1 Detection of Phytoplasma rDNA in different tissues of LD-Infected Palms by PCR

Palm tissue	DNA conc.	Intensity of Amplified Phytoplasma rDNA band			
		Palm 1	Palm 2	Palm 3	Palm 4
Disease stage		4.80	5.60	6.06	6.70
Root tips	0.5 ng	NT	-	NT	-
	5.0 ng	NT	-	NT	+(f)
	6.25 ng	NT	-	NT	+(vf)
	12.5 ng	NT	-	NT	+
	25.0 ng	NT	-	NT	+
	50.0 ng	NT	+(f)	NT	++
	100.0 ng	NT	+(f)	NT	+
Petiole +2	0.5 ng	-	NT	-	-
	5.0 ng	-	NT	+(f)	-
	6.25 ng	-	NT	+	+(vf)
	12.5 ng	-	NT	++	+(f)
	25.0 ng	-	NT	++	+(f)
	50.0 ng	-	NT	+++	+
	100.0 ng	-	NT	-	++
Petiole +1	0.5 ng	-	+(f)	-	-
	5.0 ng	-	+	-	-
	6.25 ng	-	++	-	-
	12.5 ng	-	++	+(vf)	+(vf)
	25.0 ng	-	+++	+(vf)	+(f)
	50.0 ng	-	-	-	+
	100.0 ng	-	-	-	++
Petiole -1	0.5 ng	-	-	-	-
	5.0 ng	-	-	+(vf)	+(f)
	6.25 ng	-	-	+(vf)	-
	12.5 ng	-	-	+	-
	25.0 ng	-	-	+	+

	50.0 ng	+ (vf)	-	++	++
	100.0 ng	-	+ (vf)	-	+++
Petiole -2	0.5 ng	-	-	-	+ (vf)
	5.0 ng	-	-	-	-
	6.25 ng	-	-	+ (vf)	+ (f)
	12.5 ng	-	-	+ (vf)	+
	25.0 ng	-	-	+ (f)	++
	50.0 ng	-	-	-	+++
	100.0 ng	+ (f)	+ (f)	-	++++
Petiole -3,-4	0.5 ng	-	-	-	-
	5.0 ng	-	-	-	-
	6.25 ng	-	-	-	-
	12.5 ng	-	-	+ (vf)	+ (vf)
	25.0 ng	-	-	+	+ (f)
	50.0 ng	-	-	+	+ (f)
	100.0 ng	+ (f)	+ (f)	++	-
U.G. Point	0.5 ng	+ (vf)	-	-	-
	5.0 ng	-	-	-	-
	6.25 ng	+ (f)	-	+ (vf)	-
	12.5 ng	+	-	-	-
	25.0 ng	++	-	-	+ (vf)
	50.0 ng	+++	-	+ (vf)	+ (vf)
	100.0 ng	+++	-	-	+ (f)
Spear leaf	0.5 ng	-	-	-	-
	5.0 ng	-	-	-	-
	6.25 ng	-	-	-	-
	12.5 ng	-	-	-	-
	25.0 ng	-	-	-	-
	50.0 ng	-	-	-	-
	100.0 ng	-	-	-	-

- Not detected, vf, very faint; f, faint; +, strong; ++, very strong; +++, very bright

5.3.3 Detection of Phytoplasmas in Palms Infected by Other LYD

All the primer pairs were also used in PCR reactions to screen DNA extracted from palms affected by other lethal yellowing type diseases (LYD) in Kenya and Mozambique. Each of the primer pairs, P1/ P6, P1/ LD SR, and LD16-1/Rohde reverse, amplified a ribosomal DNA fragment of appropriate size from all disease-infected samples (Plate 5.6). However, the Rohde forward/ Rohde reverse, and Rohde forward / LD SR which were the most sensitive primer pairs for detection of LD, could not detect a rDNA band in the DNA samples collected from Mozambique (Plate 5.7). This suggested a potential difference between these two diseases. These results were additional evidence for the ability of the Rohde primer pair to differentiate LD from other LYD. The primer pair had been reported to differentiate LD from the LYD in West Africa whereby they could not amplify a rDNA band from tissue infected by these diseases (Tymon, 1995). These primers, however did amplify a band from the LYD-infected palm tissue from Kenya (Plate 5.1).

To check for possible differences between LD and the LYD DNA samples from Mozambique, the latter were screened with a primer pair which has been developed for specific detection of phytoplasmas associated with the LYD from West Africa (Cape St Paul Wilt and Awka diseases, in Ghana and Nigeria respectively). The forward primer, Ghana 813 (5'-CTA AGT GTC GGG GGT TTC C), has been developed from variable sequences on the 16S rRNA gene of the Cape St Paul Wilt Disease (CSPWD) phytoplasma (Tymon, personal communication). However, the reverse primer, AK SR (5'-TTG AAT AAG AGG AAT GTG G) has been based on the 16S/23S rRNA intergenic sequences of the Awka Disease phytoplasma (Tymon, 1995). By use of this primer pair in PCR, a ribosomal DNA band of about 1.00 kbp was amplified from DNA of palms infected with LYD from Mozambique, but not from LD-infected DNA (Plate 5.8).

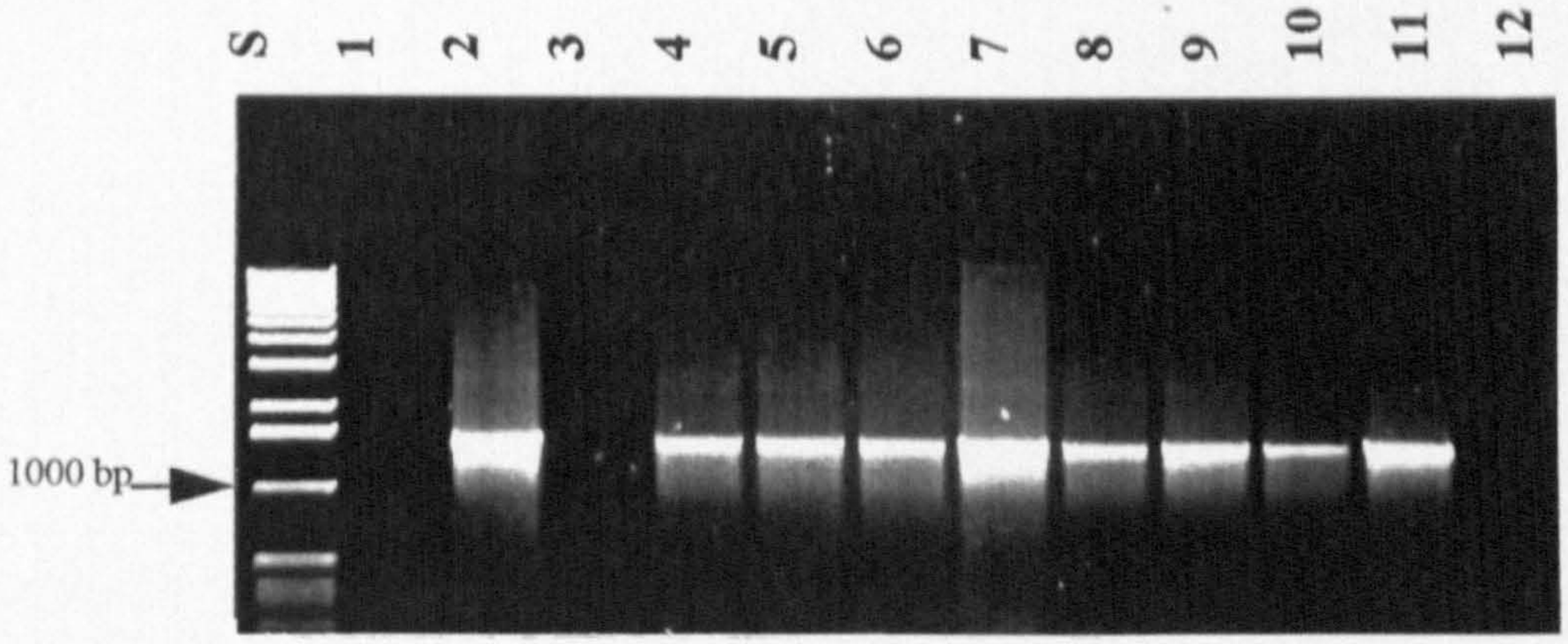


Plate 5.6: Amplification of phytoplasma rDNA from coconut DNA infected by lethal yellowing like disease (LYD) in Mozambique using mollicute specific primers, P1 and P6. S, 1Kb DNA; 1, healthy coconut (Tanzania); 2, LD DNA; 3, healthy coconut DNA (Mozambique); 4-11, LYD infected DNA-Mozambique; 12, water control.

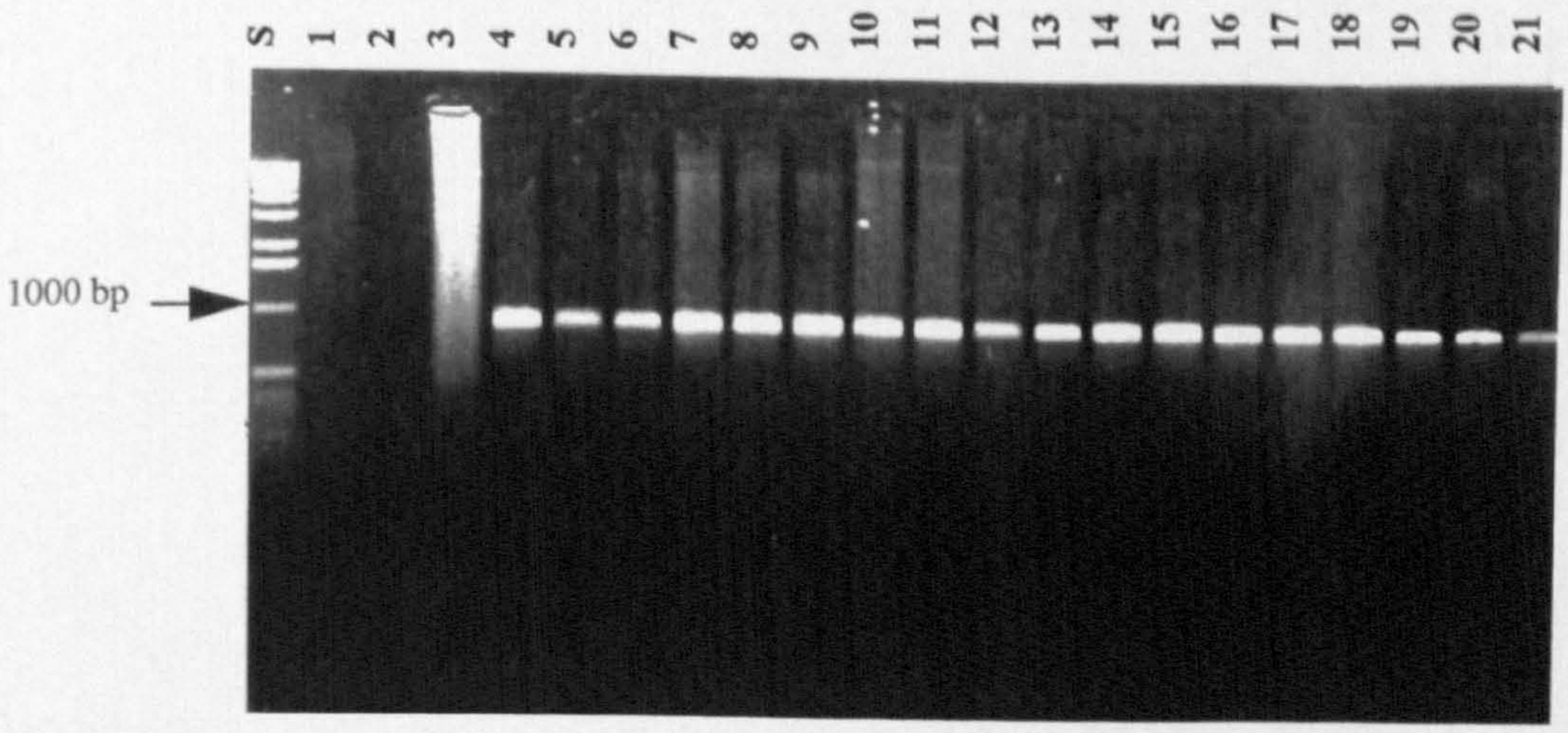


Plate 5.8: Amplification of phytoplasma rDNA from coconut DNA infected by LYD in Mozambique using primers Ghana 813 and AK SR. S, 1Kb DNA; 1-2, healthy coconut DNA (Tanzania and Mozambique); 3, LD-infected DNA; 4-21, LYD-infected DNA from Mozambique.

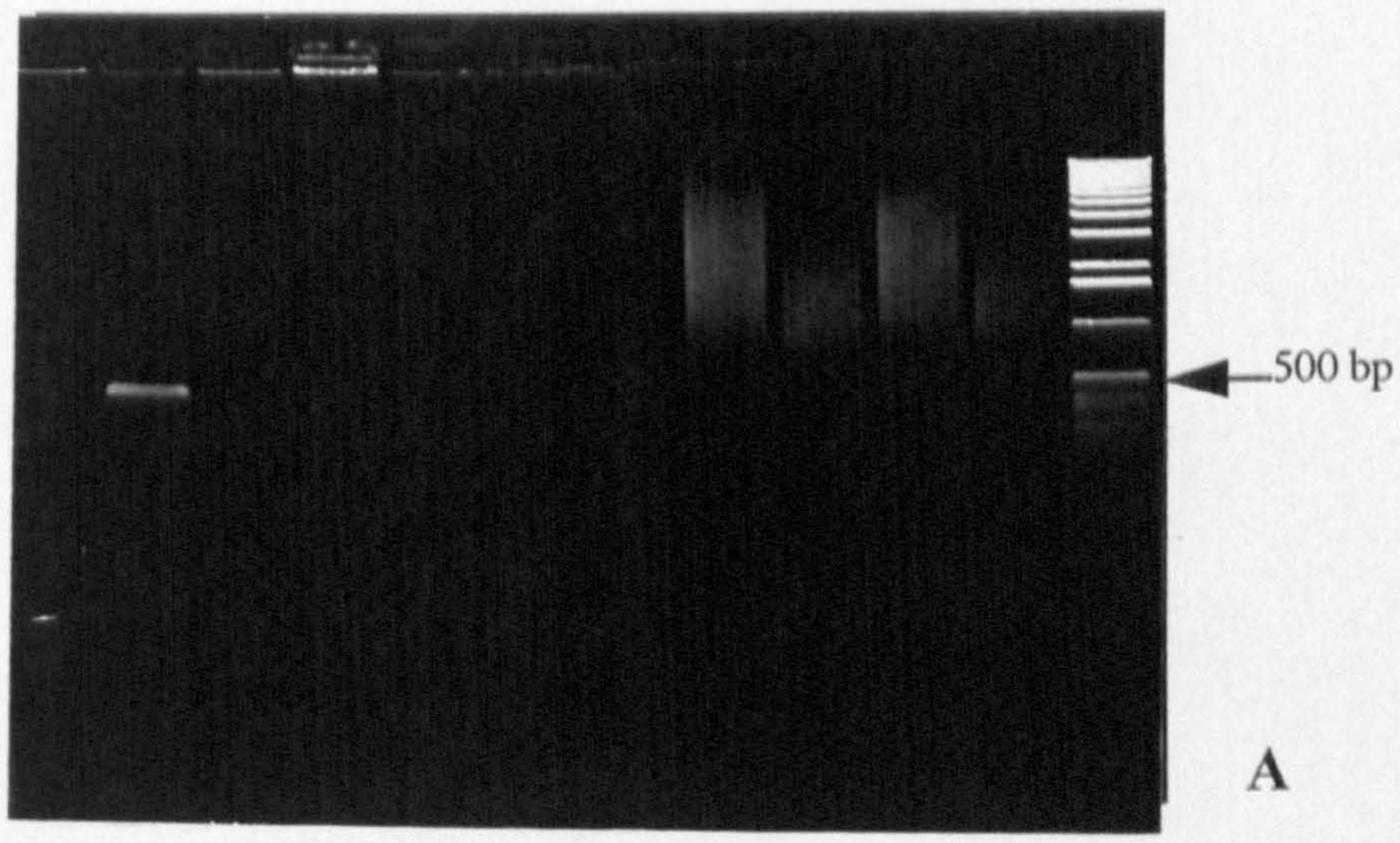


Plate 5.7: Amplification of 16S r DNA from coconut DNA infected by LYD from Mozambique by use of (A) Rohde forward and reverse primers, and (B) Rohde forward and LD SR primers. Only the LD phytoplasma was detected by either primer. S, 1 Kb; 1, Healthy coconut DNA; 2, LD DNA; 3-11, different tissues infected with LYD from Mozambique

To confirm the similarity of the LYD from Mozambique to the LYD from West Africa, PCR products amplified from LD DNA, Kenyan LYD DNA, Mozambican LYD DNA, CSPW DNA, and Awka disease DNA using the P1/P6 primer pair were digested with restriction enzymes *Rsa*I and *Alu* I. The pattern of restriction fragments was the same for LD and Kenyan LYD, but different from the pattern shown by Mozambique, CSPWD and Awka. The pattern for the last three was also the same (Plate 5.9). By digesting with *Rsa* I, each of the isolates produced two restriction fragments, the difference only occured in their respective sizes as shown on Plate 5.9. Similarly, digestion with *Alu* I produced four bands for each isolate, but the difference was in the sizes of the bands (Plate 5.9). These results confirm that the pathogens responsible for LD in Tanzania and LYD in Mozambique are genetically different, and that the LYD in Mozambique is more closely related to the West African LYDs. There was no difference detected between the phytoplasmas causing LD and LYD in Kenya.

5.4 SAMPLING FOR DETECTION OF INCUBATING INFECTIONS BY PCR

5.4.1 Detection of Phytoplasmas in Symptomless Palms

Due to the similarity of LD to LY, it has been assumed that LD has a long incubation (latent period) like LY. The incubation period of LY has been estimated to be between 114-262 days (Dabek, 1975). In addition, the LY phytoplasmas have been established by pathogen-specific PCR to reach detectable levels in the susceptible Jamaica Tall palms 47-57 days before the appearance of disease symptoms (Harrison,1996). In order to establish when incubating LD phytoplasmas can be detected, DNA was extracted from spear leaves sampled at monthly intervals, from 180 randomly selected palms. The palms were selected at 6 locations, and sampling continued for a period of 1 year (refer section 4.3.1). The DNA was screened for phytoplasma by PCR using Rohde forward/ reverse primers. Of these palms, 24 subsequently developed disease at the end of one year. Of the 24 palms, phytoplasmas had been detected in 3 (12.5%), 2 months before onset of disease, in 3 (12.5%), 1 month before onset of disease, and in 5 (21%) at the time disease symptoms appeared.

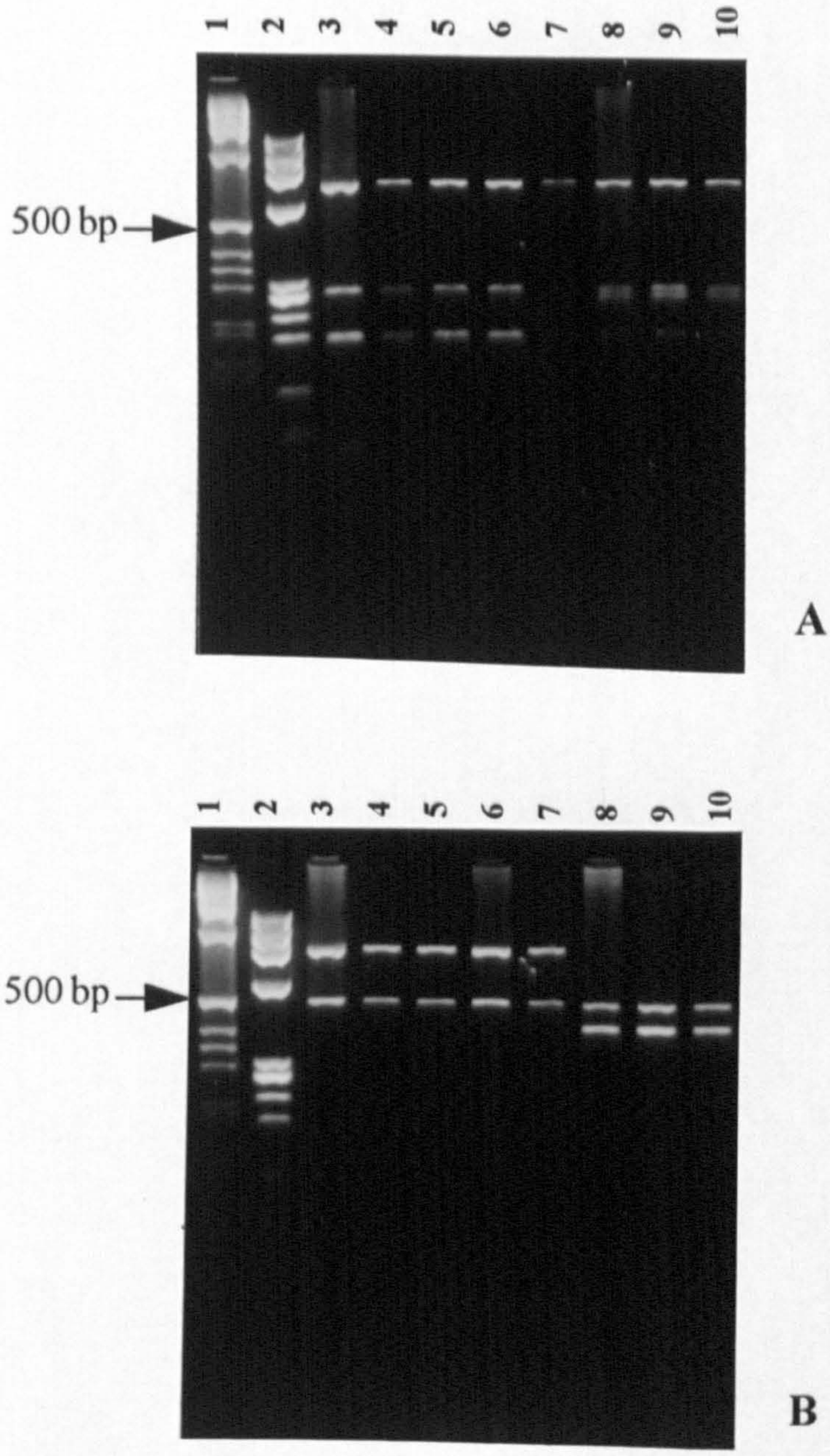


Plate 5.9: *Alu* I (A) and *Rsa* I (B) restriction profiles of 16S rDNA amplified from coconut DNA infected with LYD using primers P1 and P6. The different LYD isolates are from Ghana (lanes 3-4), Nigeria (5), Mozambique (6-7), Kenya (8) and Tanzania (9-10). Lanes 1 and 2, size makers, 1Kb and Theta x 174 DNA.

In 6 palms (25%), phytoplasmas were detected after disease symptoms had developed, and in 7 (29%) diseased palms, phytoplasmas were never detected (Table 5.2).

5.4.2 Detection of Phytoplasmas in Coconut Embryos and Offsprings of LD-Infected Palms

DNA was extracted from embryos of ten nuts collected from palms showing typical LD symptoms, and from spear leaves of eight seedlings raised from nuts that were harvested from LD-infected palms (Section 4.3.2). All DNA samples were screened by PCR for the presence of phytoplasmas using the Rohde forward/reverse primer pair. There was no rDNA band amplified from any of the samples.

5.5 SCREENING THE LD DNA FOR PHTOPLASMA STRAINS BY PCR

To determine whether strains of the LD phytoplasma exist, ribosomal DNA was amplified from palms infected by LD at three locations which represent respectivcly the high, moderate, and low incidence disease areas. The selected areas were Kifumangao, Chambezi and Kigombe respectively. The rDNA PCR fragments were amplified with Rohde forward/ reverse primer pair, cloned into the pTAg vector, then sequenced by automatic DNA sequencing. There were no differences detected in the sequences as shown in representative gels (Fig. 5.1). Since the primer pair used is only able to amplify a 560 base pair fragment, this region was considered too short to show any differences between strains. Attempts were therefore made to use another primer pair which is able to amplify the ribosomal DNA sequences in the intergenic region between the 16S and 23S ribosomal RNA genes. This region is known to be very variable among phytoplasmas. The primers, Oligo 4 (5'-GAA GTC TGC AAC TCG ACT TC) and Oligo 7 (5'-CGT CCT TCA TCG GCT CTT) have been developed based on the sequences of the 16S and 23S rRNA genes respectively (Kirkpatrick *et al.*, 1994a). However, no amplified fragments have as yet been successfully cloned for sequencing.

Table 5.2 Detection of Phytoplasma rDNA in Symptomless Palms by PCR

Palm Affected and Location	Coconut cultivar	Level of LD Tolerance	LD Symptoms observed	Phytoplasma detected
November to December (Short Rainy Season)				
#04 Kigombe	MYDxWAT	Susceptible	Dec 1994	1 month later
#24 Chambezi	EAT-S'Mnara	Tolerant	Dec 1994	Not detected
January to February (Short Dry Season)				
#18 Kigamboni	MYDxWAT	Susceptible	Jan 1995	Same month
#25 Kigombe	CRDxRLT	Susceptible	Jan 1995	1 mo later
#5 Chambezi	EAT-Chamb.	Tolerant	Jan 1995	Same month
#29 Kifumangao	Tacunan Dwf	Tolerant	Feb 1995	2 months later
#03 Kigombe	MYDxWAT	Susceptible	Feb 1995	2 months earlier
March to June (Long Rainy Season)				
#25 Chambezi	EAT-Tumaini	Susceptible	Mar 1995	Not detected
#19 Kifumangao	EAT-S'Mnara	Tolerant	Mar 1995	2 months later
#22 Kifumangao	EAT-Lamu	Tolerant	Mar 1995	1 month later
# 26 Kigombe	CRDxRLT	Susceptible	Mar 1995	Not detected
#14 Chambezi	Karkar Tall	Susceptible	April 1995	Not detected
#21 Kigamboni	MYDxWAT	Susceptible	May 1995	1 month earlier
#10 Sotele	EAT-Boma	Susceptible	May 1995	2 months earlier
#16 Kigombe	CRDxWAT	Susceptible	June 1995	2 mo earlier
July to October (Long Dry season)				
#01 Kigamboni	MYDxWAT	Susceptible	July 1995	Not detected
#29 Kigombe	MYDxWAT	Susceptible	July 1995	1 mo earlier
#27 Sotele	EAT-LBS	Tolerant	Aug 1995	1 month later
#1 Chambezi	MYD	Tolerant	Aug 1995	Same month
#2 Kigamboni	MYDxWAT	Susceptible	Sept 1995	Same month
#15 Pongwe	EAT-Boma	Susceptible	Sept 1995	Not detected
#5 Sotele	EAT-Mikindani	Susceptible	Oct 1995	Same month
#9 Sotele	EAT-Mchukwi	Susceptible	Oct 1995	1 month earlier
#30 Kigombe	MYDxWAT	Susceptible	Oct 1995	Not detected

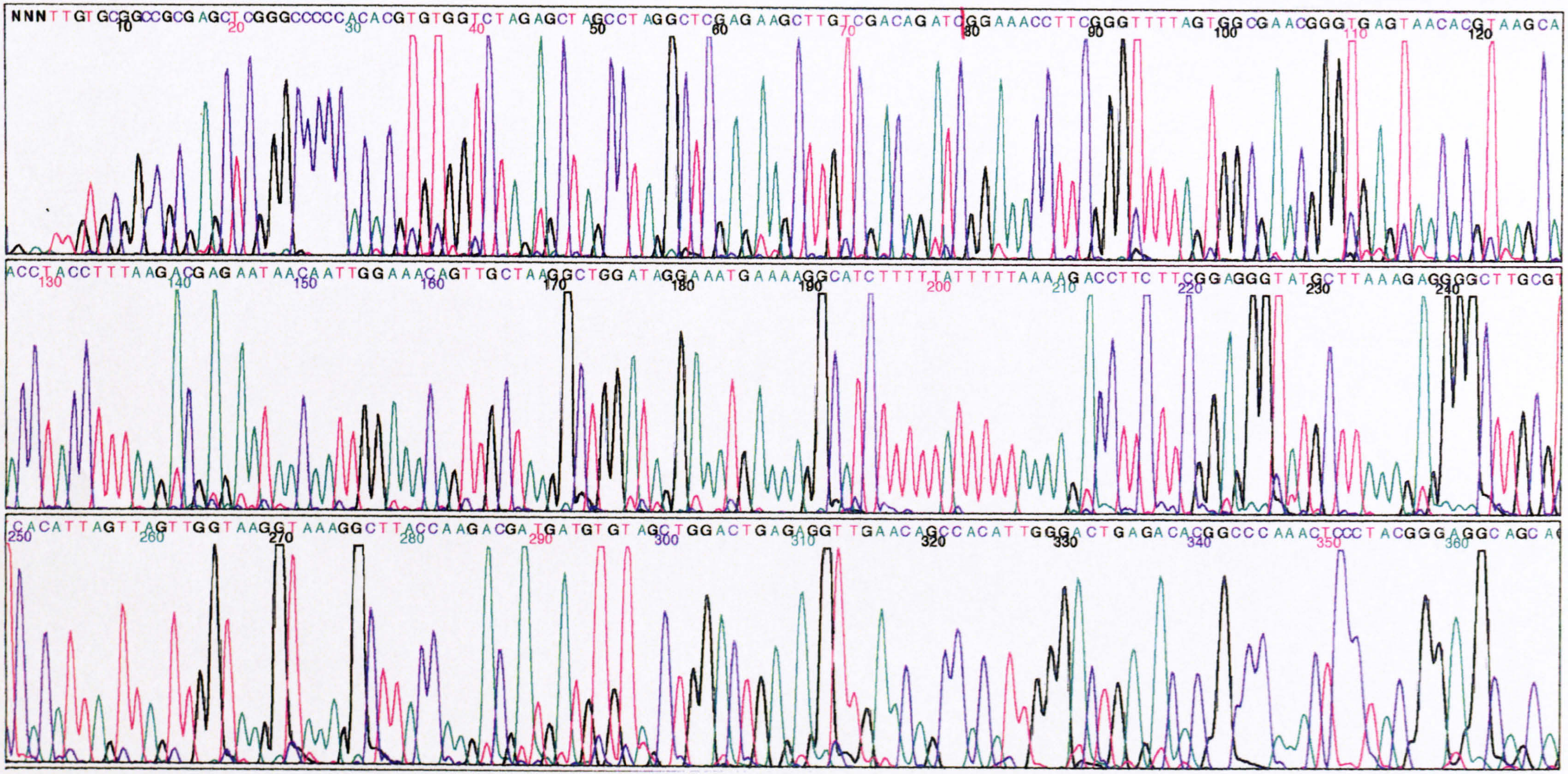


Fig. 5.1A: DNA sequence of a cloned phytoplasma rDNA fragment amplified from LD-infected coconut at Chambezi

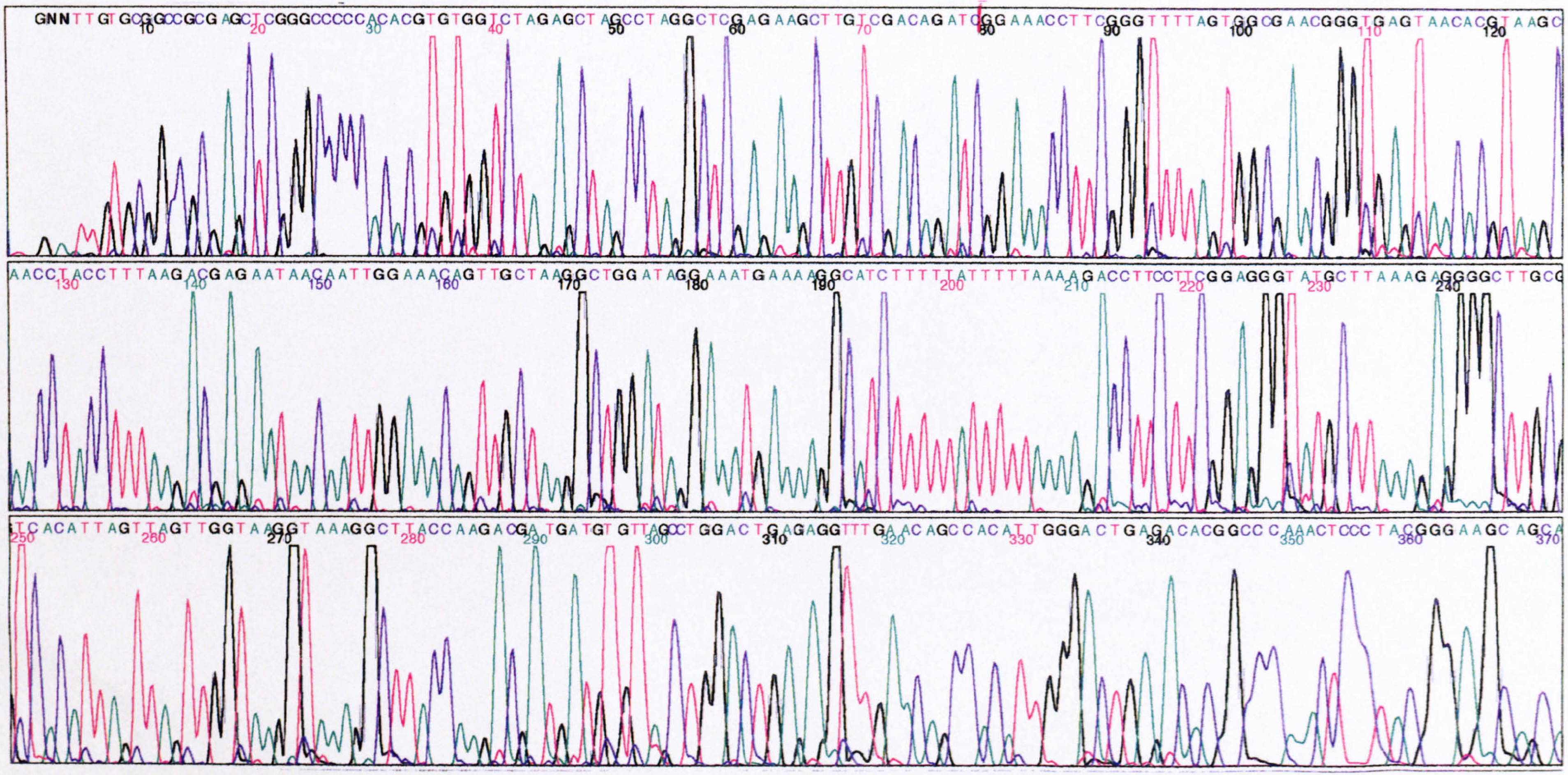


Fig. 5.1B: DNA sequence of a cloned phytoplasma rDNA fragment amplified from LD-infected coconut at Kifumangao

5.6 DISCUSSION

Detection of phytoplasmas by amplification of 16S rDNA sequences provides a rapid, sensitive and specific assay for reliable detection of these non-culturable plant pathogens (Ahrens and Seemuller, 1992). By use of the *Mollicute*-specific primers to amplify a DNA band from LD infected tissue but not from healthy coconut, this study has confirmed the phytoplasmal aetiology of LD in Tanzania and LYD in Kenya and Mozambique. The study has further demonstrated that PCR-based assays using primer pairs designed on the basis of the 16S rRNA sequences, or a combination of these with one based on the 16S/23S spacer region (SR) sequences of the LD phytoplasma, can be employed to effectively detect its presence in infected coconut tissues. Thus, the primer LD SR, based on the variable sequences flanking the universally conserved $tRNA^{ile}$ gene in the 16S/23S spacer region, when combined with a suitable forward primer, e.g Rohde forward, was shown to specifically detect the LD phytoplasma in PCR assays.

The experiments have also shown that primers which specifically detect LD, can be used to differentiate it from other closely related but genetically different organisms. The causal agent of LYD in northern Mozambique could not be amplified with the Rohde forward/ reverse nor the Rohde forward/LD SR primer combinations (Plate 5.7), whereas the Tanzanian samples could be. The other primer combinations, however, were able to amplify bands from both Mozambique and Tanzanian samples. Thus, the Rohde forward/ reverse primer pair proved very useful for differentiating the LD phytoplasma from strains of coconut LYD phytoplasmas which are similar but not genetically identical, although it does amplify rDNA from other non-coconut phytoplasmas (Tymon, personal communication). Additional tests confirmed the Mozambique phytoplasma to be more closely related to the West African LYD. There was no evidence to suggest that the phytoplasmas causing LD in Tanzania and LYD in Kenya were different.

For diseases which have a prolonged latent (incubation) phase, detection of infection prior to onset of disease symptoms is very useful for effective disease management. By sampling spear leaves, LD could be detected 2 months before symptom development. The detection period is similar to that reported for LY in Florida (Harrison, 1996).

However, differences occurred in the proportion of diseased palms in which infection was diagnosed. While phytoplasma rDNA was detected by PCR in 71% of LD infected palms, Harrison (1996) reported 100% detection in the DNA from spear leaves of LY infected palms by the same technique. Several factors maybe responsible for the low level of detection. One is the quality of chemicals that were used for DNA extraction. For some time during the screening period, chemical supplies were limiting, and DNA had to be extracted using ordinary reagent grade chemicals. The resulting poor quality DNA may have inhibited PCR amplification.

Another, more important factor, could be the interaction between pathogen, host, and environment, because LD is known to be less aggressive than LY (Schuiling *et al.*, 1992a). An indepth analysis of the coconut varieties which became infected by the disease, the seasons at which infection occurred, and whether phytoplasma rDNA was detected in the samples, provided evidence for implicating this interaction. All the 6 palms in which phytoplasmas were detected before appearance of disease symptoms were of the very susceptible hybrids MYD X WAT and CRD X WAT, or the susceptible EAT sub-populations, Mchukwi and Boma. The majority (61%) developed disease symptoms either during the rainy season, or within a month after (Table 5.2). This might imply that the rainy weather conditions which favour palm growth were also favourable for pathogen multiplication. The interaction between favourable weather and susceptible hosts, facilitated rapid phytoplasma multiplication to reach detectable levels long before the onset of disease.

The data were also in agreement with the reverse situation. Out of the seven palms (29%) in which no phytoplasma rDNA was detected, the DNA of three palms was damaged during extraction, and it was not possible to obtain a replacement. That leaves only 4 palms (16.5%) in which lack of detection could not be explained. Three of these palms (75%) developed disease symptoms during the dry season when palms are under stress, and although susceptible to infection, no phytoplasma was detected. This provided additional evidence that environmental conditions which affect both the host and pathogen, may therefore have a direct influence on the sensitivity of detection for incubating infections.

The sensitivity of phytoplasma detection was also affected by the interaction between the pathogen and disease tolerant hosts. Unlike the susceptible ecotypes in which phytoplasmas attained detectable levels before the onset of disease, the opposite was true for disease tolerant varieties. It was observed that four of the six palms (67%) in which phytoplasmas were detected after appearance of symptoms, were all LD tolerant varieties (Table 5.2). Similar results were obtained when DNA probes were used for detecting incubating infections (section 4.3.1).

The third factor that might explain inability to detect phytoplasma in palms with characteristic disease symptoms could be the pathogen itself. The possibility that strains of the LD phytoplasma do exist cannot be overruled. If present, one strain was being detected by the Rohde's primer pair, and the other was not. This argument is supported by the results in which this primer pair could not detect phytoplasma in the LYD from Mozambique, while it was the most sensitive for detection of LD. Since Tanzania and Mozambique share a common border, it is possible that the LYD strain found in Mozambique is also present in Tanzania. The only way to verify this would be to re-screen all the samples from which no band was amplified with primer pairs which can detect the Mozambique phytoplasma strain. For the isolates which can be amplified with this primer pair, sequence data from the amplified ribosomal DNA fragments did not show any indication of strain differences. This could be because the amplified region is rather short and based on the highly conserved region on the 16S ribosomal gene. Sequencing and analysis of the fragments amplified from the more variable 16S /23S intergenic region may possibly show the differences if they exist.

An interesting observation, is that all the diseased palms in which no phytoplasma was detected were located in the low and moderate-disease incidence regions. These regions are located in the north and central part of the country, further away from Mozambique. These regions have also grown coconut for a much longer time, compared to the southern regions where coconuts were introduced later, and suffered more from the disease. One possible hypothesis to explan this situation would be that LYD strains do exist in the low and moderate-incidence areas, and that they interfere with each other during acqusition and multiplication in the insect vector, and subsequent transmission to palms. If both

strains are acquired by an insect, no transmission occurs, but if only one strain is acquired, then transmission is effected. Similarly, if both strains infect a palm no disease symptoms develop, but if only one strain infects, the disease develops. This hypothesis would assume that the southern regions which are recent coconut growers do not have the second strain. Such an hypothesis could possibly explain the low incidence of disease in these regions, despite similar weather conditions to the south.

A situation of interference between phytoplasma strains has been reported among the strains of aster yellows, whereby acquisition of the severe or dwarf strains first by the vector *Macrosteles fascifrons* hindered transmission of the challenging Tule lake strain acquired later. In a reversed order, the challenging dwarf or severe strain was transmitted in favour of the first acquired Tule lake strain (Purcell, 1982).

Dot hybridization studies have demonstrated that the concentration of phytoplasmas in the spear leaves of LD infected tissue is lower than in the root tips. Inability to detect phytoplasmas in the spear leaves, may also be due to this factor. Hence, for purposes of forecasting incubating infections, it may be worthwhile to sample both roots and spears leaves in order to increase the chances of pathogen detection.

There was no rDNA band amplified from the DNA of embryos excised from LD-infected palms. This is contrary to reports from Florida, where phytoplasma rDNA was amplified from a large proportion (21%) of embryos excised from nuts on LY-infected palms (Harrison, 1996). Failure to detect phytoplasma in embryos in this study could be due to the small sample size. More embryos collected from LD infected palms should be screened in future due to the importance of these results for exchange of germplasm. None of the seedlings raised from nuts harvested from LD-infected palms were positive for phytoplasmas. These results are similar to those reported by Harrison (1996). This might imply that transmission of these organisms through seed (embryos) is not feasible. Additional studies are necessary to verify this hypothesis.

Chapter 6: RESEARCH ON POTENTIAL INSECT VECTORS OF LD

6.1 INTRODUCTION

The spread of lethal disease(LD) within coconut farms is characterized by strong gradients in the direction from wind-exposed borders towards the centre of the fields. According to Schuiling *et al.* (1992a), this pattern strongly suggests an air-borne insect vector. Insects have also been implicated as vectors of LD because the causal pathogen of the disease has been found to be phytoplasmas. Evidence for this includes the detection of phytoplasmas in the phloem of LD-infected palms (Deutsch and Nienhaus, 1983), and remission of symptoms in diseased palms after injection with tetracycline antibiotics (Kaiza, 1987). Despite this evidence it has not been possible to implicate any insect as the vector (Anonymous, 1987; Schuiling *et al.*, 1992a).

The majority of phytoplasma diseases are transmitted by insects in the Order *Homoptera* and especially Suborder *Auchenorryncha* (Nienhaus and Sikora, 1979; Nielson, 1979; Wilson, 1988). Of these, the true leafhoppers (*Ciccadellids*) have been considered the most important vector group (Nielson, 1979). These insects, however, are not the predominant group on coconut palms. In Jamaica, during the search for the vector of LY, leafhoppers were found to predominate in the undergrowth, and planthoppers (*Fulgorids*) were the most predominant group on palms (Dabek, 1981). The cixiid, *Myndus crudus* van Duzee, was the most predominant planthopper, and it remained the prime suspect vector of LY in Jamaica although extensive transmission trials failed to implicate it (Schuiling *et al.*, 1976; Eden-Green, 1978; Eden-Green and Schuiling, 1978; Dabek, 1981). This planthopper has been shown in transmission trials to be the vector of LY in Florida (Howard *et al.*, 1983).

Studies on the Homoptera insects associated with palms in Tanzania, have revealed similar findings to those in Jamaica, although the planthopper *Myndus crudus* has not been found in Tanzania. The *Auchenorrhyncha* insects found on palms in Tanzania

have been predominantly *Fulgoroidae* (planthoppers), with species in the family *Derbidae* being most abundant (Kaiza, 1987). Similarly, studies in Ghana have found the planthoppers *Myndus adiopodumeensis* (Cixiidae) and *Nzinga palmivora* (Typhlocibinae) to be the most predominant insects, but no *Myndus crudus* (Dery *et al.*, 1997). In both cases, however, transmission trials have so far failed to implicate any of the predominant insects as vectors (Schuling *et al.*, 1992a; Dery *et al.*, 1997).

The transmission experiments for LD were conducted with insects collected directly from the field on disease infected palms. This was because the breeding habits of these insects were unknown. The experimental insects were also not assayed to confirm whether they could ingest the phytoplasmas, or whether the phytoplasmas could multiply in them. The main hindrance to assaying was the lack of rapid and sensitive techniques for detecting the phytoplasmas in the suspected insects. Although this could have been done by electron microscopy, the expenses involved in preparation of samples made the technique impractical. These two aspects may have contributed significantly to the failure of the transmission trials.

In contrast, LY, which is similar to LD, has been successfully transmitted in Florida although similarly field-collected insects were used in the trials (Howard *et al.*, 1983). Their success may have been facilitated by the aggressive nature of the LY pathogen, or may be a more efficient vector. For the less aggressive LD, success of transmission trials, and hence identification of the insect vector might be facilitated by a technique which makes it possible to monitor the suspected insects for ingestion of phytoplasma during feeding, and whether the phytoplasma multiplies in them, before they are used in the trials.

Until recently, detection of phytoplasmas in the insect vectors was almost impossible. It could be achieved only in a few cases by use of serological assays (Lin and Chen, 1985). However, the DNA recombinant technology has made available quick and sensitive techniques for detecting phytoplasma infections both in the plant hosts and insect vectors (Kirkpatrick *et al.*, 1987; Davis *et al.*, 1988a; Vega *et al.*, 1992; Rahardija *et al.*, 1992; Harrison, 1996). By use of cloned random fragments of

phytoplasma DNA as probes in DNA-DNA hybridization assays, the Western X-disease phytoplasma of stone fruits has been detected in plant and insect hosts, and demonstrated to multiply in the leafhopper vector, *Colladonas montanus*, and in celery, the herbaceous host plant (Kirkpatrick *et al.*, 1987).

Similarly, by use of the polymerase chain reaction (PCR), ribosomal phytoplasma DNA has been amplified from both vector and non-vector insects fed on phytoplasma infected plants (Vega *et al.*, 1992; Harrison, 1996), implying that even non-vector insects can acquire the phytoplasma during feeding. However, by use of PCR and specific DNA probes to monitor insects, multiplication of the LY phytoplasma has been demonstrated to occur only in the vector planthopper *M. crudus* van Duzee, but not in the nonvector planthopper, *Peregrinus maidis* Ashmead (Harrison, 1996).

In contrast, these techniques have been used to demonstrate that the severe strain aster yellows (SAY) phytoplasma multiplies in both its leafhopper vector *Macrosteles fascifrons*, and the nonvector leafhopper *Dalbulus maidis* De Long & Wolcott (Vega *et al.*, 1992). These research findings have provided the stimulus for attempting similar studies on the LD phytoplasma. Although detection of the phytoplasma in the insects does not necessarily prove the vector status, it might help to narrow down the number of species to a few that could be conveniently screened in biological assays, and increases the probability of tracing the real vector(s).

Knowledge about the epidemiology of a disease often provides useful information which leads to the identification of the vector(s). For example the observed jump spread pattern of LY in Jamaica provided the first evidence for suspecting an air-borne insect as a possible vector of the disease (Carter and Suah, 1964). Later, the discovery of phytoplasmas in infected tissue (Plavsic-Banjac *et al.*, 1972) assisted to direct the search specifically to phloem feeding insects. The spread of LD within infection foci has generally shown a strong border effect with the gradients diffusing towards the centre of the field (Schuiling *et al.*, 1992a). Since the pathogen is a phytoplasma whose most likely vectors are phloem sucking insects, the implication of this pattern would be that the vector is probably not breeding within the fields, but

rather flying into the fields from neighbouring shrubs and bushes. This makes it necessary to investigate the flight behaviour of different phloem feeding insects which predominate in the LD affected fields, and to relate the numbers to the incidence of disease. Another characteristic feature of LD is that it spreads very fast within young coconut fields, but relatively slowly in the tall palms (Schuiling *et al.*, 1992a). This could suggest that the insect vector does not fly higher up the tall palms but rather feeds on the shorter, young palms. Alternatively, the vector may feed equally on all palms but palm age could be influencing susceptibility of the palms to infection. All these factors were investigated concurrently with the sampling and detection of the phytoplasmas in the suspected insects in an attempt to understand the possible role of insect vector(s) in the spread of LD.

6.2 FLUCTUATION OF INSECT POPULATIONS AND ASSOCIATION TO DISEASE INCIDENCE IN SELECTED COCONUT FIELDS.

Homoptera insects were continuously trapped on twenty five selected palms at two sites for a period of one year. The proportions of insects trapped on palms at specific positions in the field at Chambezi and Kifumangao, are shown in Fig. 6.1 and 6.8 respectively.

There were big differences in the numbers of insects trapped during different seasons at the two sites. At Chambezi, peak insect populations were trapped during the dry months of August to October 1995. This was followed by a sharp decline in November (short wet season), but the numbers again increased slightly, and a small peak was attained in January 1996 (short dry season). The numbers declined again in February, and trappings remained generally low until the end of the wet season in June, 1996.

Among the groups of palms at Chambezi, tall palms near the edge of the fields trapped more insects during August to October than all the other groups (Fig. 6.1). In contrast, short palms near the edge of the field, trapped the least number of insects throughout the year, except for the small peak trapped at the beginning of the long

dry season in June 1996. Surprisingly, short palms in the middle of the field trapped comparatively large insect numbers throughout the year. Tall palms in the middle of the field also trapped reasonably high numbers of insects throughout the year.

Relatively more insects were trapped at Kifumangao, than at Chambezi. A peak insect population was also trapped during the dry months between August and October, after which there was a sharp decline (Fig. 6.8). After this time, however, the pattern was different from that observed at Chambezi. The insect numbers dropped at the begining of the short rainy season (Nov.), but increased again and attained a small peak in Dec. Similarly, the number of trapped insects decreased during the short dry season in Jan.- Feb., and increased during the long rainy season between March and June. A striking similarlity to Chambezi was that at Kifumangao as well, more insects were trapped on palms in the middle of the field than those near the edges throughout the year, particularly on the short palms.

Figs. 6.2 to 6.7 shows the variation in the relative abundances of the most common auchenorrhynchous insects at Chambezi, between August 1995 and July 1996. The corresponding information for Kifumangao is shown in Fig. 6.9 to 6.14. The trapped insect species were predominantly planthoppers in the family *Derbidae*, except for four species, one each for families *Cercopidae*, *Nogodinidae*, *Lophodidae*, and *Meenoplidae*. They were desgnated code letters which appear in figures 6.2 - 6.7, and 6.9- 6.14 as follows: A = *Diastrombus abdominalis*; B/C = *Diastrombus mkurangai*; D = *Phenice pongwei / Paraphenice mawae*; E = *Robigus magawae*; F = *Diastrombus schuilingi*; G = *Bandusia erythrostena*; H = *Zoraida fuligipennis*; I = *Elasmosceles cimicoides*; J = unidentified grass-green *cicadelid*; K = *Amania angustifrons / Nesodryas antiope*; L = *Kamendaka kordofana*; M = *Meenoplus* spp; N = *Lydda woodi;* O = *Diazanus spp.;* P = *Zorabana* spp. The species desgnated Q, R, S, T, V, and Z are all undescribed planthoppers.

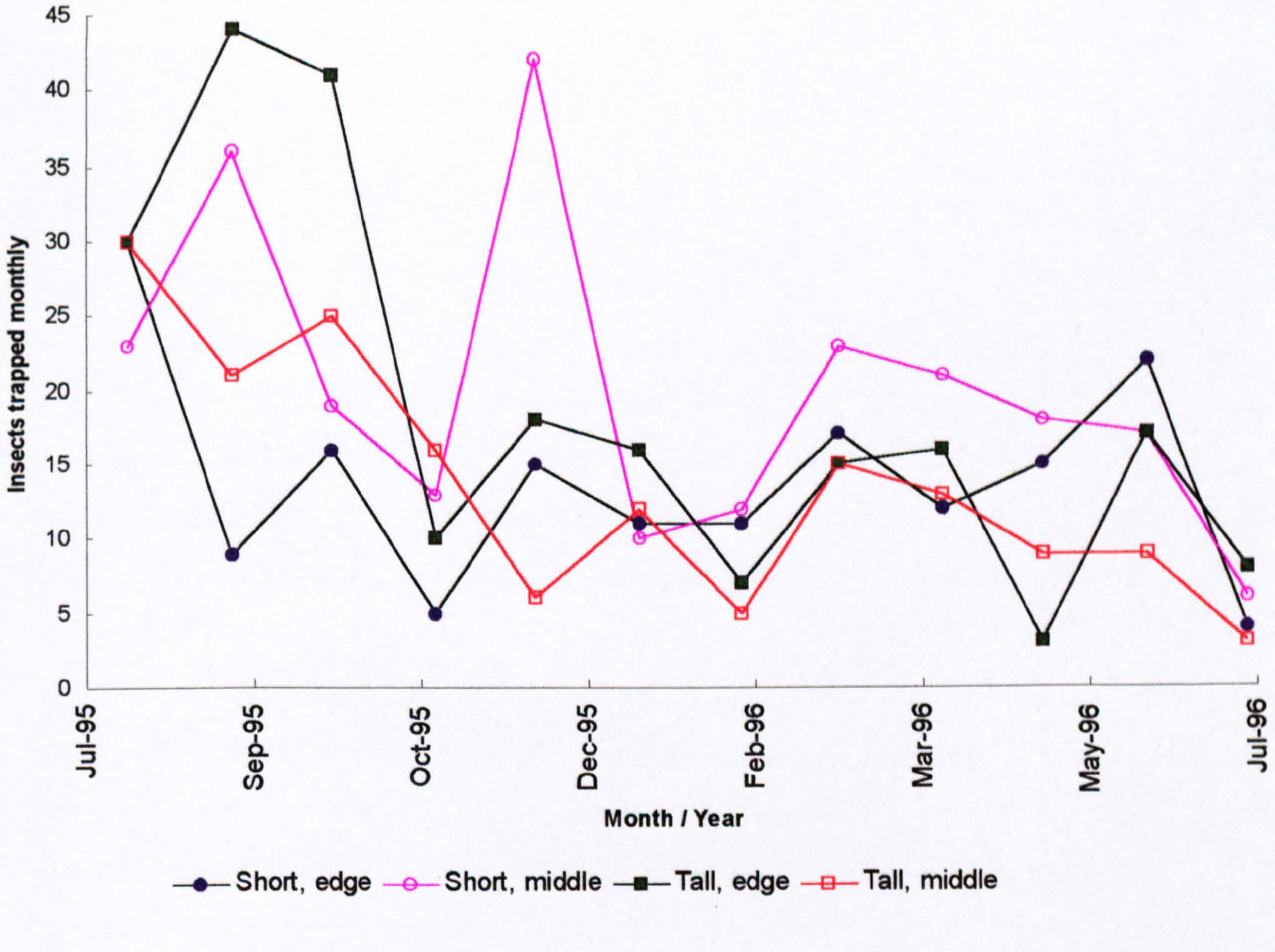


Fig. 6.1 **Fluctuations in the population of homoptera insects trapped on selected palms at Chambezi between July 1995 and July 1996.**

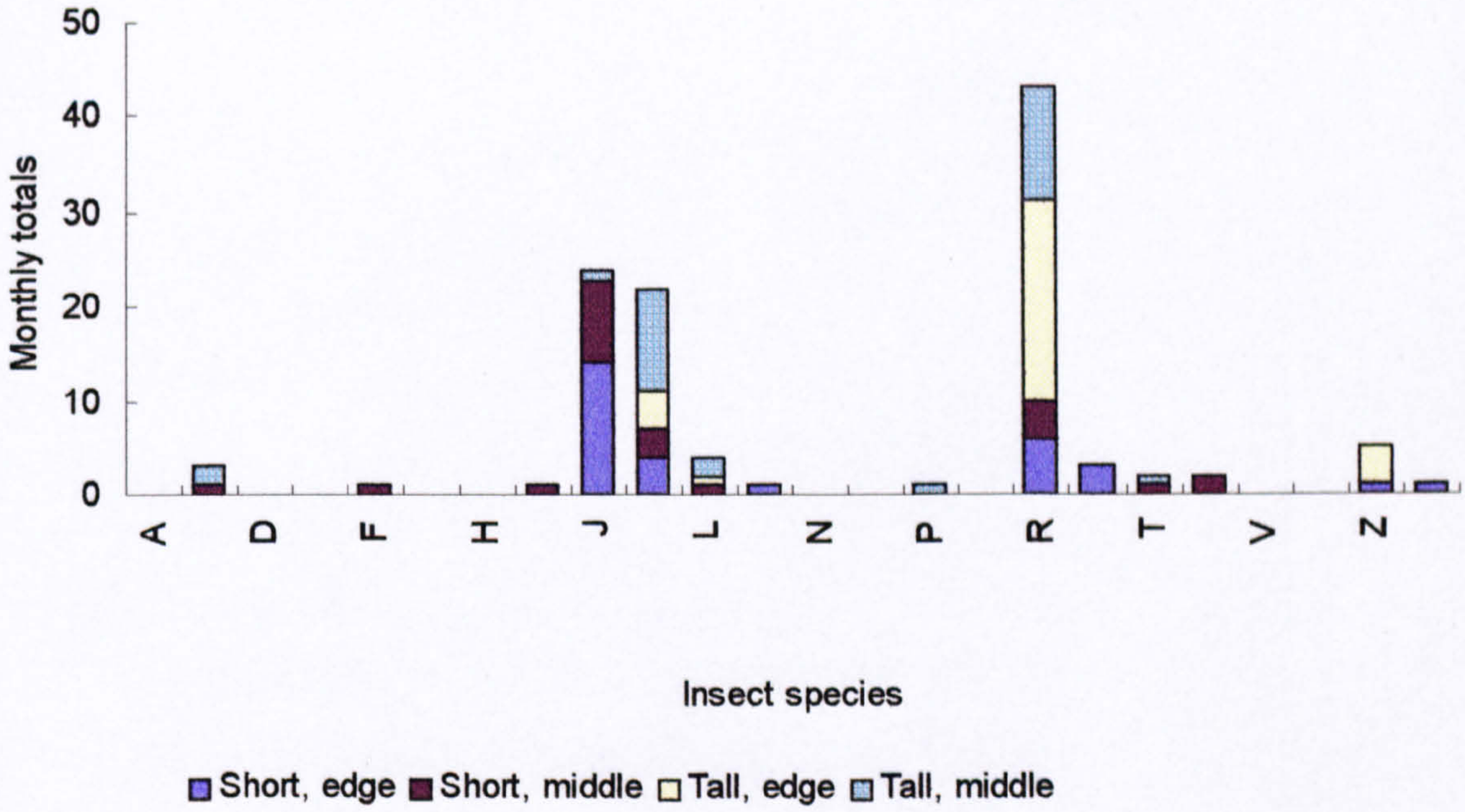


Fig. 6.2a Homoptera insects trapped at Chambezi during August 1995

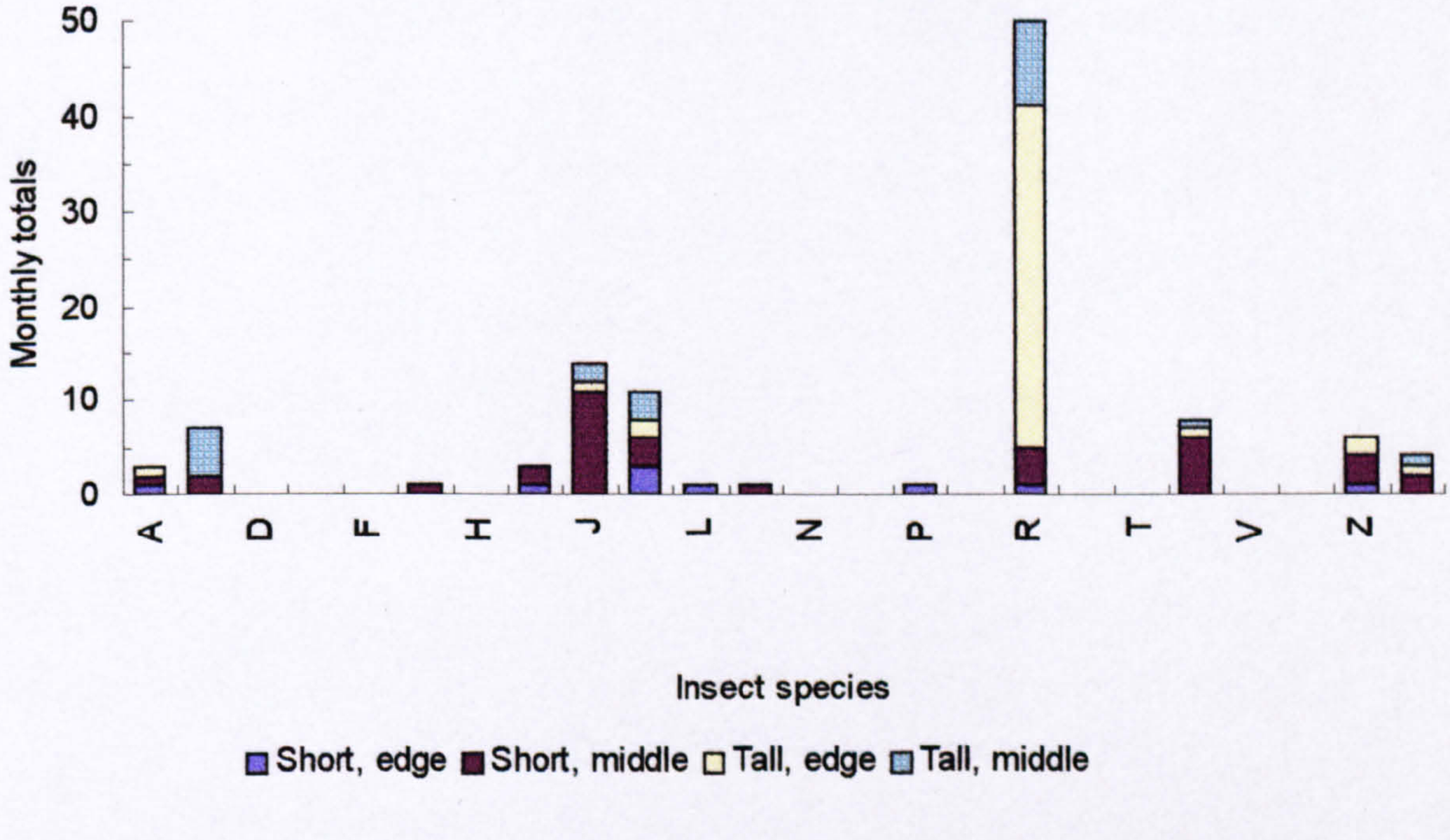


Fig. 6.2b Homoptera insects trapped at Chambezi during September 1995

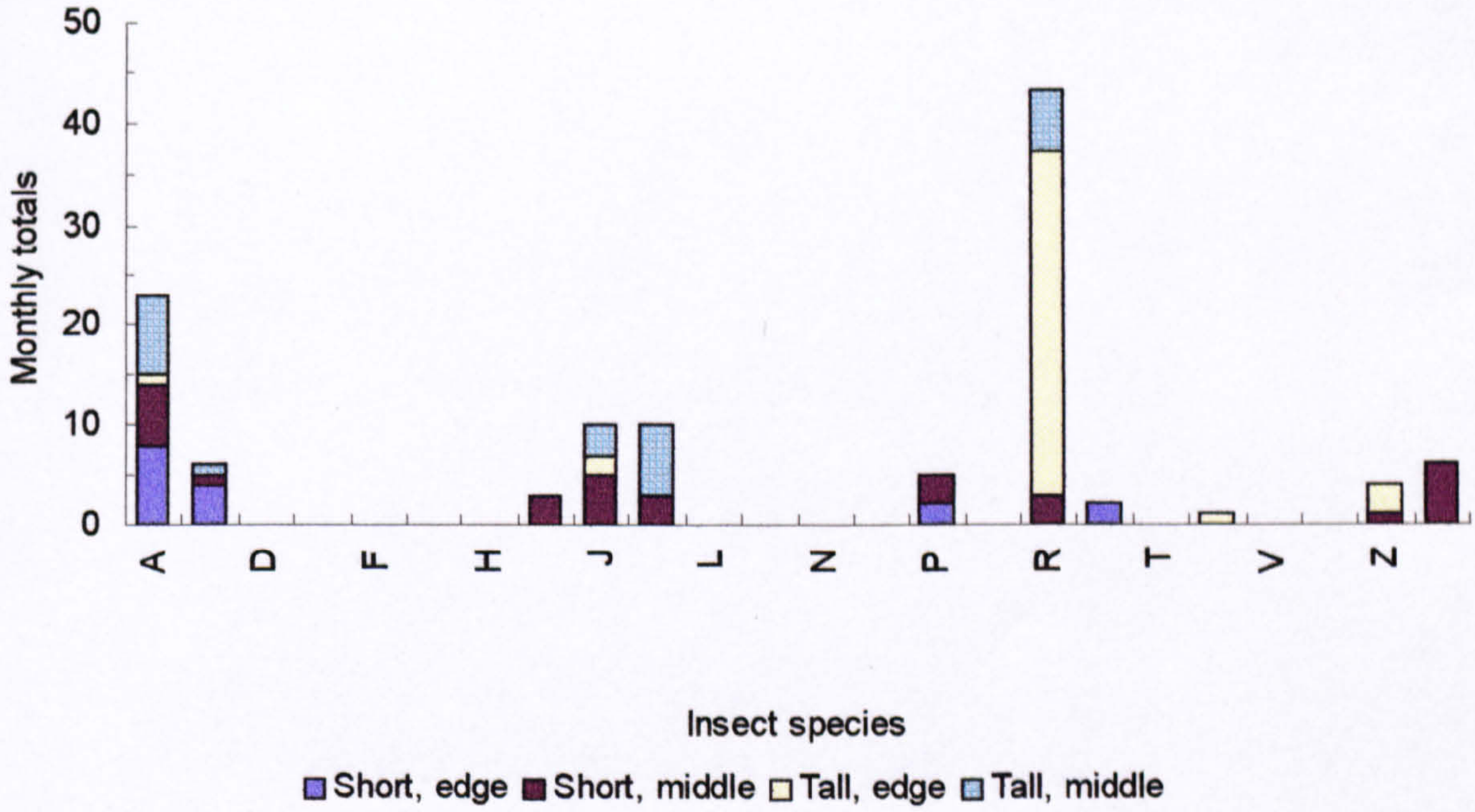


Fig. 6.3a Homoptera insects trapped at Chambezi during October 1995

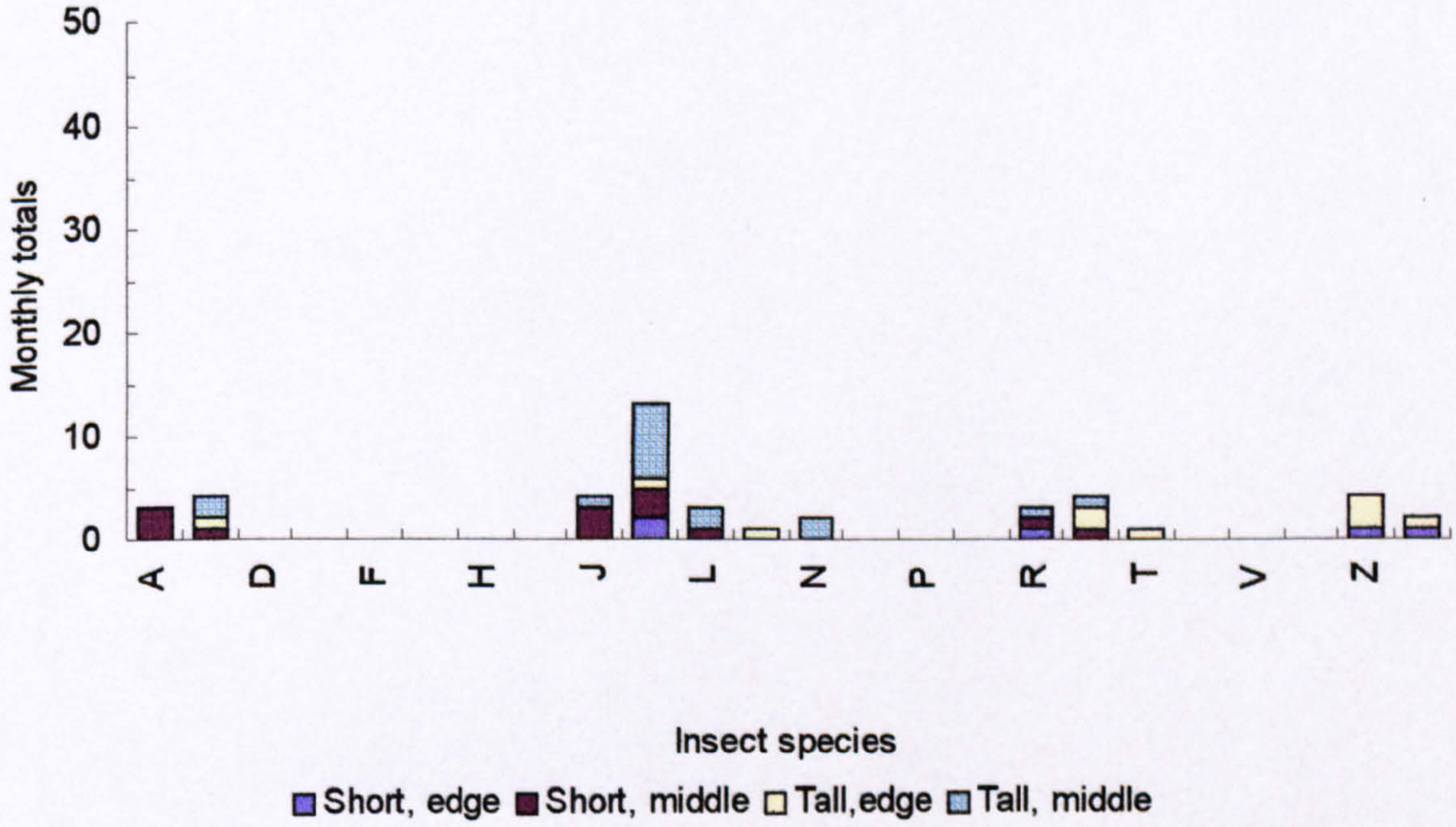


Fig. 6.3b Homoptera insects trapped at Chambezi during November 1995

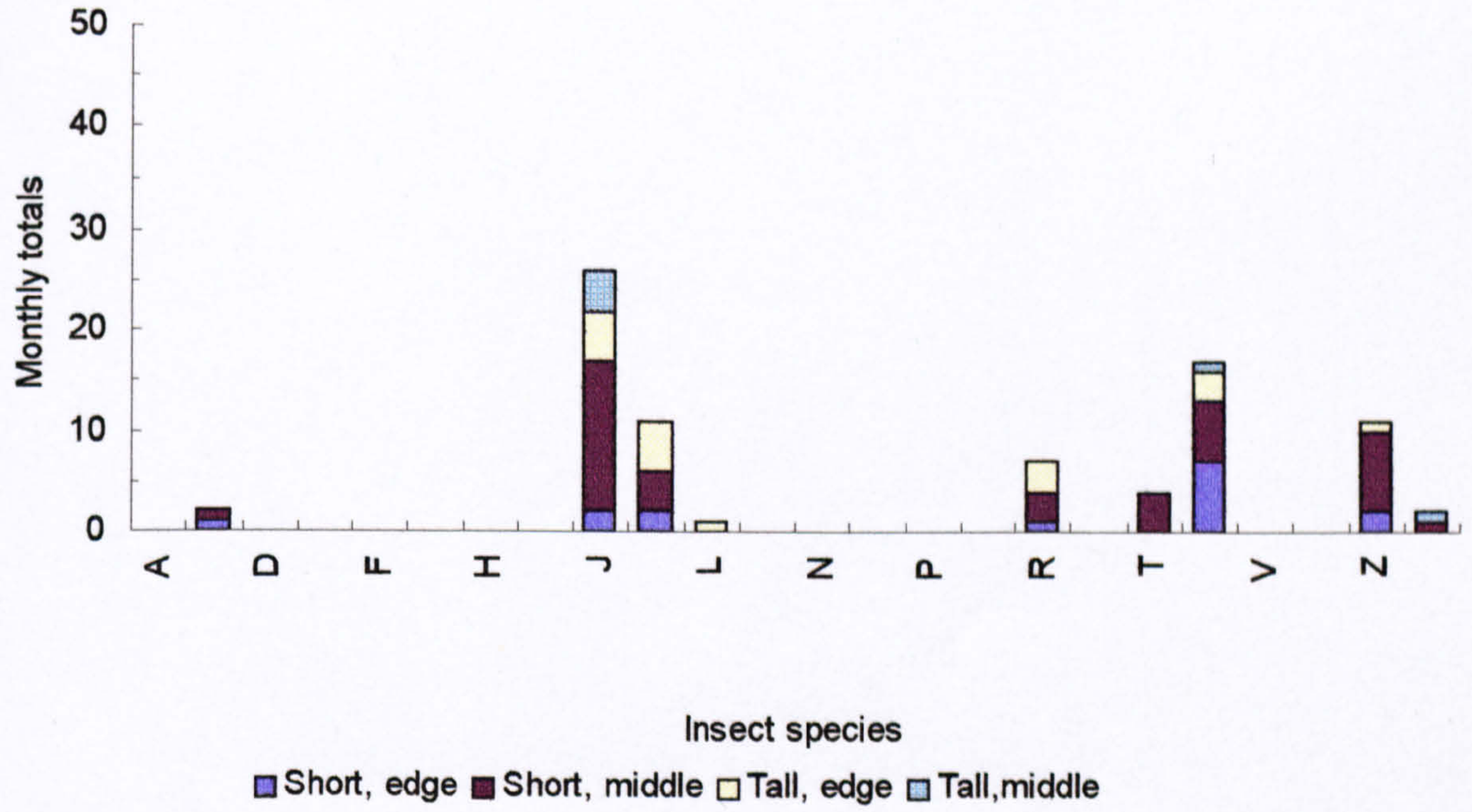


Fig. 6.4a Homoptera insects trapped at Chambezi during December 1995

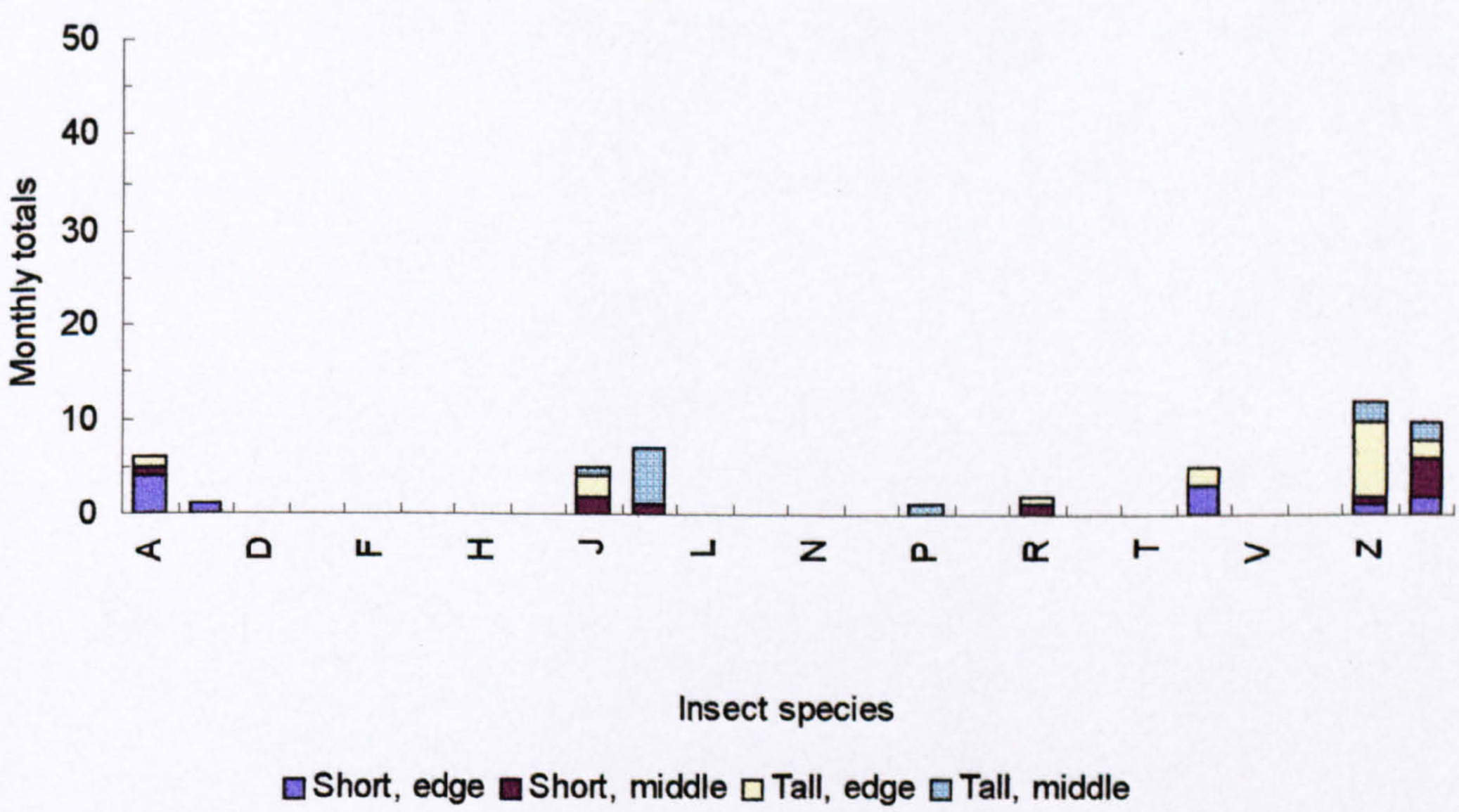


Fig. 6.4b Homoptera insects trapped at Chambezi during January 1996

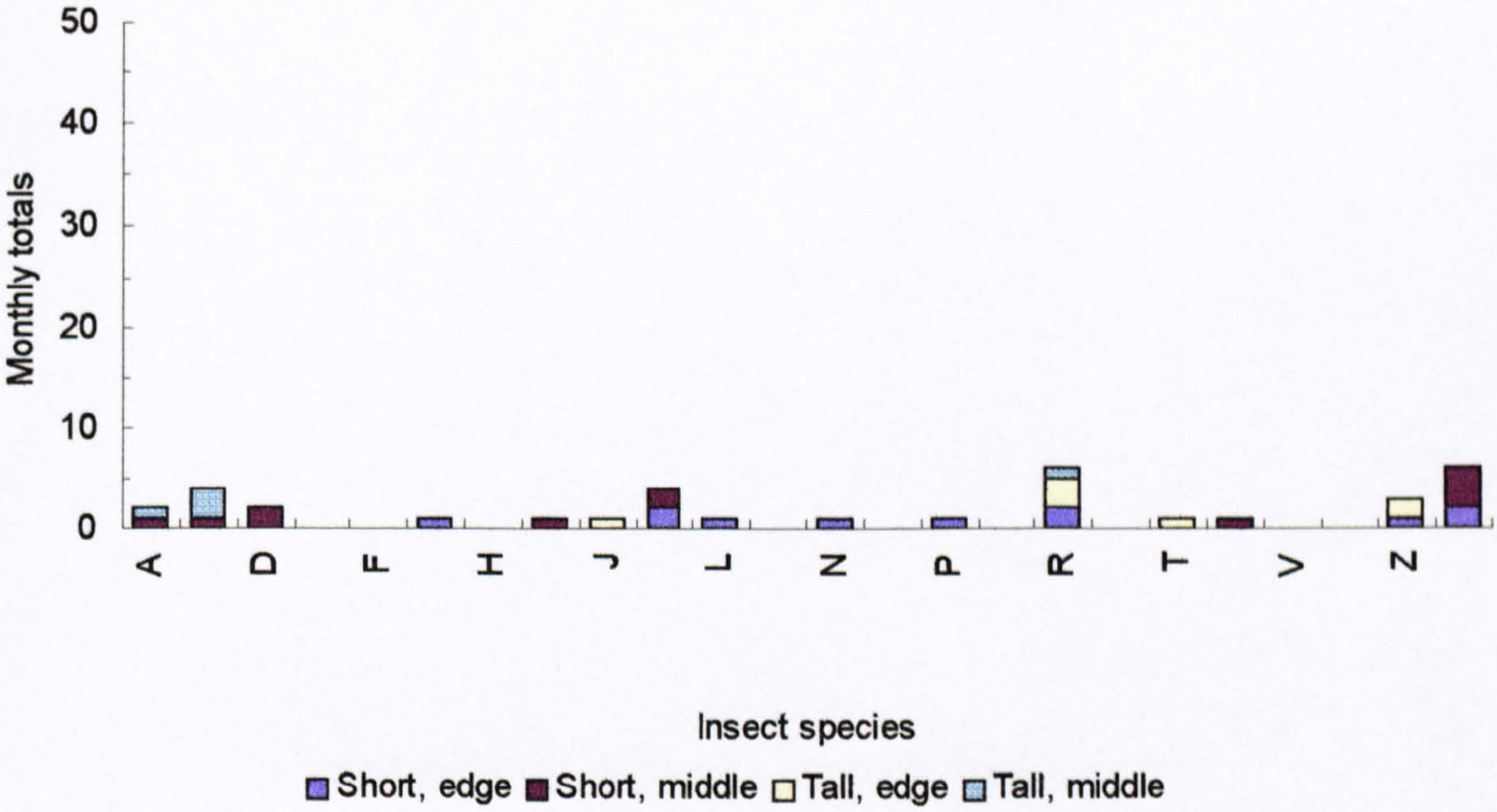


Fig. 6.5a Homoptera insects trapped at Chambezi during February 1996

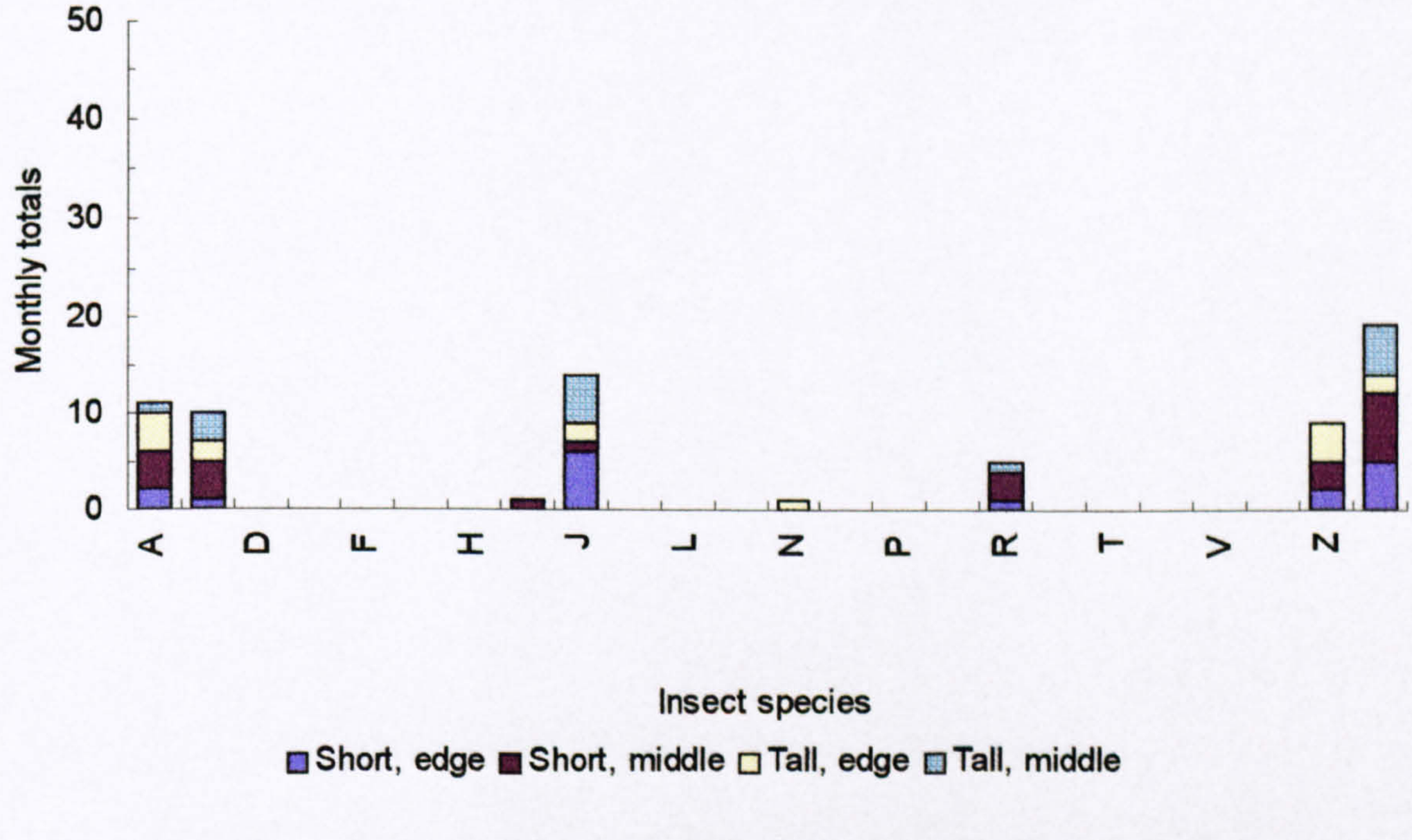


Fig. 6.5b Homoptera insects trapped at Chambezi during March 1996

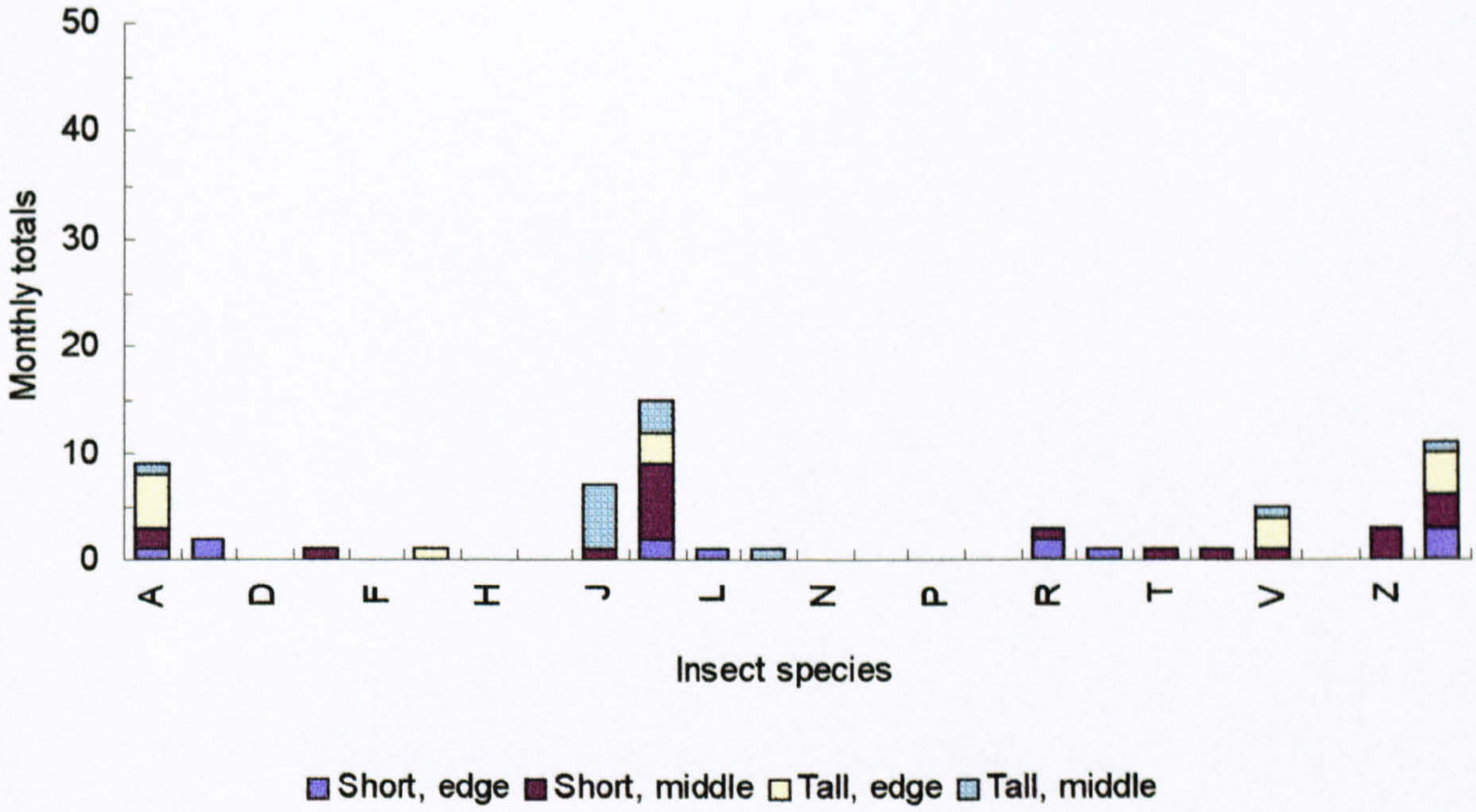


Fig. 6.6a Homoptera insects trapped at Chambezi during April 1996

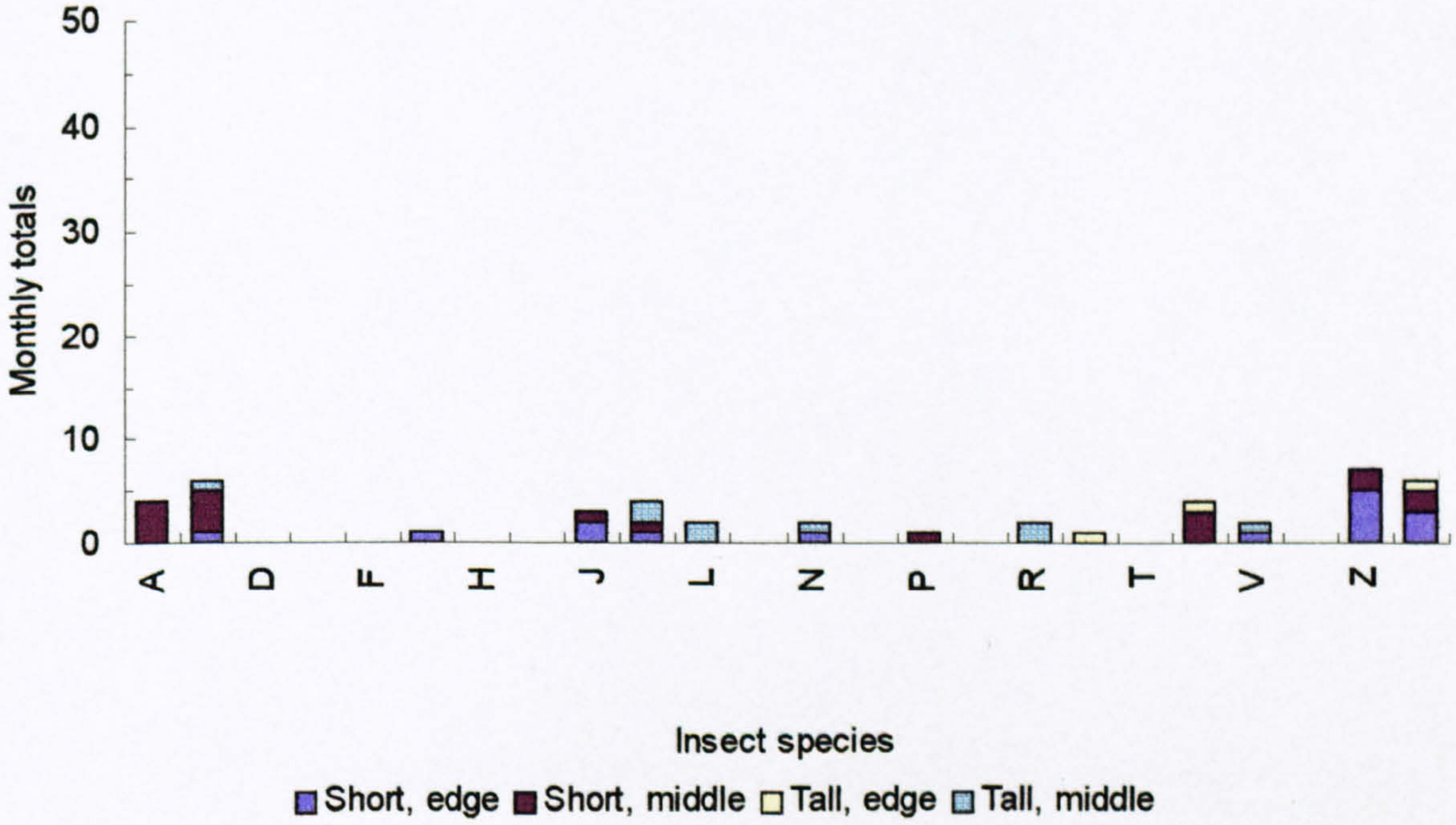


Fig. 6.6b Homoptera insects trapped at Chambezi during May 1996

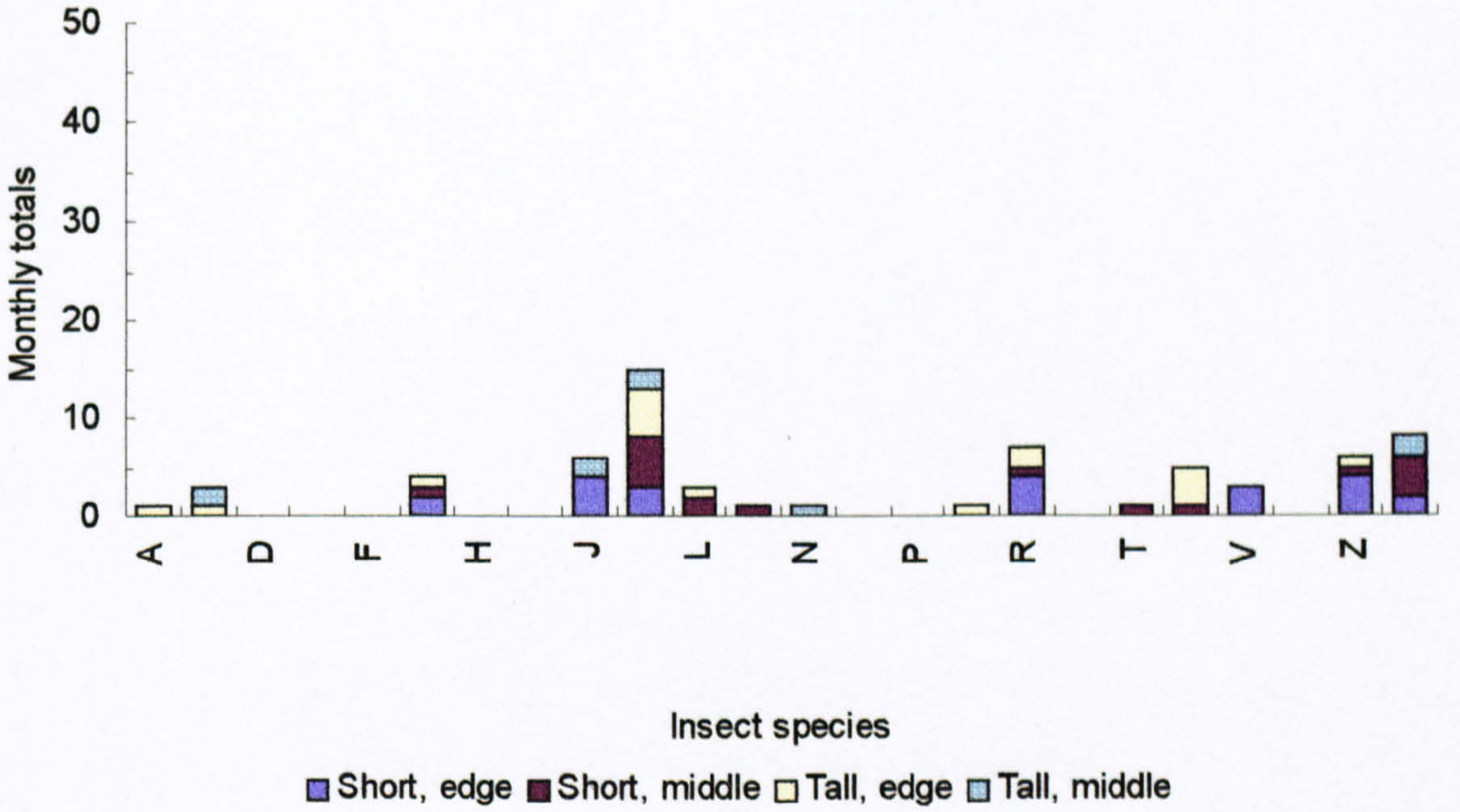


Fig. 6.7a Homoptera insects trapped at Chambezi during June 1996

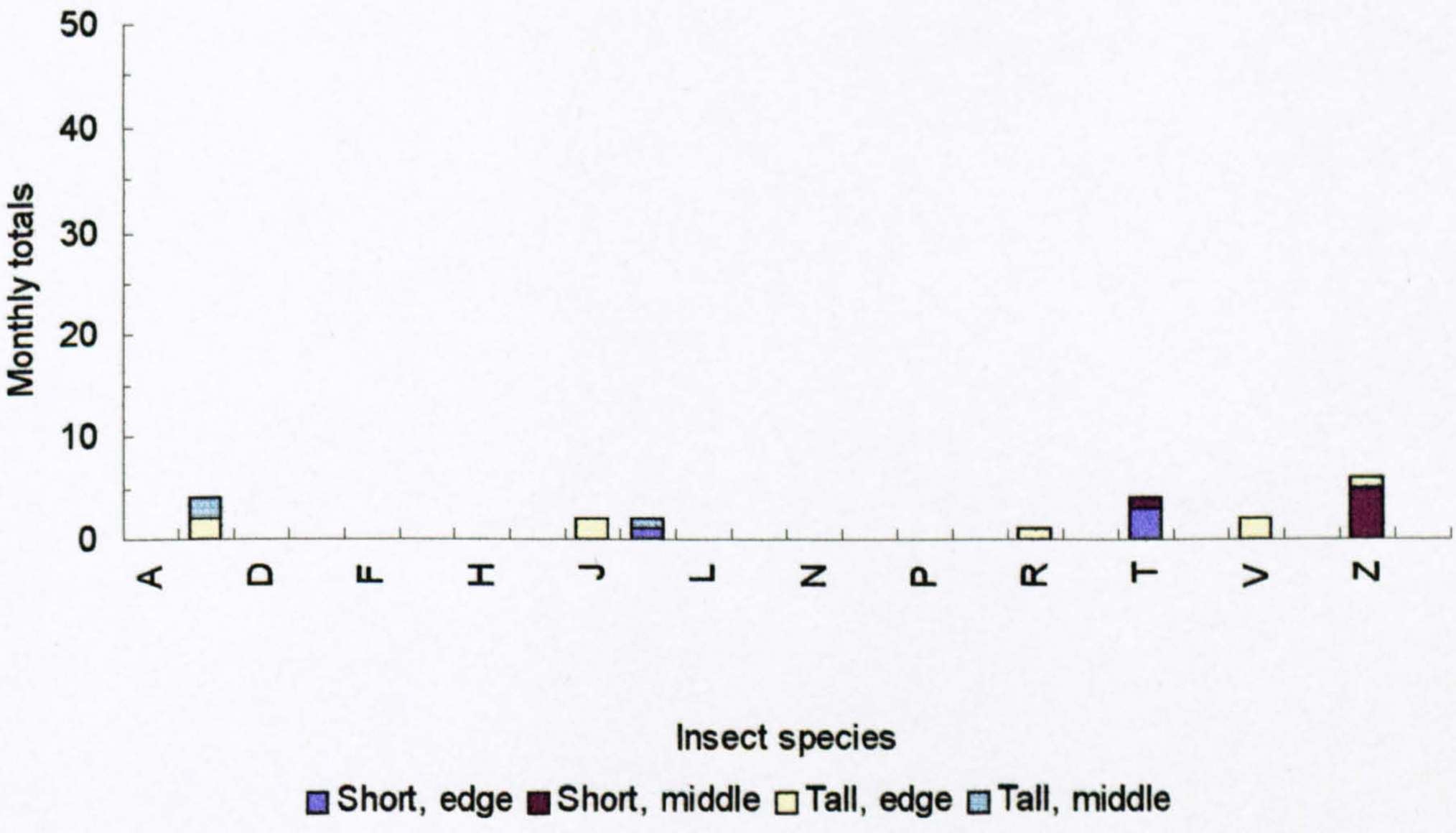


Fig. 6.7b Homoptera insects trapped at Chambezi during July 1996

Generally, very few insects were trapped at both sites in comparison to previous studies (Kaiza, 1987). The differences could have been due to the trapping methodology. In the previous studies, insect suction traps were complemented by counting insects on the underside of leaves on selected palms. Trapping of insects from palms for DNA extraction during this study also indicated that more insects could be collected manually than from traps. A total of 15,000 insects were manually collected within a period of less than one year (Table 6.2), whereas the total number collected from the traps at the two sites in a year were only 2410. This may imply that not many insects are attracted to the traps, and that a combination of methods may be necessary to give a better representation of insect numbers in coconut fields.

Despite the low numbers, there were clear differences in the populations of different species on palms at different times of the year. The two indistinguishable derbids, *Nesodryas antiope* and *Amania angustifrons* (species K), which were previously suspected to breed on palms (Kaiza, 1987) were relatively more abundant than the other species. The other abundant insect species were the undescribed grass-green leafhopper (species J), and yet another undescribed green planthopper (species R). The green planthopper (R) was more abundant at Kifumangao than at Chambezi (Fig. 6.2).

Specific observation on the behaviour of insect species that have been suspected to be putative vectors of LD for long time, that is, *Diastrombus abdominalis* and *D. mkurangai*, (Anonymous, 1987; Kaiza, 1987), revealed that these insects were not trapped in big numbers, although they are generally abundant. Despite this, they were present throught the year, especially, *D. mkurangai*. The behaviour of *D. mkurangai* was particularly striking, because the distribution in the field varied with specific months. At Chambezi, for example, these insects were caught on palms near the edge of the field mainly during the months of October (Fig. 6.3a), January (Fig. 6.4b), and March to April (Fig. 6.5b and 6.6a). In June and July, they were caught only on tall palms, both at the edges and in the middle of the field (Fig. 6.7a and 6.7b). Their predominance during dry months would suggest that their flight into the field from outside was favourable during those months. For the rest of the year, they were

caught in the middle of the field, and predominantly on short palms, which suggests breeding within the field.

The behaviour of these insects was different at Kifumangao. Although the general picture was that more insects were trapped on short palms in the middle of the field (Fig. 6.8), none of these were *D. nkurangai*. For the whole year, these insects were predominantly trapped on tall palms, especially near the edges of the field (Fig. 6.9 - 6.14). In a few months when they were trapped on short palms, it was only on those located near the field edge (Fig. 6.10b, 6.11a, 6.12b, 6.14a). These results suggest that at this location, these insects were breeding outside, and continuously flying into the field. If these insects are the true vectors of LD, then this behaviour is in agreement with the strong edge effects that have been observed in the disease infected fields (Schuiling *et al.*, 1992a), with higher disease incidence near the borders.

The other insect species highly suspected to be a putative vector of LD based on the results of this study (sections 6.3 and 6.4), that is *Meenoplus spp* was found to be less abundant. At Chambezi it was trapped only during the months of August, September, April and June (Fig. 6.2a &b, 6.6a and 6.7a). Except for August, it was trapped on palms in the middle of the field. Similarly at Kifumangao, it was trapped during the months of November, January and February (Fig. 6.10b, 6.11b and 6.12a) and in all cases on tall palms, either in the middle of the field or near the edge. These data seem to suggest breeding both outside and within the field at Chambezi, and predominatly outside the field at Kifumangao. From the relative abundance of this species, it can be deduced that if it is also the vector of LD, then its contribution to disease transmission would be restricted to short periods within a year.

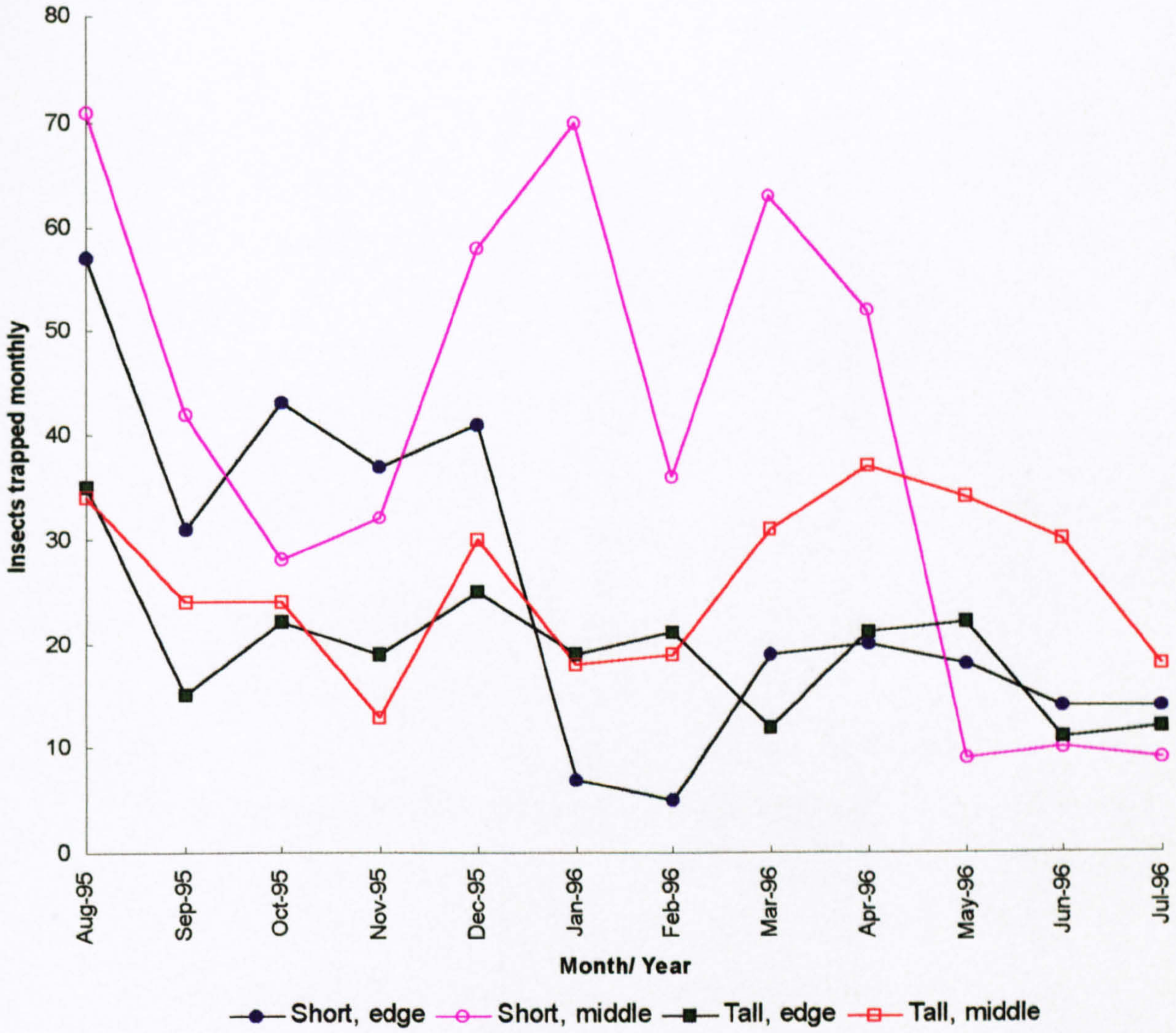


Fig. 6.8 **Fluctuations in the population of homoptera insects trapped on selected palms at Kifumangao between July 1995 and July 1996**

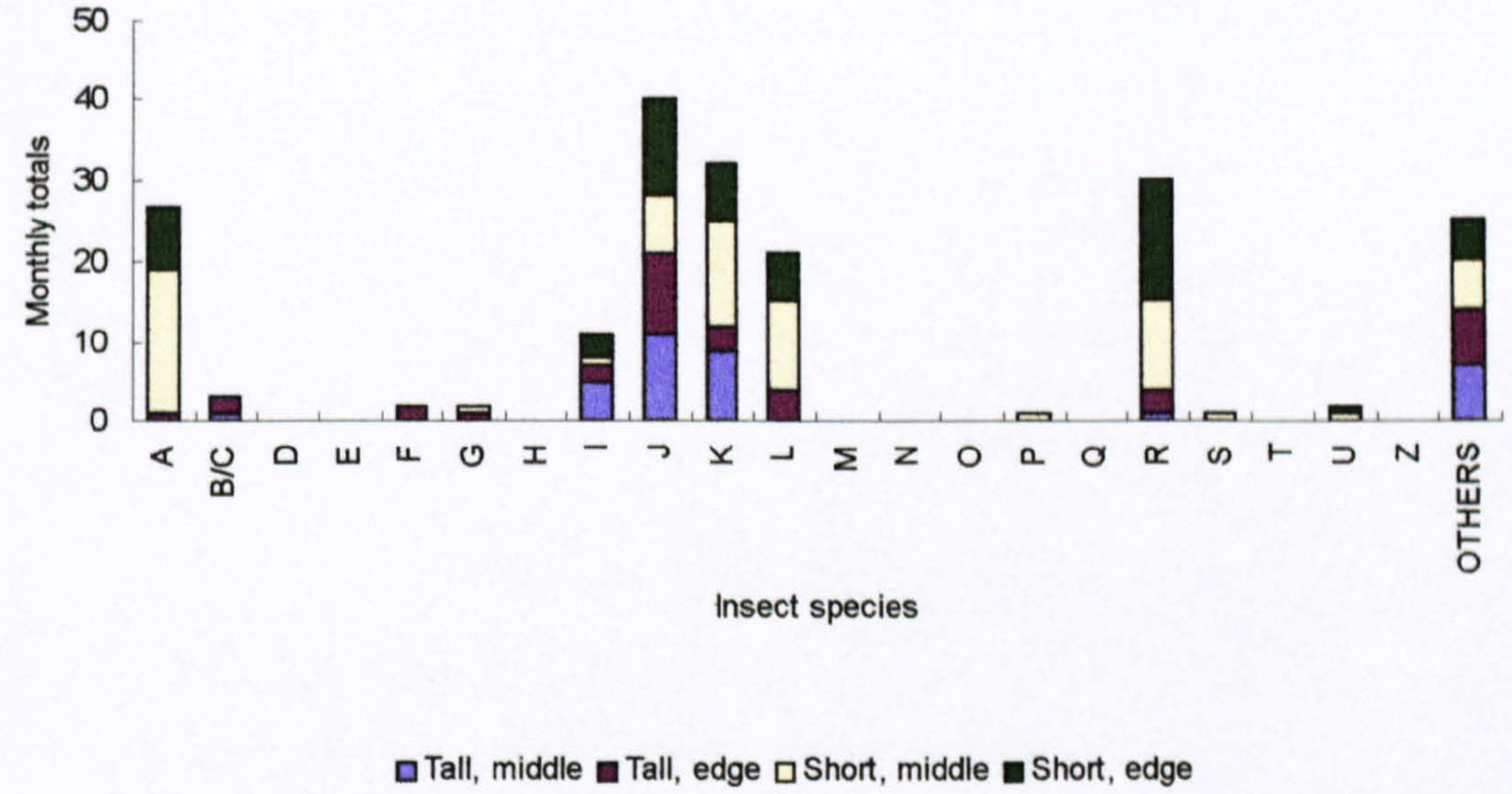


Fig. 6.9a Homoptera insects trapped at Kifumangao during August 1995

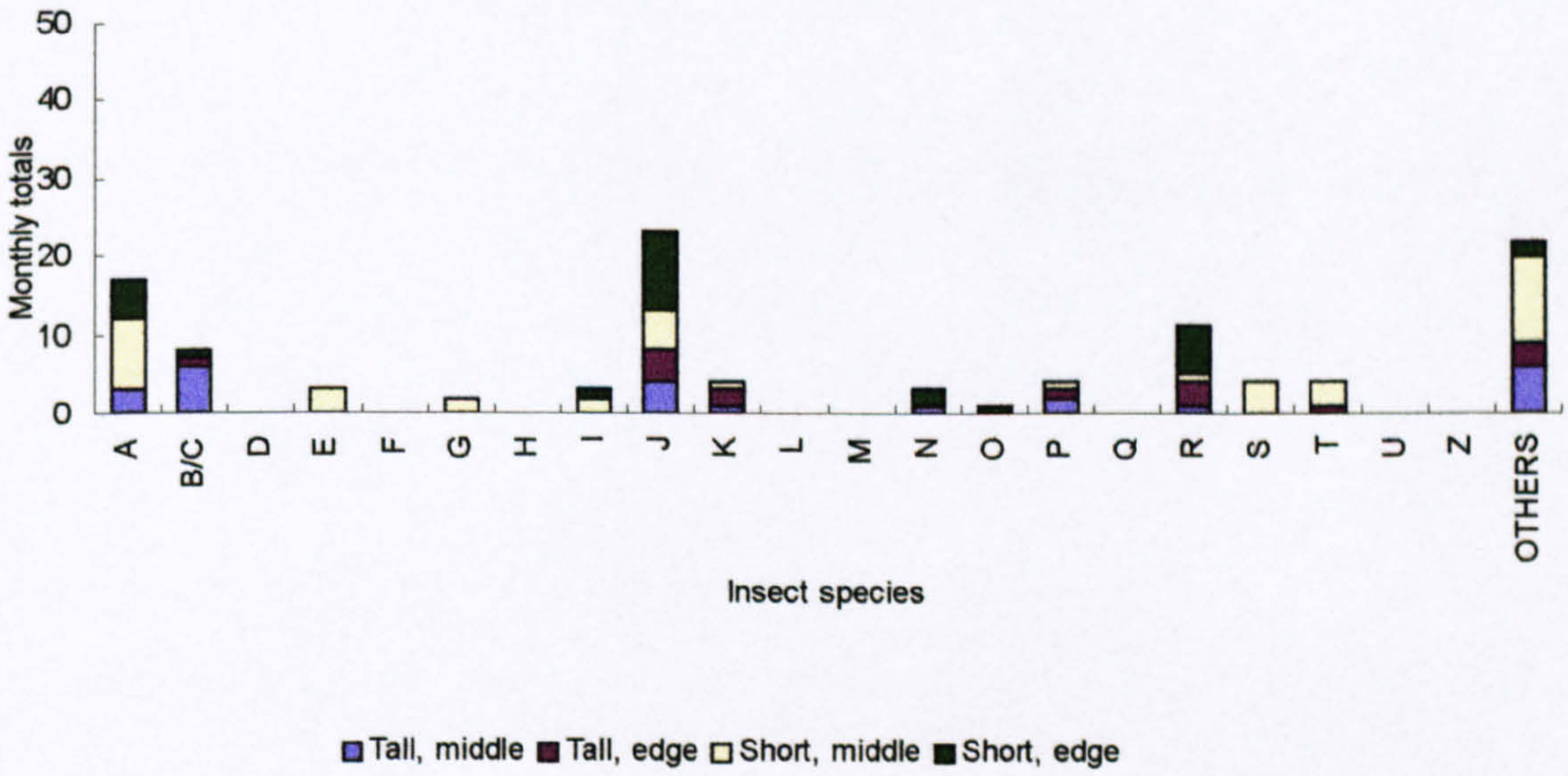


Fig. 6.9b Homoptera insects trapped at Kifumangao during September 1995

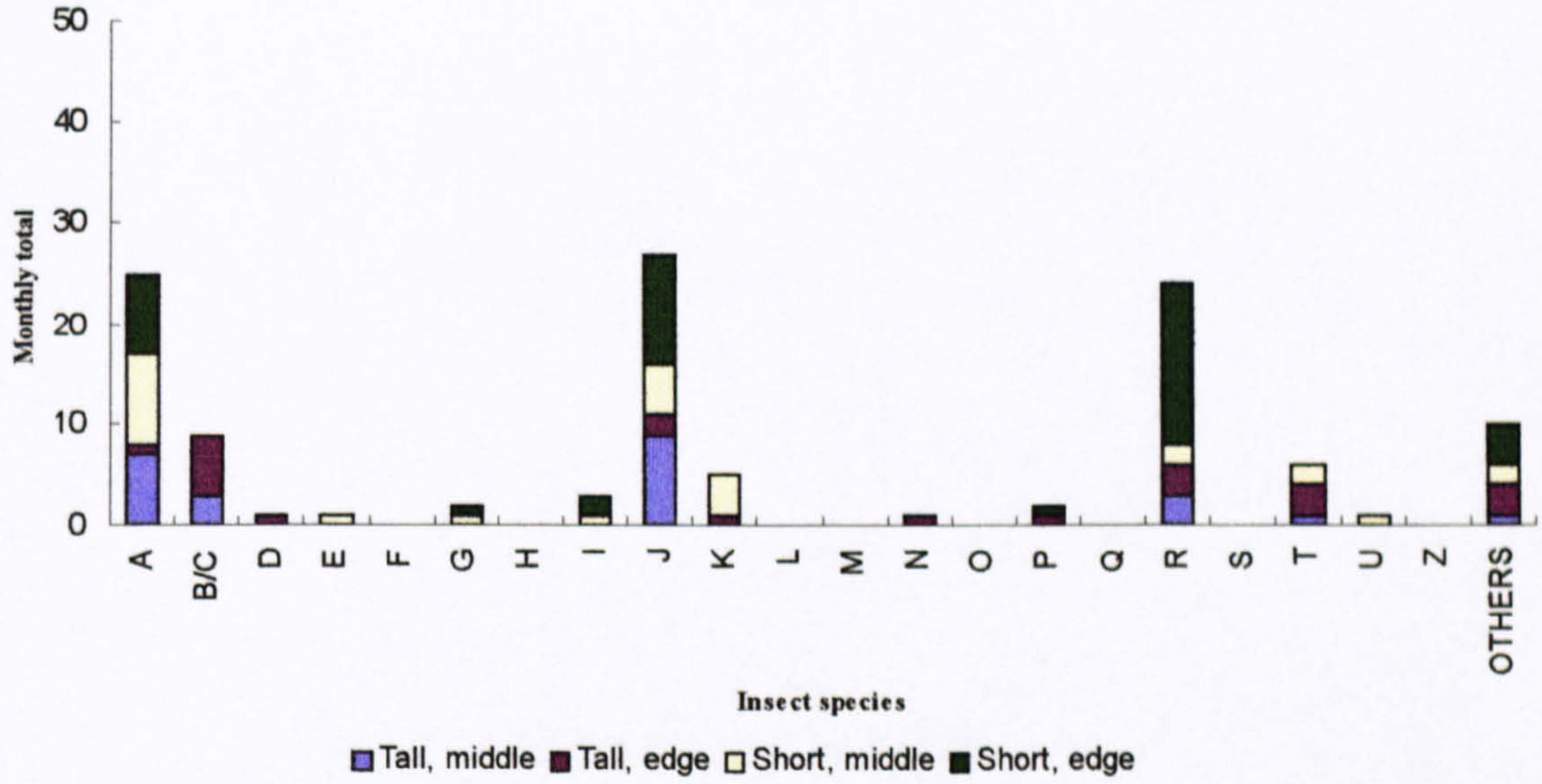


Fig. 6.10a Homoptera insects trapped at Kifumangao during October 1995

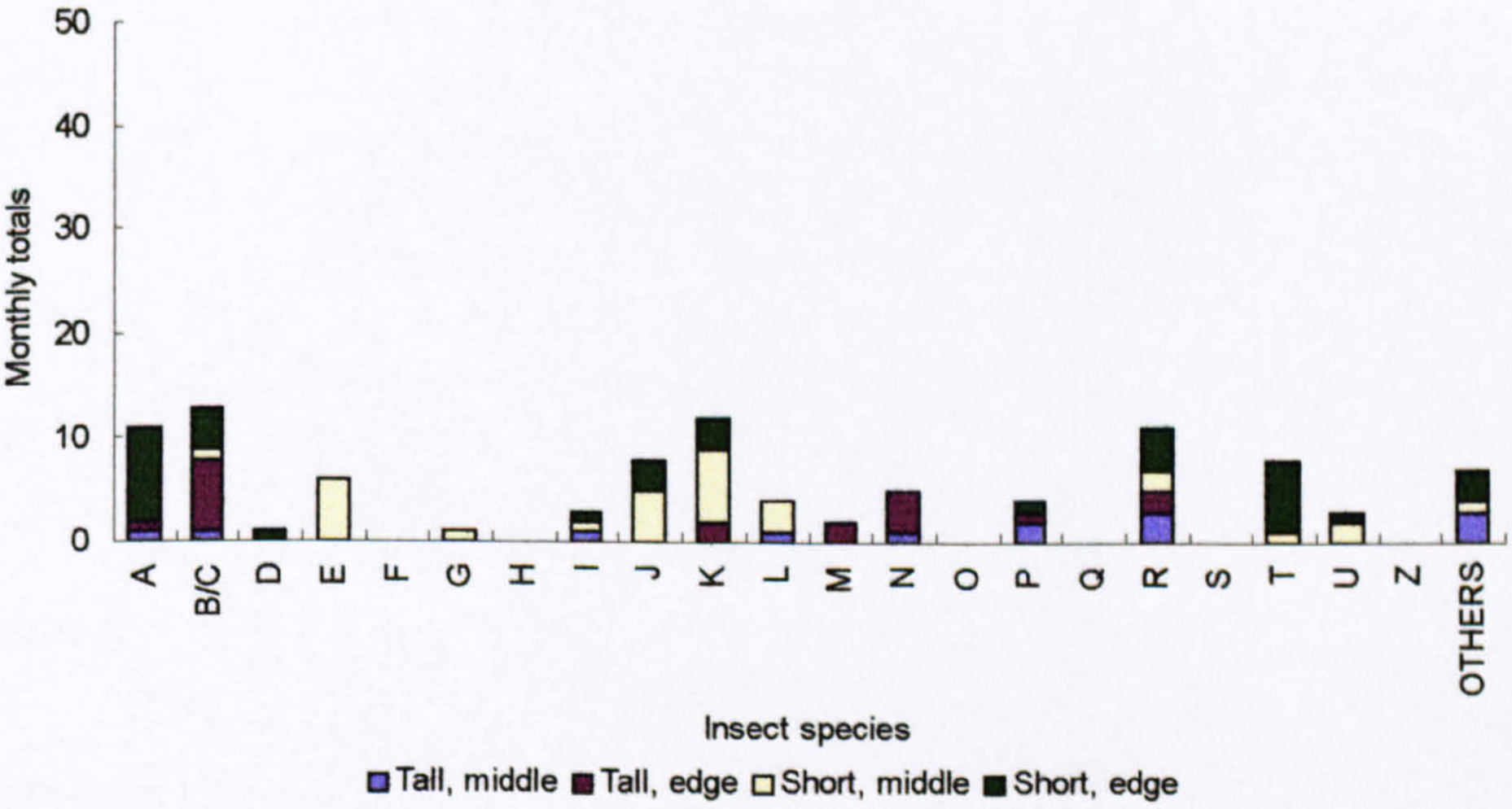


Fig. 6.10b Homoptera insects trapped at Kifumangao during November 1995

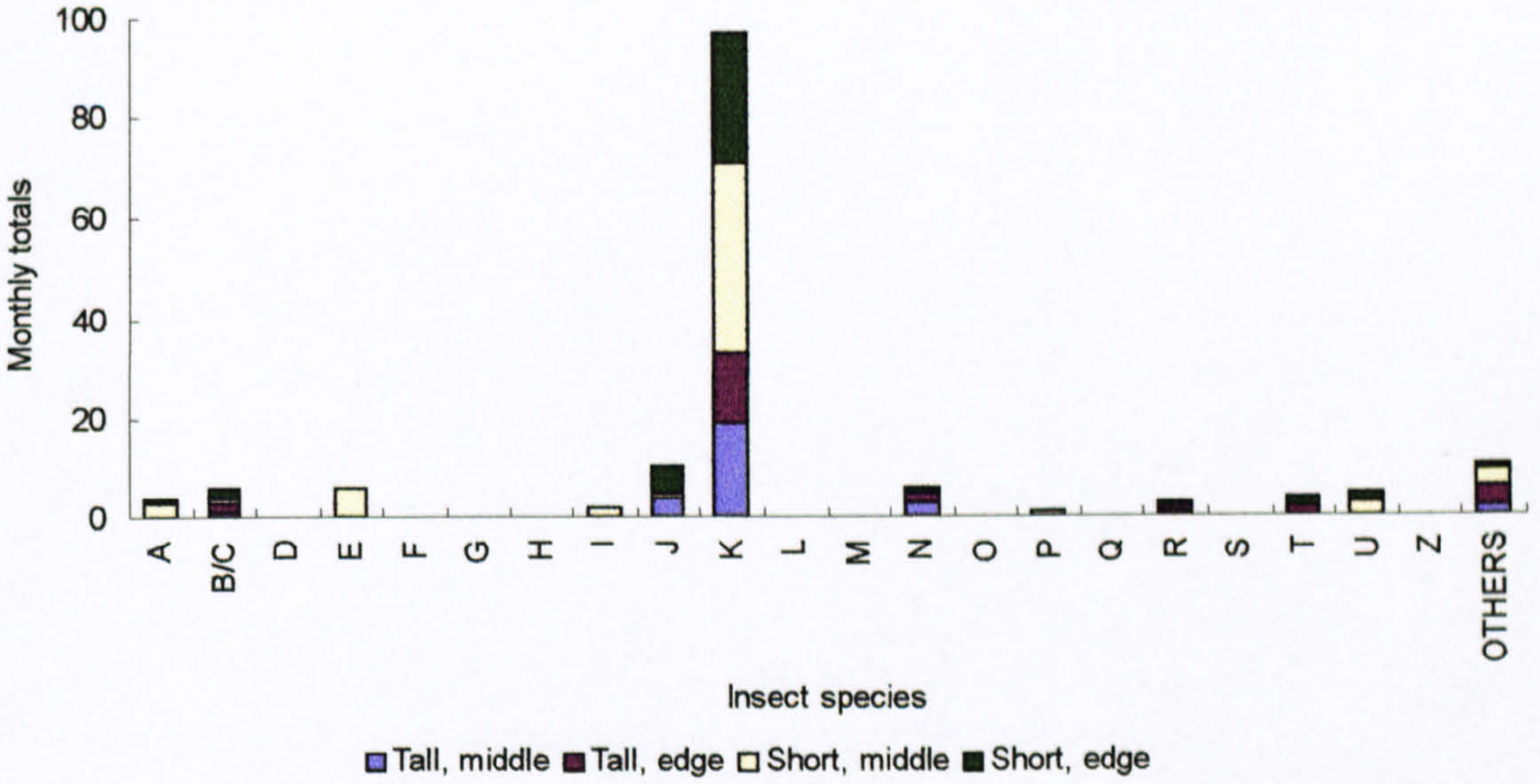


Fig. 6.11a Homoptera insects trapped at Kifumangao during December 1995

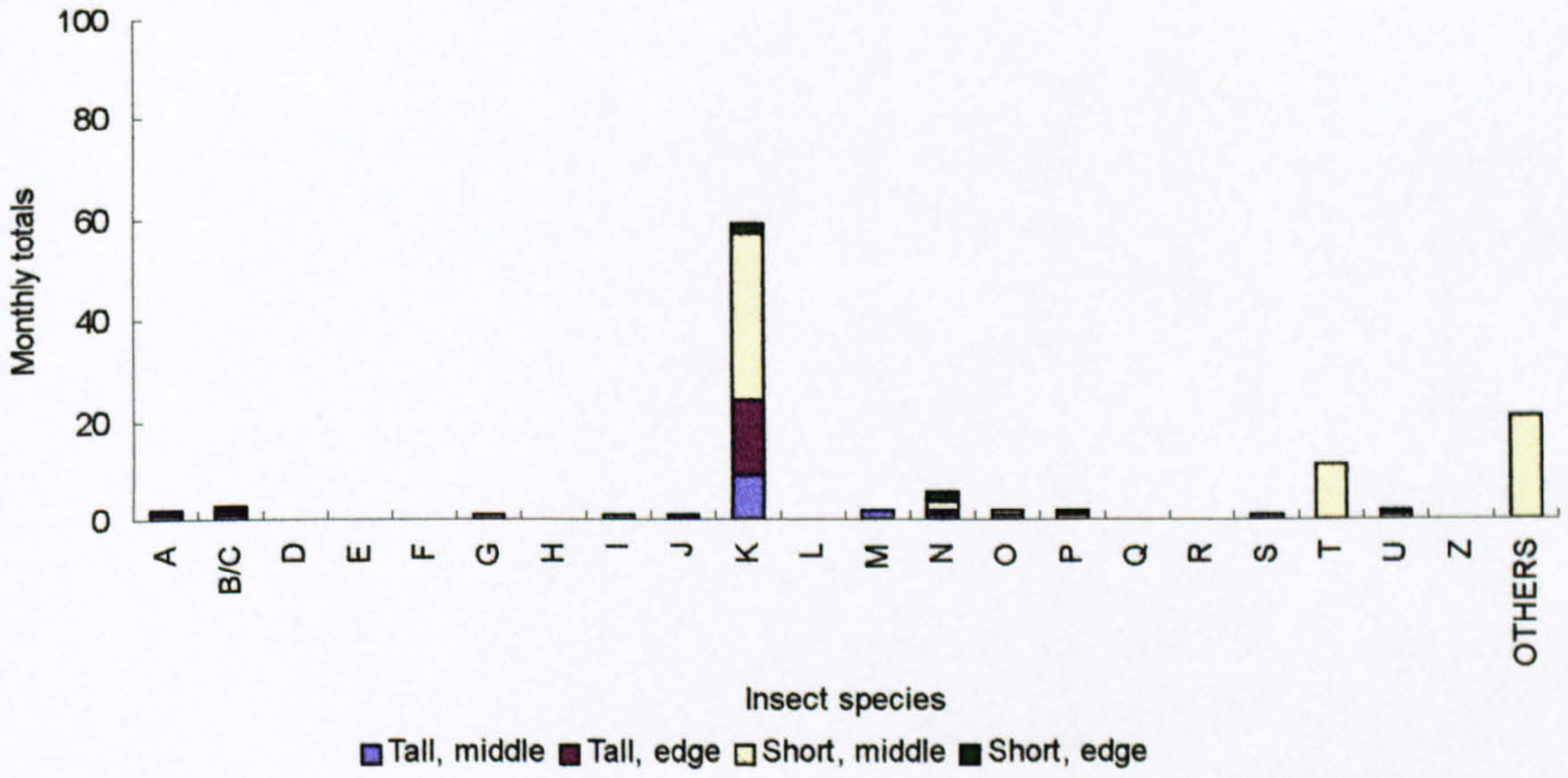


Fig. 6.11b Homoptera insects trapped at Kifumangao during January 1996

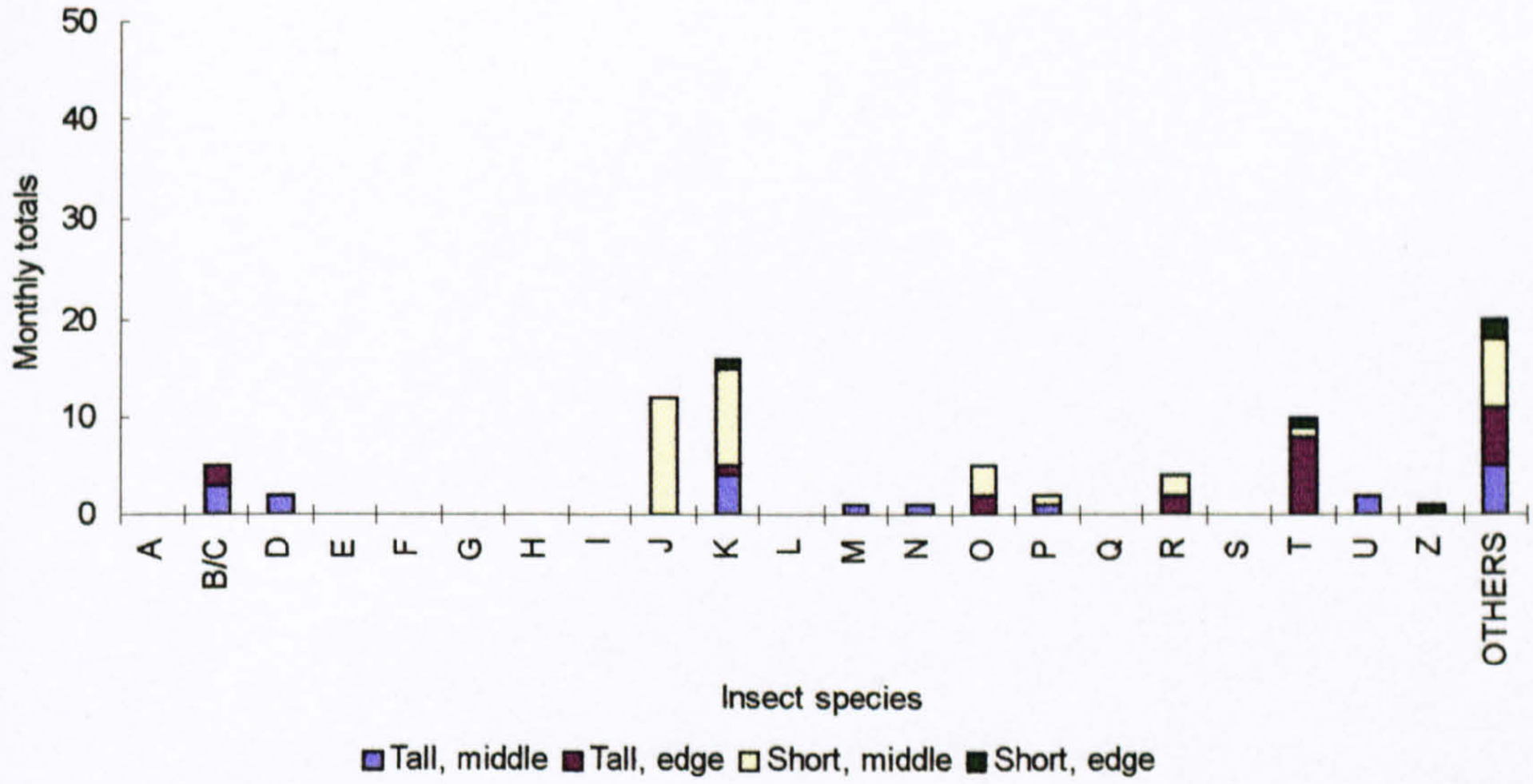


Fig. 6.12a Homoptera insects trapped at Kifumangao during February 1996

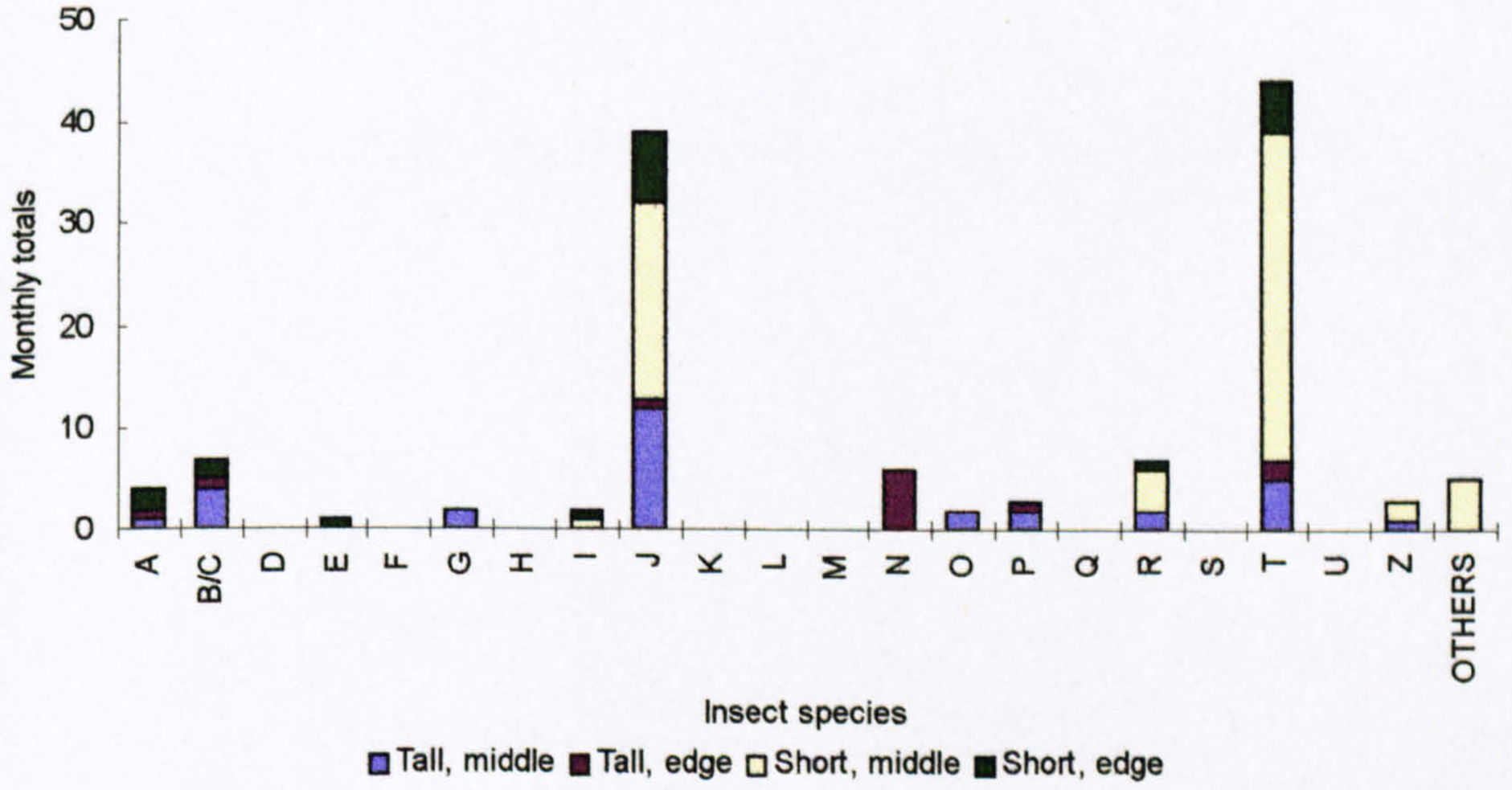


Fig. 6.12b Homoptera insects trapped at Kifumangao during March 1996

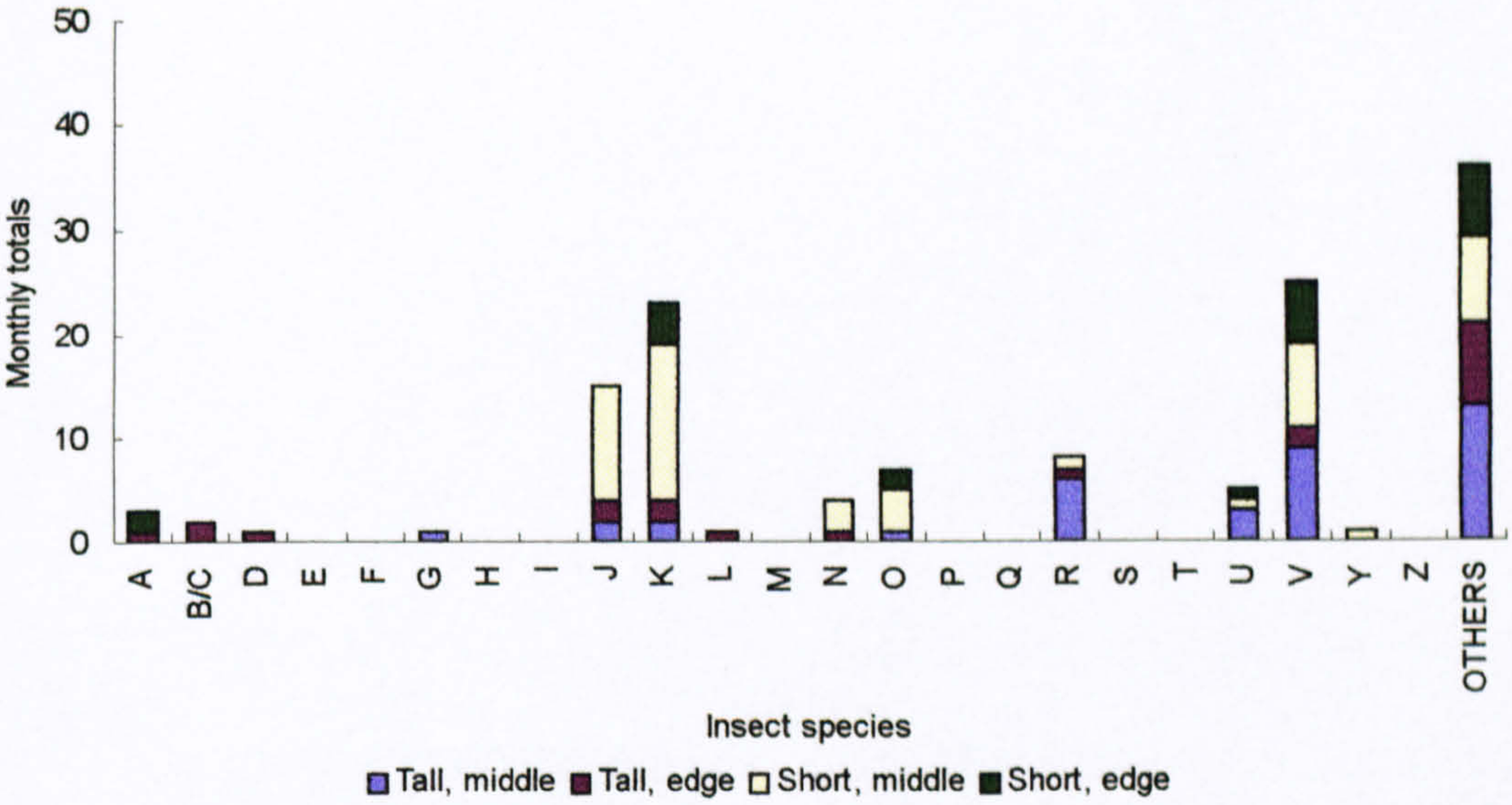


Fig. 6.13a Homoptera insects trapped at Kifumangao during April 1996

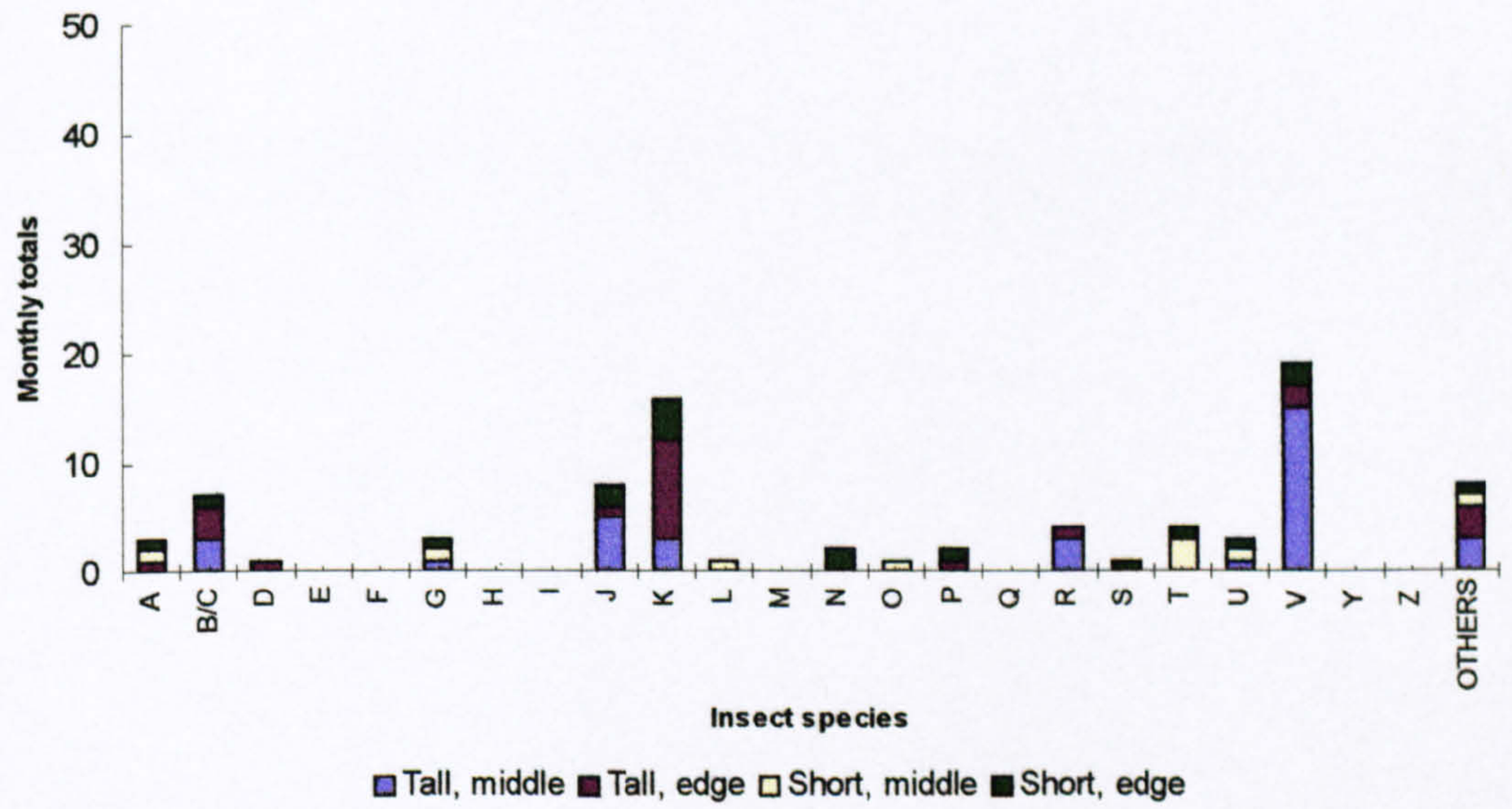


Fig. 6.13b Homoptera insects trapped at Kifumangao during May 1996

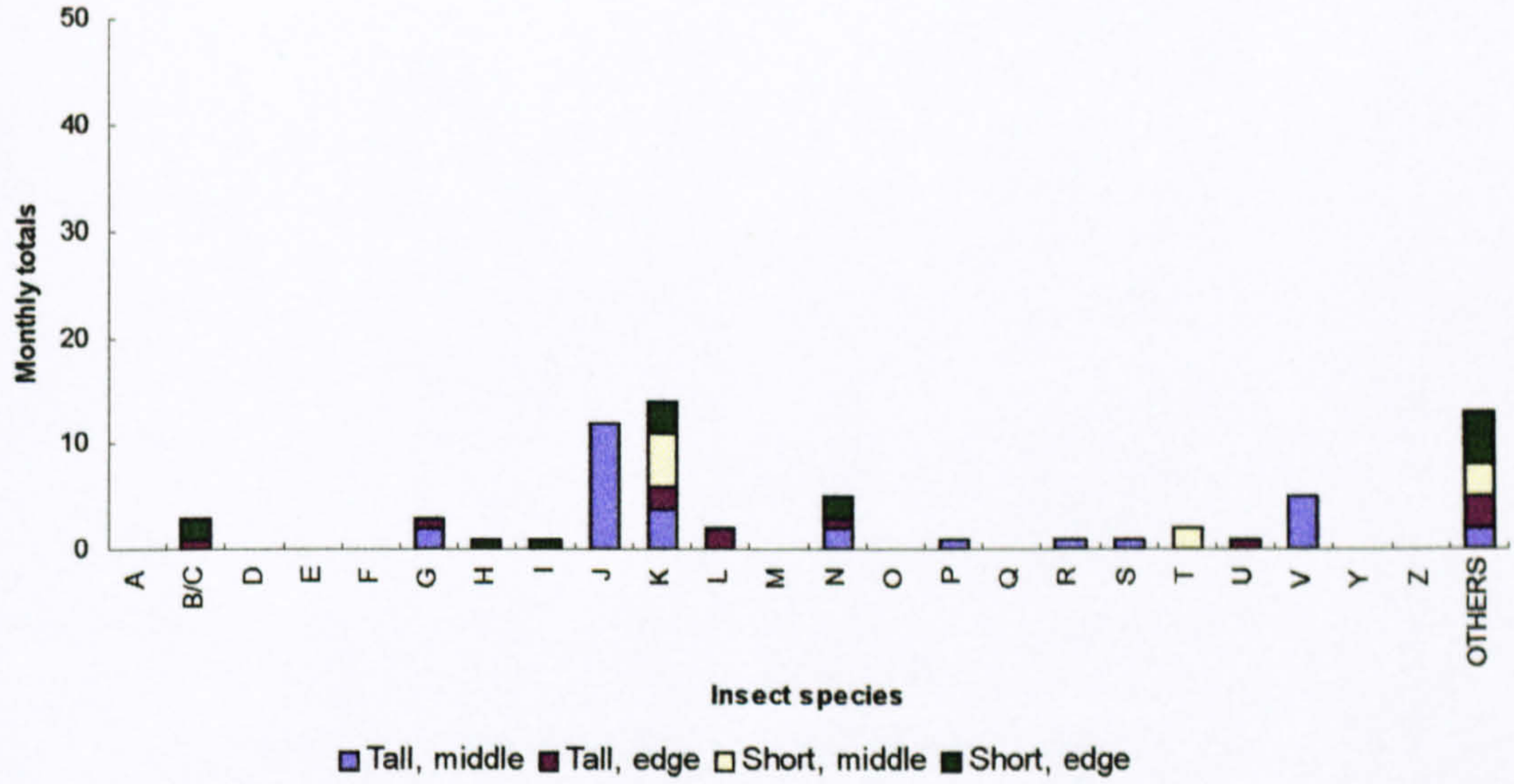


Fig. 6.14a Homoptera insects trapped at Kifumangao during June 1996

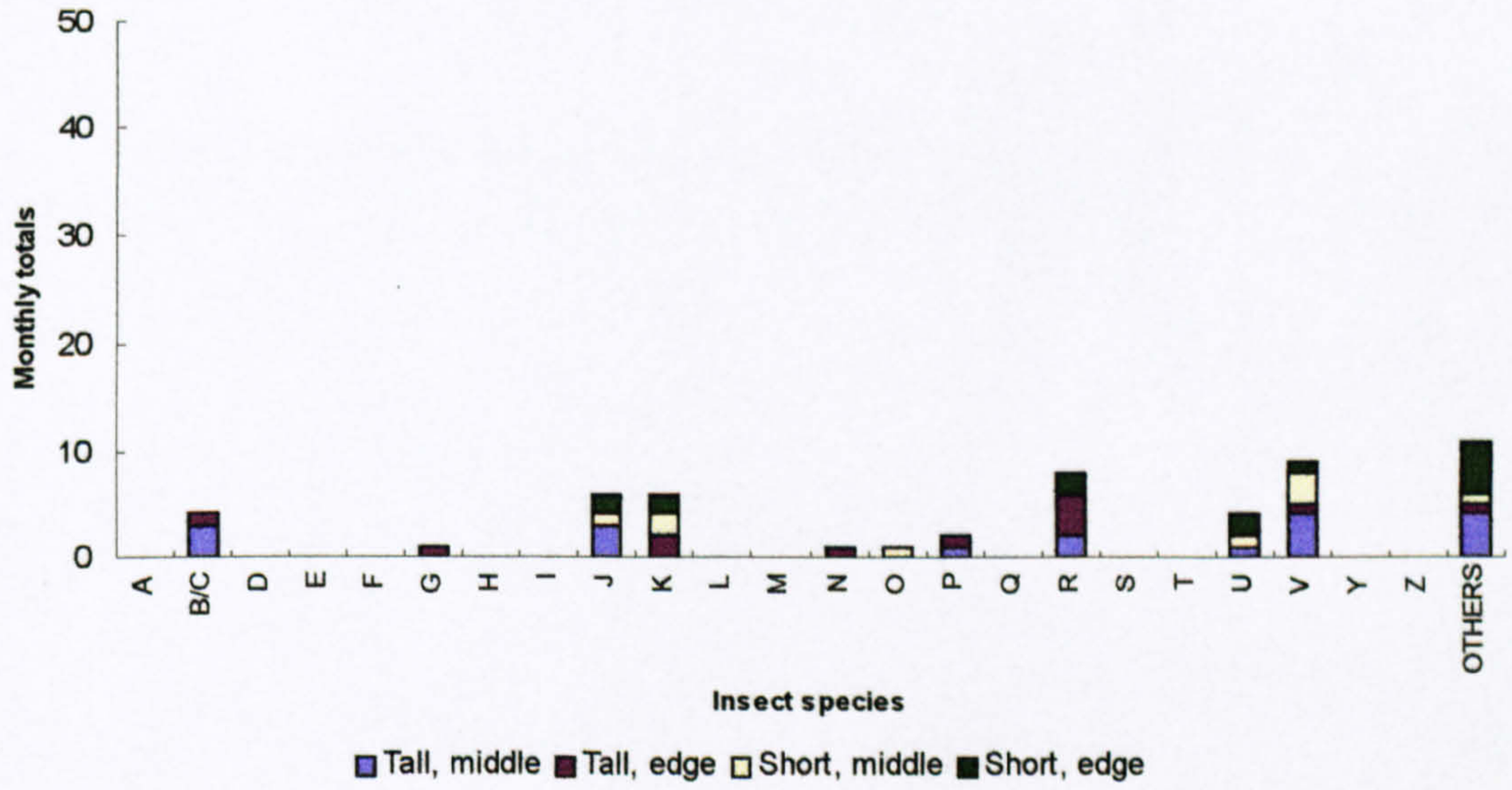


Fig. 6.14b Homoptera insects trapped at Kifumangao during July 1996

The incidence of disease at each site was recorded monthly. The regression of disease incidence on the insect populations at the two sites, is shown in Fig. 6.15, and 6.16, for Chambezi and Kifumangao respectively. The results showed a strong linear relationship (correlation) between the total number of trapped insects and disease incidence at both sites. At Chambezi, the correlation coefficient, r was 0.75 (R^2 = -0.56, data not shown). The correlation was stronger when the incidence of disease was regressed against the number of trapped *D. mkurangai* and *Meenoplus* sp. alone at Chambezi, with the correlation coeffient increasing to 0.87 (Fig. 6.15a; R^2= -0.75). The coefficient of correlation, r, was further increased to 1.0 (R^2 = -1.02), when the disease incidence data was regressed against the numbers of these two insect species trapped during the previous year (Fig 6.15b). When the disease incidence data was regressed on the numbers of these insects tapped nine months before, the correlation was weaker, with a correlation coefficient of 0.66 (R^2 = -0.43).

The data from Kifumangao showed a similar trend to Chambezi. The correlation coefficient, r, between disease incidence and total trapped insects was 0.78 (R^2 = -0.61, Fig. 6.16a). However, the regression of disease incidence on the numbers of *Diastrombus mkurangai* and *Meenoplus* sp. together, produced a weaker linear relationship with the correlation coefficient, r decreasing to 0.60 (R^2 = -0.36). The correlation coefficient decreased further to 0.37 (R^2 = - 0.14) when the incidence of disease was regressed on the numbers of these two insect species trapped the previous year. However, a stronger linear relationship was detected when the disease incidence was regressed on the numbers of *D. mkurangai* (alone) trapped one year before, with the correlation coefficient of 0.75 (R^2 = -0.56, Fig. 6.16b). These results suggest strongly that the suspect insect species, *Diastrombus mkurangai* and *Meenoplus* sp., may be the vectors of LD, and that the incubation period of the disease is about one year. The data from Kifumangao particularly pinpointed *D. mkurangai* as the prime suspect vector.

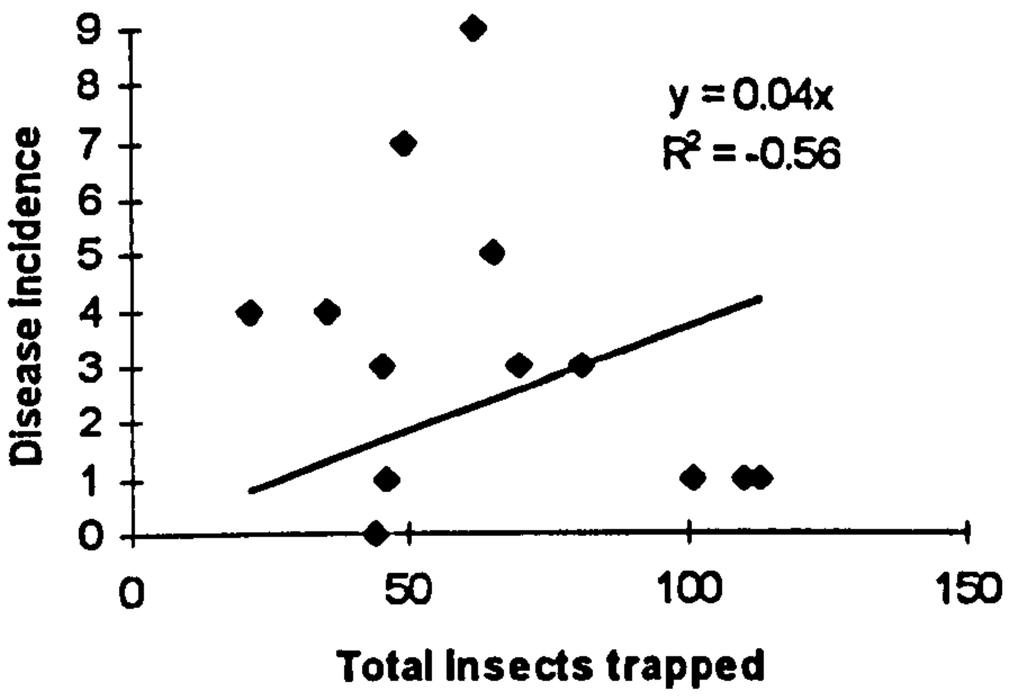


Fig. 6.15a **Regression of disease incidence at Chambezi during 1995-96 on the total insects trapped**

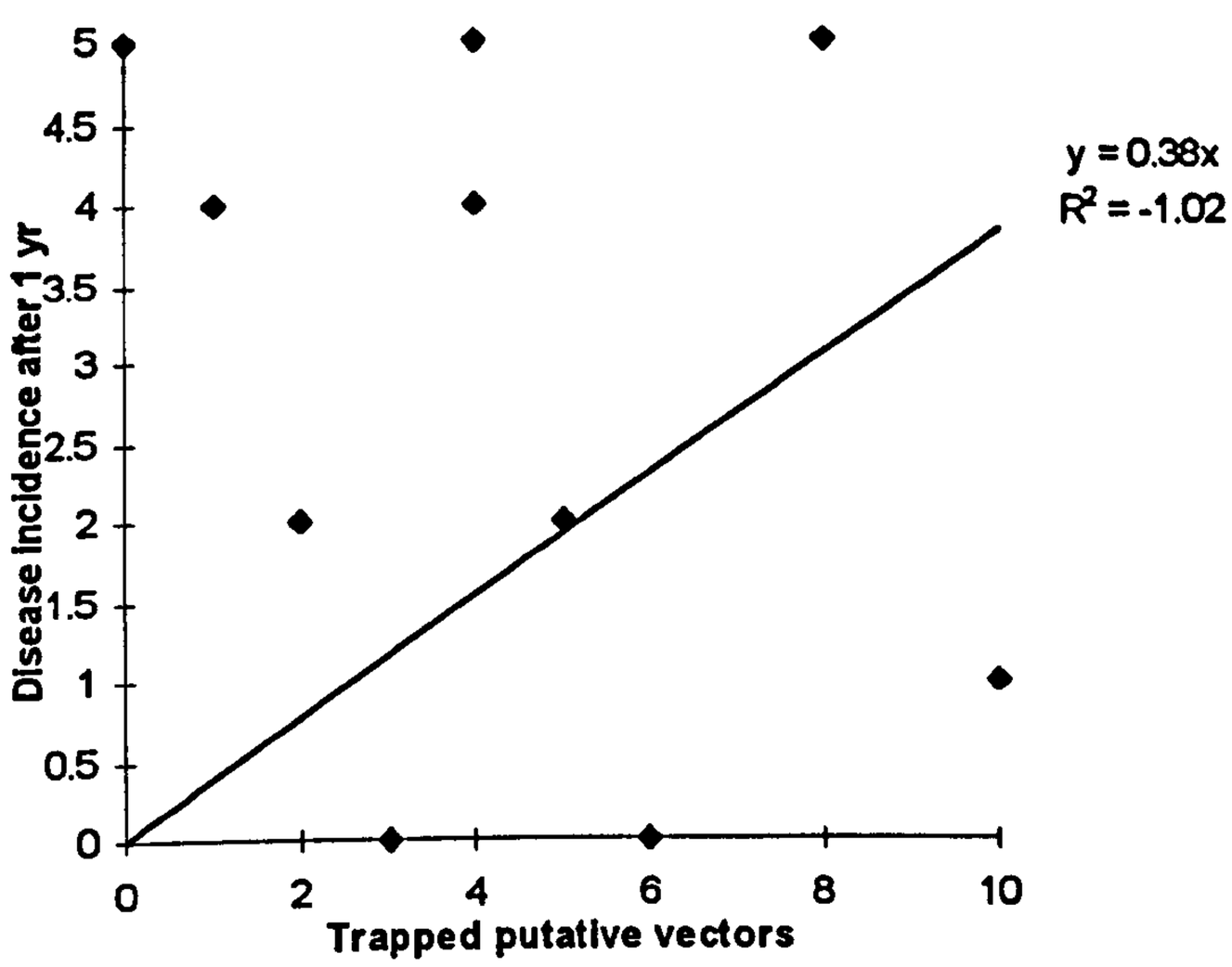


Fig. 6.15b **Regression of disease incidence at Chambezi during 1996-97 on the population of *D. mkurangai* and *Meenoplus* spp trapped during the previous year.**

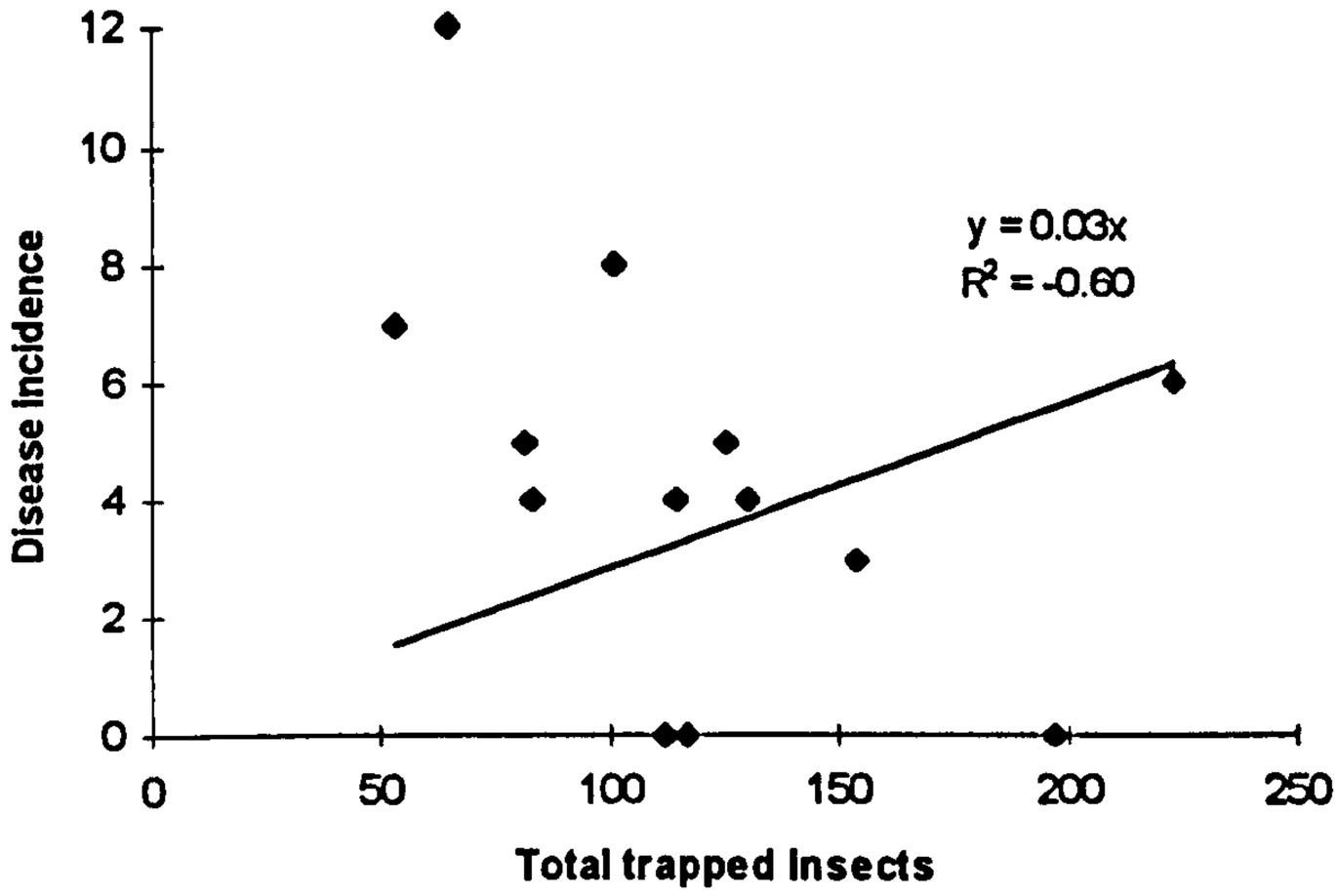


Fig. 6.16a **Regression of disease incidence at Kifumangao during 1995-96 on the total insects trapped.**

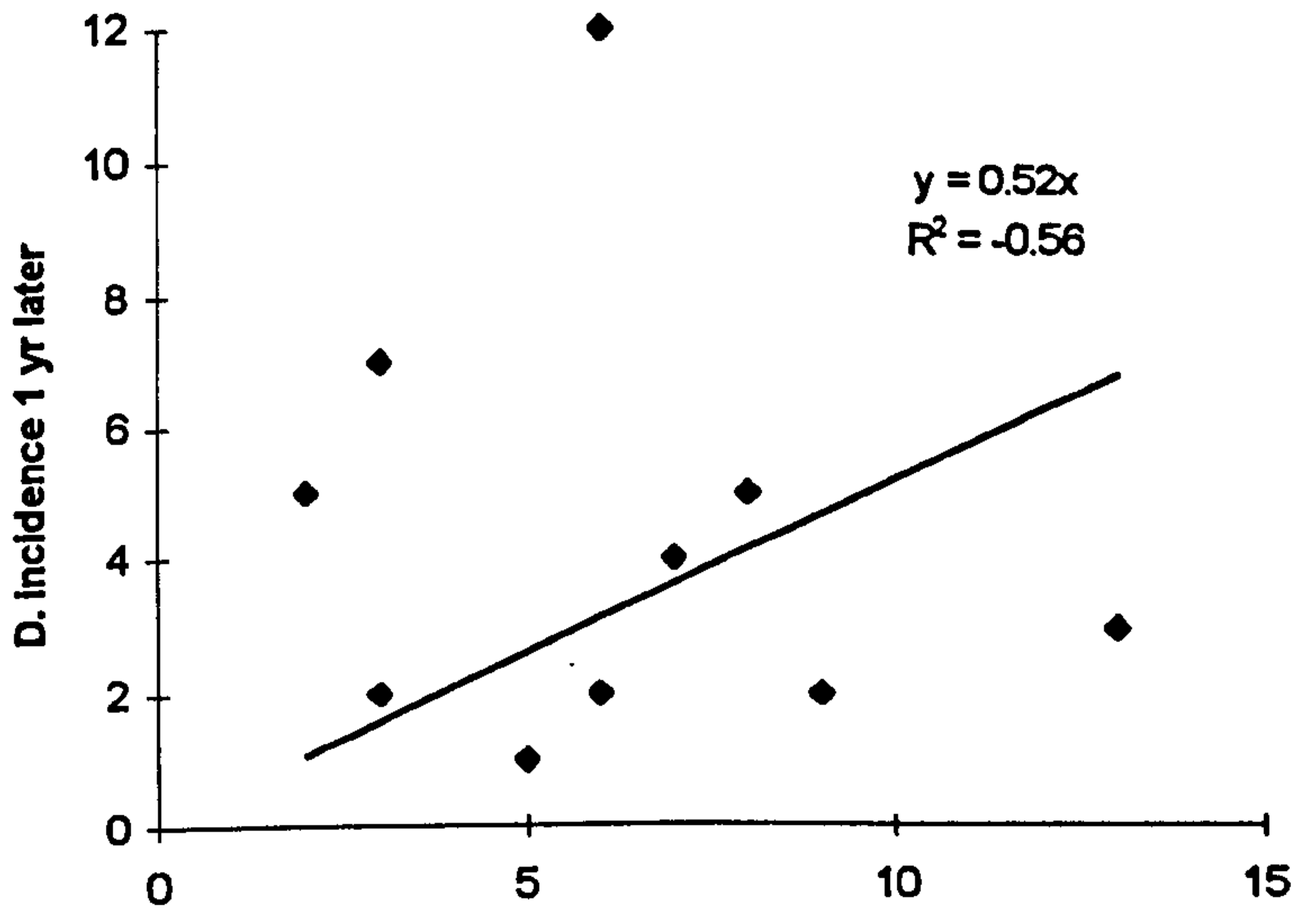


Fig. 6.16b **Regression of disease incidence at Kifumangao during 1996-97 on the population of *D. mkurangai* trapped the previous year.**

6.3 USE OF PROBES TO DETECT PHYTOPLASMA rDNA IN INSECTS SUSPECTED TO BE LD VECTORS

The sensitivity of DNA probes for detection of phytoplasma in the DNA extracted from insects suspected to be potential vectors of LD was investigated with probe LD 12-66. Insects for this study were all collected by aspiration from the underside of LD-infected palms using large flasks. DNA was extracted from single insects, and blotted onto nylon membranes as single spots. The probe DNA was labelled with DIG. A summary of insect species screened, and the numbers per species is shown in Table 6.1.

Phytoplasma DNA was detected in 37 insects out of 137 screened. The positive insects were distributed over three families and six species. They include:

1) Family *Derbidae* (Species *Diastrombus abdominalis*, *D. mkurangai*, *Phenice pongweil* *Paraphenice* spp, and *Amania angustifrons*).
2) Family *Lophodidae* (Species *Elasmosceles cimicoides*).
3) Family *Meenoplidae* (Species *Meenoplus*).

However, it was only insects of the species *D. mkurangai* and *Meenoplus* spp respectively which were found to have a phytoplasma DNA band when screened by PCR (section 6.4). The highest number of insects testing positive for phytoplasma by DNA probes, (but not PCR) were collected from Mpiji, an area where the incidence of disease was very high at the time of insect collection.

Table 6.1 Detection of phytoplasma DNA in potential insect vectors by use of the LD 12-66 DNA probe

Insect species	Location from which insects were collected**				
	Mpiji	Chambezi	Kigombe	Miteja	Ndarara
Family *Derbidae*					
Diastrombus abdominalis	10 (9)	5 (0)	8 (1)	8 (3)	2 (1)
Diastrombus mkurangai	0 (0)	0 (0)	4 (0)	23 (5)	3 (0)
Phenice pongwei / *Paraphenice mawae*	1 (1)	0 (0)	2 (0)	0 (0)	0 (0)
Diastrombus schuilingi	0 (0)	0 (0)	7 (0)	4 (0)	0 (0)
Zoraida fuligipennis	0 (0)	0 (0)	4 (0)	1 (0)	0 (0)
Amania angustifrons	2 (2)	0 (0)	8 (0)	0 (0)	1 (0)
Lydda woodi	0 (0)	0 (0)	0 (0)	0 (0)	14 (0)
Zorabana spp	0 (0)	0 (0)	0 (0)	0 (0)	7 (0)
Undescribed (Spp Z)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)
Family *Lophodidae*					
Elasmosceles cimicoides	14 (13)	5 (4)	0 (0)	0 (0)	0 (0)
Family *Meenoplidae*					
Meenoplus spp	0 (0)	1 (1)	0 (0)	2 (0)	0 (0)

** Total number of insects per species screened by dot blotting indicated. The number showing strong hybridization to the probe is shown in brackets.

6.4 DETECTION OF PHYTOPLASMA DNA IN POTENTIAL INSECT VECTORS BY PCR

The most sensitive primer pairs, that is Rohde forward / reverse, and Rohde forward/ LD SR were routinely used to screen field collected insects suspected to be potential vectors of LD. The possibility of inhibitors in insect DNA was always eliminated by including control reactions in which insect DNA was spiked with LD-infected DNA for each PCR reaction. PCR products of the same size as from infected plants were detected in 12 out of more than 5,000 insects screened using the Rohde forward/ reverse primer pair (Plate 6.1). All the insects from which rDNA was amplified are members of *Derbidae* family, and belong to either *Diastrombus mkurangai* species, (8) or *Meenoplus* spp (4).

PCR products from the insects and LD-infected coconut were digested with the restriction enzymes *Alu*I, *Taq*I and *Tru*9 *I*, and the banding patterns of restriction fragments from both sources were indistinguishable (Plate 6.2). These tests therefore established that the PCR product from insects is the same phytoplasma DNA as that detected in palms.

The use of restriction fragment length polymorphisms to confirm that the DNA amplified from insects is the same phytoplasma as in LD-infected palms (Plate 6.2), indicates that these two insect species are able to ingest phytoplasma from infected palms during feeding. This, however, does not prove that they are genuine vectors of LD. More trials will have to be conducted to establish whether they can transmit the pathogen from infected to healthy palms.

Table 6.2 The use of PCR to screen leaf sucking insects (homoptera) for phytoplasma rDNA

Insect species	Total collected	Total screened	DNA band (+)
Family *Derbidae*			
Diastrombus abdominalis Distant	7529	2191	0
Diastrombus mkurangai	3415	1270	8
Diastrombus schuilingi	776	339	0
Phenice pongwei / Paraphenice mawae	31	23	0
Robigus magawae	2	0	0
Zoraida fuligipennis	40	0	0
Amania angustifrons Melchar / *Nesodryas antiope* Fennah	222	222	0
Kamendaka kordofana Synave	12	3	0
Lydda woodi Muir	3521	684	0
Zorabana spp = Pamendanga spp.	160	87	0
S (undescribed)	5	5	0
T (undescribed)	15	15	0
Y (undescribed)	61	50	0
Z (undescribed)	4	2	0
Family *Cercopidae*			
Bandusia erythrostena	6	0	0
Family *Lophodidae*			
Elasmosceles cimicoides Spinola	236	163	0
Family *Meenoplidae*			
Meenoplus Sensu latu	29	14	4
Family *Nogodinidae*			
Diazanus spp.	249	47	0
Others (unclassified)	58	20	0

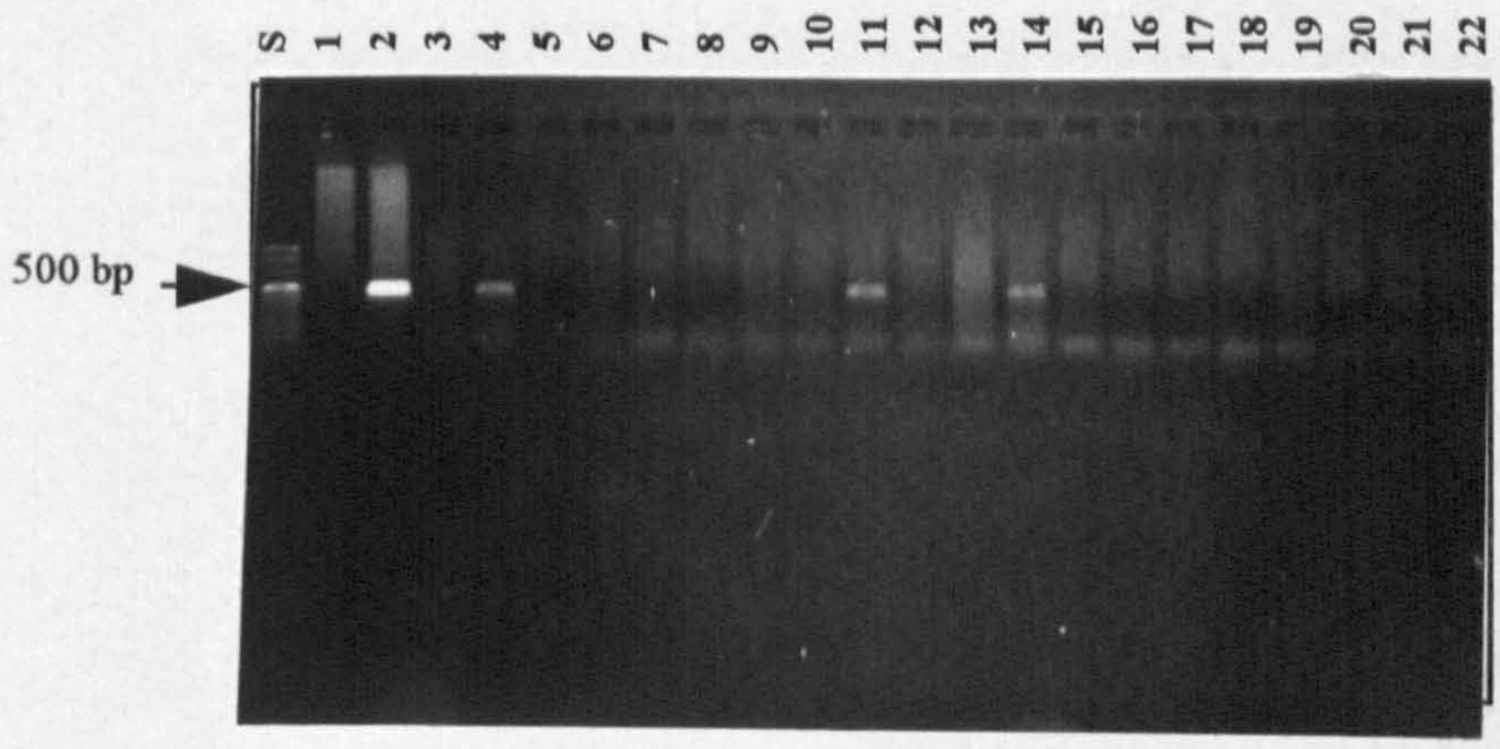


Plate 6.1: Amplification of phytoplasma rDNA from the DNA of putative insect vectors of LD. Example of amplification from DNA of *Diastrombus mkurangai*. A 500 bp band was amplified from LD DNA and several insects of this species by use of Rohde's forward and reverse primers. The samples are healthy coconut DNA (lane 1); LD DNA (2); insect DNA (3-22). Each sample of insect DNA was extracted from 3 insects. rDNA was amplified from samples 2, 4, 11, 14 and 21. S, 1 Kb DNA.

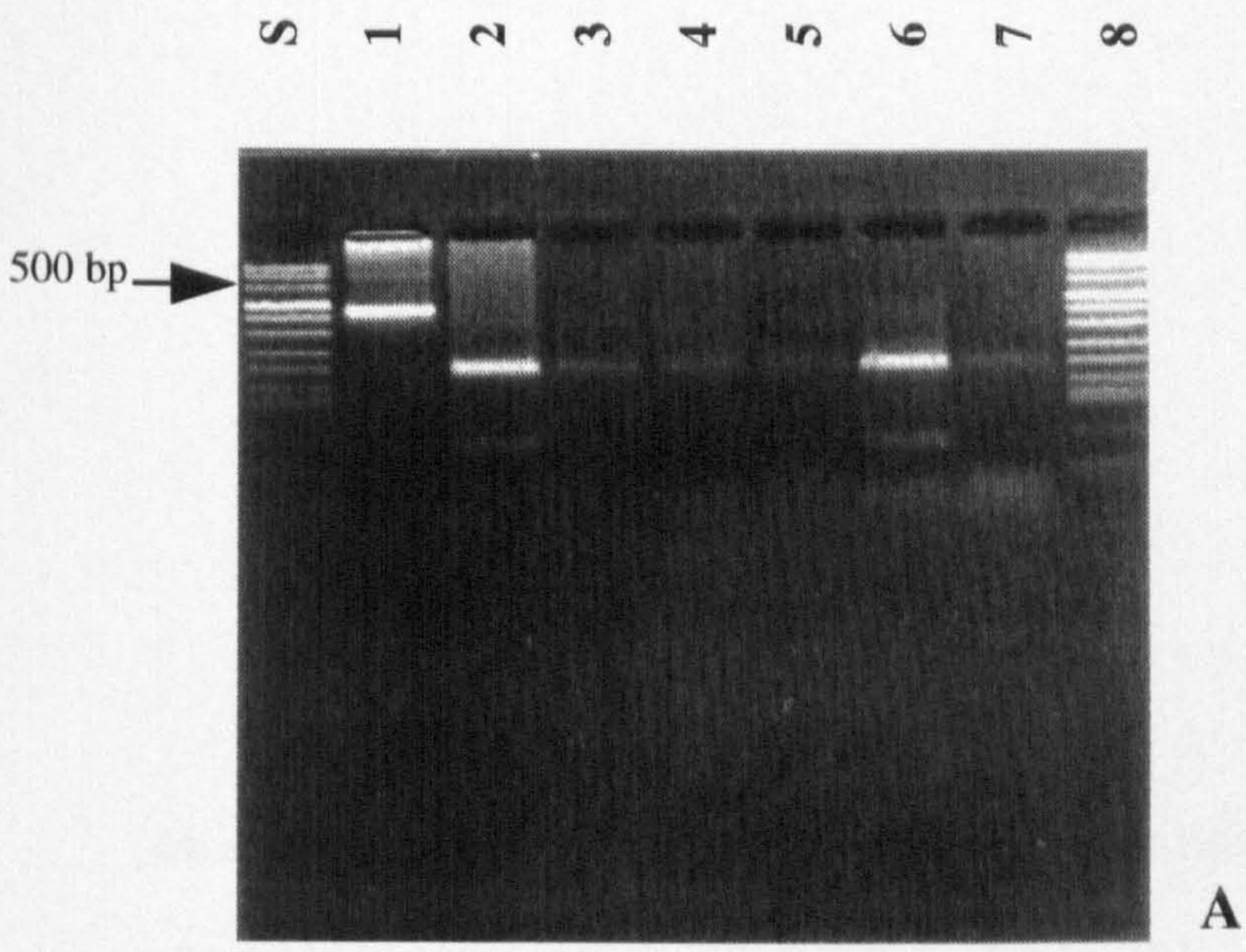


Plate 6.2: Restriction enzyme digestion of 16S rDNA amplified from LD-infected coconut DNA and DNA of putative insect vector, using *Alu* I (A) and *Tru* 9. (B) enzymes. S, 1 Kb DNA; 1, undigested LD rDNA (in A) or healthy coconut DNA (in B); 2, digested LD rDNA; 3-7, rDNA amplified from different insects. The rDNA was amplified with Rohde's primer pair.

6.5 DISCUSSION

Results from studies on the flight behaviour of auchenorrhynchous insects in LD-infected fields indicate a strong relationship between seasons and the breeding and flight of insects into the fields. The fact that peak populations were trapped near the edges of fields during the dry months, suggests that adult insects were emerging during these months and flying into the fields from outside at this time of the year. This time of the year would be the ideal time in future for surveying the vegetation surrounding disease-infected fields, in attempts to trace their alternate hosts, or breeding grounds. The different flight behaviour observed at Kifumangao may have been due to changes in the rainfall pattern during the second half of 1995. In that year, the months of August to October received more rain than normal for other years, but December was exceptionally dry. This may have triggered a different breeding behaviour for the insects, as seen by a sharp increase in numbers during the month of December, which was drier than normal. Such a relationship would suggest that the local environmental conditions per site may have a much stronger influence on vectors, and may be indirectly responsible for the differences in disease incidence that is reported in different regions of the country. It calls for intensified efforts into investigating the role of environmental factors in the epidemiology of the lethal disease with regard to their influences on palm growth and insect vectors.

The results also suggest that, although a large number of insects fly into the field from outside at certain times in the year, a large proportion of these insects do breed within the fields. Since different insects have different breeding habits, this information may be very useful in the search for the putative vectors by combining it with data from sensitive detection of phytoplasma in field collected insects. For instance, it was revealed by the data at Kifumangao (Figs. 6.9 -6.14), that one of the insect species which is a prime suspect based on results of DNA detection in insects (Plate 6.2; Table 6.2), was not trapped on short palms within the field. This implies it does not breed predominantly within the field, and that efforts to locate its alternate hosts and breeding grounds should target sources outside the field. At Chambezi the approach would be different since this insect was traced inside the fields during certain seasons.

The flight pattern of the trapped auchenorrynchous insects in general seemed to agree with the reported pattern of LD spread, showing strong gradients from the borders (Schuiling *et al.*, 1992a). This was more apparent at Chambezi than at Kifumangao, especially during the dry months of August to October (Figs. 6.1 and 6.8). However, by basing comparisons on specific insect species, utilising the evidence that these species were the prime suspects (Table 6.2), flight patterns obtained at Kifumangao provided the best fit to the pattern of disease spread. The insect species, *Diastrombus mkurangai* was trapped only on palms near the edge of the field, or on tall palms in the middle of the field. This strongly suggests that the insects fly into the field from their breeding habitats outside, and because their flight behaviour coincides with the pattern of disease spread, they are, by implication the most probable vector of LD.

Schuiling *et al.* (1992a) reported that the rate of LD spread in younger palms was much higher than on older palms. The flight pattern of the suspect species *D. mkurangai* at Kifumangao, would be contrary to expectations since more of these insects were trapped on taller, older palms. However, the traps on short palms near the edges also trapped substantial numbers, from which they could fly to other short palms nearby. The higher rate of spread may also be related to greater susceptibility of younger palms. The other suspect vector, *Meenoplus* spp. was trapped on short palms inside the field, so if it is another vector, it could be contributing to spread in younger palms.

The comparatively poor correlation obtained by regressing the disease incidence data on the numbers of trapped auchenorrhynchous insects in general, and a very good correlation when the same data was regressed on the numbers of suspected insect species only, provided additional evidence that these species are the most probable vectors of LD.

Results of dot hybridization to probes for detection of phytoplasmas in potential insect vectors, were of limited value, essentially because results did not correlate with those obtained by the more sensitive PCR test. Among the insects whose DNA showed a positive hybridization signal to probe LD 12-66 in dot blots, only one was also amplified in PCR reactions. Lack of detection in insects where the phytoplasma was detected by probes would suggest that these insects might have contained some other insect

mycoplasmas or related organisms, which are not coconut infecting phytoplasmas. An interesting observation, however, was the higher frequency of detection by probes in most insects collected at Mpiji site. At this site, the incidence of disease was very high at the time of insect sampling. Harrison (1996) reported that the LY phytoplasma could be readily ingested by a non-vector insect species, *Peregrinus maidis*, which was shown to be unable to transmit the pathogen. Considering that there were many disease affected palms at Mpiji at the time, there is a possibility that the insects randomly ingested the phytoplasmas, and that the detection signal was due to phytoplasmas. It is also possible that phytoplasma at this site was the strain that cannot be detected by the Rohde primer pair used in the analysis. This possibility will be investigated in future.

Specific primers were used to screen potential insect vectors for the presence of the LD phytoplasma, and PCR products were amplified in 8 individuals of the species *Diastrombus mkurangai* and 4 of *Meenoplus* spp, out of more than 5,000. This very low proportion of insects that tested positive for phytoplasma (0.16%) indicates that the LD phytoplasma is not readily acquired by the putative insect vectors. On the contrary, similar studies in Florida reported a comparatively high proportion (40%) of insects captured on LY-infected palms that tested positive for phytoplasma by PCR (Harrison *et al.*, 1994c). The low level of LD acquisition may be due to low pathogen titre in infected plants, which is in turn influenced by weather conditions.

Davies *et al.* (1994) reported that by application of nested PCR, detection of the pear decline phytoplasma in field captured psyllid vectors was improved from previously inconsistent results to 10% positive. The PCR reaction involved a preliminary amplification of 15 to 20 cycles using phytoplasma specific primers (Deng & Hiruki, 1991a), while the rest of the cycles utilised the pear decline specific primers. A similar approach will be adopted in future in attempt to overcome the problem of low levels of LD phytoplasma detection in insects. It will also be necessary to establish the extent by which weather conditions are influencing the process of phytoplasma acquisition by insects. Since the initial tests have narrowed the number of suspects for potential LD vectors to two species, and confirmed that these insects can acquire the LD phytoplasma, trials will also be set up in future to determine whether they can transmit the disease.

A combined approach of laboratory detection of phytoplasmas in insects, with field studies on the behavour of these insects, suggests very strongly that the insect species *D. mkurangai* and *Meenoplus spp.* are the vectors of LD. This interdisplinary approach to the study of the disease and its putative vectors may assist in vector identification and the understanding of disease epidemiology.

Chapter 7: GENERAL DISCUSSION

Lethal disease is the major threat to coconut production in Tanzania because of its destructive mode of action. The disease, which cannot be controlled by chemicals or cultural practices, is estimated to cause an annual palm loss of between 0.5 -2.0 % (Schuiling *et al.*, 1992a). If left unchecked, it could completely destroy the whole palm population in the country within 200 years. The disease has spread unchallenged during the past 36 years, mainly due to lack of resistant coconut varieties and limited knowledge about its epidemiology and insect vectors. The main factor undermining control efforts, and limiting the understanding of disease epidemiology has been lack of a quick, specific, and sensitive method of pathogen detection. An integrated approach combining breeding for disease resistance with early accurate diagnosis and identification of insect vectors could be useful for effective disease management. This thesis has investigated the potential use of molecular detection techniques for sensitive and specific detection of the lethal disease phytoplasma and their utilisation for disease diagnosis and epidemiological studies.

Two molecular techniques have been developed. Deoxyribonucleic acid (DNA) probes were developed by cloning random fragments of the lethal disease phytoplasma DNA extracted from disease infected palms (Table 3.2, Plates 3.2-3.4). As shown in Plates 3.5 , 3.6 and 4.5, these probes were screened for specificity of pathogen detection, and found to be specific for detection of LD and other phytoplasmas associated with lethal declines of coconut palms. The second technique used oligonucleotide primers for the conserved regions of the 16S rRNA gene, and for the variable spacer region between 16S and 23S rRNA genes in the polymerase chain reaction (PCR). Amplification of rDNA coded by the rRNA genes was primed fom LD-infected coconut plams, but no amplification products were obtained from healthy coconut palm tissues (Plates 5. 1 and 5.2). Some sets of primers were able to detect differences between the pathogens associated with LD and LYD in neighbouring Mozambique (Plate 5.7).

The use of cloned phytoplasma DNA sequences as molecular probes for specific and sensitive detection, and identification of phytoplasmas in their hosts has been extensively investigated and is well documented (Kirkpatrick *et al*, 1987; Davis *et al.*, 1988b; Bertaccini *et al.*, 1990b; Bonnet *et al.*, 1990; Kollar *et al.*, 1990; Harrison *et al.*, 1992; Nakashima *et al.*, 1991; Ko and Lin, 1994). Harrison *et al.* (1992) reported that hybridization analyses using probes developed from cloned random fragments of phytoplasma DNA extracted from infected palms reliably and specifically detect the LY phytoplasma in infected palm hosts. The DNA probes developed in this study, especially clones LD 12-66 and LD 19-87 were used extensively for detection of phytoplasma in LD-infected coconut palms. They were shown to detect the pathogen in all meristematic tissues of infected palms, often in as little as 7.8 ng of total palm DNA. However, background hybridization to healthy coconut DNA considerably reduced sensitivity, and was the main problem associated with the use of DIG-labelled probes (Plate 4.2). The observed problem of hybridization to healthy host DNA and low sensitivity is contrary to the other reports mentioned above. In comparable experiments with a ^{32}P labelled LY-specific probe, the LY phytoplasma was detected in 1.9 ng of total DNA from infected tissue (Harrison *et al.*, 1994b). The low sensitivity may be related to the low pathogen titre in coconut tissues and difficulties experienced in separating phytoplasma DNA from palm DNA (Plate 3.2). In the case of LY, this problem has been overcome by extracting DNA for probe production from hosts other than coconut (Harrison *et al.*, 1992, 1995). However, no alternative host for LD is known to date.

The influence of native enzyme activity in coconut tissues on the sensitivity of DIG detection procedures requires investigation. Randles *et al.* (1992) reported that phosphatase activity in coconut tissue can affect the sensitivity of DIG probes in DNA hybridization assays, and recommended that incubating the DNA in 0.5N NaOH for 2 hrs at 37 ^{0}C before blotting eliminated this problem. It is possible that this activity was also contributing to the non-specific hybridization of probes, because some probes showed this characteristic only when labelled non-radioactively with DIG. Since radioactive labelling is not a method of choice in our environment, the suggested remedies to improve on the sensitivity of non-radiactive DIG-detection will be investigated in future. Although the background hybridization signal may be considerably reduced by treating the DNA with

proteinase K during extraction according to the procedure of Davis *et al.* (1993) as shown in Plate 4.2 (lane 2), or by cross-hybridizing the labelled probe to blots of healthy coconut DNA before hybridization to the test blot (Plate 4.3), these are only short term solutions. They are also expensive and time consuming.

Another alternative approach that could fully improve the sensitivity of these probes for disease detection is to improve the quality of the cloned DNA inserts. Each of the two clones has been shown to contain more than one DNA insert (Tables 3.2 and 3.5). As shown in Plate 3.4, it appears that only one of the inserts in clone LD 19.87 was responsible for hybridization to healthy sequences. The inserts in clone LD 12-66 were very difficult to separate from vector sequences (section 3.4.2, Table 3.5). The vector sequences may also be contributing to the non-specific hybridization signal. In order to increase probe sensitivity, it will be necessary to separate the inserts from each other and from vector sequences, re-screen each separately, and sub-clone the fragments which show affinity to only phytoplasma DNA. For clone LD 12-66, this will involve double digesting the plasmid DNA with the enzyme *Eco* RI, and another enzyme that does not have a recognition sequence in the vector cloning site, but cuts the insert DNA.

Pathogen-specific DNA probes are very useful for epidemiological studies of phytoplasma diseases (Kirkpatrick *et al.*, 1990; Kuske & Kirkpatrick, 1992b; Chen *et al.*, 1992b), particularly in the monitoring of pathogen populations in infected hosts, and disease forecasting. The developed LD DNA probes provided valuable information on the distribution of LD phytoplasmas in the tissues of infected palms. Results showed high phytoplasma concentrations in the sink areas of affected palms, i.e. in the root tips, the petioles of young, unemerged leaves, the unopened inflorescences, spear leaves and the area below the growing point. There were, however, variations between tissues and different stages of disease progression as shown in Plate 4.2. These results are in agreement with reports by Thomas (1979) and Deutsch and Nienhaus (1983) that the distribution of phytoplasmas in the tissues of coconut palms infected by LY and LD respectively, is uneven. The observed pattern of LD phytoplasma distribution in infected coconut tissues is very similar to the pattern reported for the LY phytoplasma in Florida (Harrison *et al.*, 1995). However, the concentration of phytoplasma in the spear leaves of

LD infected coconut palms was found to be lower than that reported for the spear leaves of LY infected coconut. Since the DNA probes have a low detection sensitivity due to the problem of background hybridization, studies which aim at quantifying the phytoplasmas in infected tissues could produce better results by use of quantitative PCR. In this approach, a competitive PCR scheme is used to co-amplify the target DNA in the presence of known quantities of a synthetic competitive DNA which contains the same primer annealing sites as, and competes with the target DNA (Wang *et al.*, 1989; Mahuku *et al.*, 1995). The technique is capable of detection and quantification of plant pathogenic micro-organisms present in limited amounts in their hosts, and may be useful for studies like screening for resistance.

The ability to use these probes to monitor the distribution of the LD phytoplasma in different parts of the infected palms and at different stages of disease progression is promising, particularly in understanding the epidemiology of the disease. The information provided by DNA hybridization analyses, about which tissues and what stages of disease progression have the highest phytoplasma concentration, has increased the prospects for success in disease transmission trials. The possibility to synchronise the stage of disease at which phytoplasma concentration in the infected palm is at its peak, together with the tissues likely to contain the highest concentration, allows for selective acquisition feeding tests with putative vectors, and increases the probability of success in any future transmission experiments. Limited success in earlier transmission trials was partly attributed to lack of knowledge about such vital information.

When compared to DNA probes, the polymerase chain reaction (PCR) was more sensitive for LD phytoplasma detection. By use of several primers including the *Mollicute* -specific 16S rRNA primer pair, P1 and P6 (Deng and Hiruki, 1991a), primer pairs specifically based on the 16S ribosomal DNA sequences of LD phytoplasma (Rohde *et al.*, 1993, Harrison, 1994), and one based on the 16S/23S intergenic sequence of LD phytoplasma (Tymon, 1995), phytoplasma ribosomal DNA (rDNA) sequences were amplified from reaction mixtures containing LD infected palm DNA. No such bands were amplified from healthy coconut DNA. These results support the conclusions of the numerous other investigators (Ahrens and Seemuller, 1992; Deng and Hiruki, 1991; Rohde *et al.*, 1993;

Harrison *et al.*, 1994b; Lee *et al.*, 1993a), that detection of phytoplasmas by amplification of 16S rDNA sequences provides a rapid, sensitive and specific assay for reliable detection of these non-culturable plant pathogens.

Phytoplasma rDNA was amplified from tissues infected by LYD from Kenya and Mozambique. By use of the *Mollicute*-specific primers to amplify a rDNA band from LYD infected tissue but not from healthy coconut, this study has therefore confirmed the phytoplasmal aetiology of LD in Tanzania and LYD in Kenya and Mozambique. The study has also demonstrated that PCR-based assays using primer pairs designed on the basis of the 16S rRNA sequences, or a combination of these with one based on the 16S/23S spacer region (SR) sequences of the LD phytoplasma, can be employed to effectively detect its presence in infected coconut tissues. Thus, the primer LD SR, based on the variable sequences flanking the universally conserved $tRNA^{ile}$ gene in the 16S/23S spacer region, when combined with a suitable forward primer, e.g Rohde forward, can be used to specifically detect the LD phytoplasma in PCR assays.

For diseases which have a prolonged latent (incubation) phase, detection of infection prior to onset of disease symptoms is essential for effective disease management. Chen *et al.* (1992b) reported that by use of DNA probes in hybridization analyses to monitor the distribution of the pathogen in host tissues, incubating phytoplasma infections of walnut witches'-broom were detected one month before development of disease symptoms, and this helped timely enforcement of control measures. During this study, the LD phytoplasma was detected in the DNA of sampled spear leaves by DNA probes and PCR, respectively 1 and 2 months before disease symptoms developed (Tables 4.1 and 5.2). The DNA probes were again less sensitive than PCR due to the problems of background hybridization to healthy DNA. The higher PCR sensitivity is particularly useful because it enhances the programme for routine sampling and indexing of palms for phytoplasma infections whose titre is generally low.

The time before symptom development at which incubating LD infections were detected by PCR is similar to the one reported for LY in Florida (Harrison *et al.*, 1994c, 1995). However, large differences were observed in the proportions of diseased palms in which

infection was detected. Whereas phytoplasma rDNA was detected by PCR in 71% of LD infected palms, Harrison *et al.* (1994c, 1995) reported 100% detection in the DNA from spear leaves of LY infected palms by the same technique. The quality of chemicals used for DNA extraction could have contributed partly to the anomaly. The main reason, however, is most likely due to the differences observed in the distribution of phytoplasmas in the spear leaf tissues of coconuts infected by the two diseases. Dot blot hybridization analyses indicated that the concentration of phytoplasmas in the spear leaves of LD-infected coconuts was comparatively low (Plate 4.2). The root tips, however, instead found to have a much higher phytoplasma concentration. These results have important implications for the sampling scheme for indexing palms for LD phytoplasma infections. The meristematic tissue which normally has a high concentration of the pathogen cannot be sampled, because that would result in death of the palm (destructive sampling). Thus, for non-destructive sampling for LD detection, roots are recommended to be sampled together with spear leaves in order to avoid incidental false negatives. This differs from the LY phytoplasma, whose concentration in the spear leaves of infected palms was reported to be sufficiently high for reliable disease diagnosis (Harrison *et al.*, 1995).

Both dot blot analyses using DNA probes and PCR assays suggested a strong interaction between the LD pathogen, hosts, and the environment. The sensitivity of phytoplasma detection in palms which developed LD symptoms was strongly influenced by the season (weather conditions) during which they succumbed, and the type of coconut material (Tables 4.1 and 5.2). This implies that environmental conditions which affect host growth, may also affect pathogen multiplication and distribution, and hence the sensitivity of pathogen detection. Harrison, (1996) reported similar results whereby incubating LY infections were detected much earlier before onset of symptoms during the cooler winter months, and shortly before symptoms during the summer months. The influence of the host on detection sensitivity became evident in this study because the investigations were conducted on a mixture of coconut varieties with different levels of disease susceptibility. On the contrary, the LY investigations were based on a homogenous population of susceptible Atlantic tall coconut.

These results suggest the need to investigate further the role of host- pathogen-

environment interactions in the epidemiology of LD. Schaper and Converse (1985) and Schaper and Seemuller (1982) reported that the distribution of respectively the blueberry stunt and apple proliferation phytoplasmas in their host tissues were influenced by seasons. They observed that the organisms migrated to the roots during winter, and were re-distributed to the young shoots in the spring. A similar situation may be operating in the coconut palm during the wet and dry seasons, and this may be strongly linked to pathogen acquisition and spread by insect vectors. It would be useful to establish which tissues are suitable for preferential sampling during specific seasons. In the meantime, it is worthwhile to sample roots in addition to spears to increase the chances of detection, because dot hybridizations have demonstrated that the concentration of phytoplamas in the spear leaves of LD infected tissue is lower than in the root tips.

There was no rDNA band amplified from the DNA of embryos excised from LD-infected palms. This contrasts with reports from Florida, where phytoplasma rDNA was amplified from a large proportion (21%) of embryos excised from nuts on LY-infected palms (Harrison *et al.*, 1995). Inability to detect phytoplasma during this study could be due to the small size of the investigated sample. Detection of the pathogen in the embryos of seed nuts and in the young seedlings is particularly important for the exchange of potentially resistant coconut material, which would otherwise not be possible. In view of the importance of these results for exchange of germplasm for purposes of breeding, these studies will have to be intensified in future. All seedlings raised from nuts harvested from LD-infected palms tested negative for phytoplasma by PCR. These results are similar to those obtained in Florida where germinated nuts among those collected from LY infected palms also tested negative for phytoplasma infection by PCR (Harrison *et al.*, 1995). This may suggest that the phytoplasmas are not transmitted through seed. However, the few offsprings tested cannot fully justify these conclusions, and so the tests will be repeated.

The importance of detecting incubating infections for disease forecasting and crop improvement cannot be over-emphasized. Assays which ensure accurate assessment of potential infections are essential both for screening resistant breeding material, and disease management as a whole. Inability to detect the pathogen in palms which eventually developed disease symptoms, and in seednut embryos, where researchers on a similar

pathogen are able to detect it, could imply that the technique is not sufficiently sensitive for the purpose. The PCR technique is a powerful tool that should provide the desired sensitivity, provided the appropriate primers are used. Higher specificity and sensitivity of phytoplasma detection in PCR assays have been attained by exploiting sequences of cloned phytoplasma DNA as primers (Deng and Hiruki, 1991b; Schaff *et al.*, 1992; Lee *et al.*, 1993b, Harrison *et al.*, 1994b). Harrison *et al.* (1994c), reported that by using primers based on the sequences of a cloned fragment of LY phytoplasma DNA, the sensitivity of LY detection was increased 100,000 times over that of the LD-specific probe. Such a level of sensitivity has not been attained with primers based on the ribosomal RNA genes. Therefore, the alternative way to increase the sensitivity of PCR assays for LD detection is to attempt to sequence the LD-specific probes and design PCR primers based on these sequences.

Attempts to find more LD-specific primers, will have to be complemented by procedures that enhance the efficiency of PCR using the same primer pairs screened in this study. One method is a "nested PCR", which uses two sets of primers. One set of primers recognises a long sequence of the phytoplasma 16S rRNA, i.e. primers P1 and P6 (Deng and Hiruki, 1991a), are used in an initial round of PCR (about 15-20 cycles), and a second round is completed with a primer pair which recognizes regions within the previously amplified region, e.g the Rohde forward and reverse primer pair. This approach has been reported to provide an additional level of specificity and to increase amplification efficiency by minimizing nonspecific primer annealing (Haqqi *et al.*, 1988; Steffan and Atlas, 1991). This approach which has not been attempted during this study, may provide a short term solution in situations which require high detection sensitivity, like screening incubating infections in seednuts, and germinating seedlings, and for screening coconut material for disease resistance.

Pathogen-specific DNA probes are useful for differentiation of phytoplasmas (Davis *et al.*, 1993, Kuske *et al.*, 1991b) and for determination of genetic interrelatedness among phytoplasmas for their classification (Lee *et al.*, 1991; Lee *et al.*, 1992b; Lee and Davis, 1992). The LD 12-66 probe which was tested against different phytoplasmas could differentiate the LD and related coconut lethal decline phytoplasmas from those which

infect other crops (Plates 3.5 and 3.6). However, the level of genetic relatedness among the LYD phytoplasmas could not be determined. Similarly, no differences were observed between the different isolates of LD collected from regions showing differences in disease incidence. This is not surprising, because determination of genetic relatedness between closely related phytoplasma strains, requires screening the isolates against several random DNA probes of the respective phytoplasmas to determine polymorphisms (Lee *et al.*, 1991; Lee *et al.*, 1992b; Davis *et al.*, 1993; Harrison, 1996). The dot hybridization analyses only serve to detect which phytoplasmas are genetically related. This was demonstrated to be the case for LD and other LYD phytoplasmas by use of the developed LD probes (Table 3.4). Thus, it will be necessary in future to test the remaining probes against the different LYD phytoplasmas on southern blots in order to determine possible polymorphisms, and hence establish the level of genetic relatedness to the LD phytoplasma. This same procedure will determine whether there exists any strains of the LD phytoplasma in different regions of the country.

The polymerase chain reaction assays are equally useful for differentiation of, and determination of genetic interrelatedness between phytoplasmas. Pathogen-specific primers can readily detect and differentiate particular phytoplasmas from closely related strains (Harrison *et al.*, 1994b). Similarly, by use of *Mollicute*- specific primers to amplify rDNA from infected plants, followed by restriction enzyme digestion of the amplified products, polymorphisms can be detected which differentiate the phytoplasmas (Lee *et al.*, 1993a; Namba *et al.*, 1993). The present experiments have shown that primers which specifically detect LD, can be used to differentiate it from other similar but different LYD organisms. The causal agent of LYD in northern Mozambique could not be amplified with the Rohde forward/ reverse nor the Rohde forward/LD SR primer combinations, whereas all Tanzanian samples could be (Plate 5.7). The other primer combinations, however, were able to amplify bands from both Mozambique and Tanzanian samples. The Rohde forward/ reverse primers also do not amplify a rDNA band from LYD infected coconut tissue from West Africa (Tymon, 1995). Lack of amplification of rDNA from the LYD from Mozambique, led to tests which confirmed that this phytoplasma is more closely related to the West African LYD than to LD phytoplasma (Plate 5.9). There was no evidence to suggest that the phytoplasmas causing LD in Tanzania and LYD in Kenya

were different. This study has therefore demonstrated that the Rohde forward/ reverse primers are useful for differentiating the LD phytoplasma from strains of phytoplasma which are similar, but not genetically identical to it. However, these primers amplify only a small region of the 16S rRNA gene, which could not show any differences between LD isolates collected from areas that differ significantly in disease incidence when sequenced (Figure 5.1). Further attempts to detect possible strains in future will utilise primers based on the spacer region between the 16S and 23S genes, which are more variable

The Rohde forward /reverse primers are not specific to LD alone because they can amplify rDNA from LY and other unrelated phytoplasma diseases (Tymon, 1995). Despite the reported non-specificity, this primer pair was the most sensitive for LD detection among the screened primer combinations. Combining the Rohde forward primer with the LD SR primer produced specific detection, but was associated with reduced sensitivity, especially in detecting incubating infections. The implication of this is that although all the tested primers can detect LD in infected palms, there is still a need for a primer pair which combines both specificity and high sensitivity for LD detection. The sensitivity may be enhanced in future by using these primers in nested PCR in combination with the Mollicute-specific primers. The other alternative is to develop primers based on phytoplasma chromosomal DNA sequences.

Reliable detection of phytoplasmas in insect vectors has been reported, both by use of DNA probes (Kirkpatrick *et al.*, 1987; Nakashima *et al.*, 1991; Rahardja *et al.*, 1992), and PCR (Vega *et al.*, 1993; Harison *et al.*, 1995). The LD 12-66 probe that was tested for detection of phytoplasmas in putative insect vectors proved less effective, due to the low sensitivity associated with nonspecific background hybridization. The comparatively high sensitivity of PCR assays, however, was useful in the detection of insects containing the LD phytoplasma. By use of PCR to screen the DNA of putative insect vectors for the presence of the LD phytoplasma, rDNA was amplified from 8 individuals of the species *Diastrombus mkurangai* and 4 of *Meenoplus* spp, out of more than 5,000 different insects (Table 6.2, Plate 6.1). Thus, the convenience and sensitivity of the PCR technique provide a means for studying the LD-vector relationships which were not possible before.

The very low proportion of insects that tested positive for phytoplasma (0.24%) indicates that the LD phytoplasma is not readily acquired by the putative insect vectors. In Florida, a relatively high proportion (40%) of insects captured on LY-infected palms tested positive for phytoplasma by use of LY-specific primers in PCR (Harrison *et al.*, 1994c). The low level of LD detection may be due to low pathogen titre in infected plants, or comparatively low sensitivity of PCR primers. Davies *et al.* (1994) reported that detection of phytoplasmas in field captured psyllid vectors of pear decline was inconsistent, but application of nested PCR improved detection by 10%. This procedure which seems promising in overcoming the problem of low levels of LD phytoplasma detection in insects, will be tested in future. Other aspects that need to be investigated are whether phytoplasma acquisition by putative vectors is related to changes in weather conditions, and whether this has any relationship to disease incidence. Since the initial tests have narrowed the number of suspects for putative LD vectors to two species, and confirmed that these insects can acquire the LD phytoplasma, further trials will be set up to determine whether these insects are the true vectors of LD.

Studies on the fluctuations in insect populations at selected sites have indicated that the pattern of insect flight is similar to the reported pattern of disease spread, with strong gradients from the edges of the field. This was particularly true for the highly suspected insect species *Diastrombus mkurangai* at Kifumangao, suggesting that the insects come into the field from the neighbouring bushes. Since it was not possible to screen the trapped insects by PCR, the information obtained suggests that collection of insects for phytoplasmas screening in future should target palms near the edges of fields. This could provide additional data on whether they fly into the fields already infected with the phytoplasmas, and give a better indication of where to search for alternative hosts for the LD phytoplasma. The fact that these studies indicated the particular seasons during which the insects predominantly fly into the fields, should help with improving the tests in order to reduce unnecessary costs. The studies have also shown that local environmental conditions may strongly influence flight and feeding behaviour of the insects. This has important consequences for pathogen acquisition and disease spread. The trapping experiments will therefore be continued for a few more years to provide more information, and attempts will be made to relate this to the incidence of disease over the same time

span. This may help us to understand the big differences in disease incidence in the different regions which so far remain unexplained.

LD remains the most serious threat to coconut cultivation in Tanzania. The availability of rapid and sensitive methods for the detection of the LD phytoplasmas in their hosts should facilitate disease diagnosis, an understanding of the influence of the insect vectors and possible alternate hosts on disease spread, and enhance selection of resistant coconut varieties. The results presented here have paved the way for efforts to establish an integrated approach for effectively controlling the disease.

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Appendix 1

Separation of phytoplasma LD DNA from palm DNA: Fractionating the gradient columns after ultracentrifugation in CsCl-bis-benzimide gradients for one batch of LD DNA .

First run:

Tube No.	Band position	Description	Refractive index	Density (g cm^{-3})
1 &2	2.2 cm	Sharp main band at 2.2 cm from top	1.3950	1.658
	2.5 cm	Faint band immediately below	1.3975	1.6778
	2.9 cm	Very small band further down	1.401	1.72
		Fraction (1 ml) above main band	1.3920	1.619
3&4	2.2 cm	Sharp main band	1.3960	1.658
	2.5 cm	Faint band immediately below	1.3970	1.6778
	3.0 cm	Very small band further down	1.40	1.72
		Fraction (1 ml) above main band	1.3925	1.619
4&6	2.3 cm	Sharp main band	1.395	1.658
	2.5 cm	Faint band immediately below	1.395	1.658
	2.8 cm	Very small band further below	1.402	1.72
		Fraction (1 ml) above the main band	1.389	1.583
2nd Run				
1	2.9 cm	Very sharp band	1.3972	1.6778
		Fraction (1 ml) above main band	1.3950	1.658
2	2.9 cm	Very sharp band	1.3971	1.6778
	2.4 cm	Top very faint band	1.3911	1.619
3	3.0 cm	Sharp band	1.3970	1.6778

	2.3 cm	Top very faint band	1.3919	1.619
4	2.9 cm	Sharp band	1.3965	1.6778
	2.4 cm	Top very faint band	1.3911	1.616
3rd run				
1	3.0 cm	Top faint band (ca 1 mm above next)	1.3931	1.619
	3.2 cm	Lower band, also faint	1.3970	1.6778
2	2.6 cm	Top sharp band	1.3970	1.6778
	2.9 cm	Lower faint band	1.3981	1.6778
3	2.6 cm	Top sharp band	1.3970	1.6778
	2.9 cm	Lower faint band	1.3981	1.6778

A faint putative phytoplasma DNA band was separated from palm DNA after three rounds of pooling and ultracentrifugation of the fraction above the main band (tube no.1, 3rd run).

Appendix 2

Examples for determination of annealing temperature for PCR primers

1) Primer pair, Rohde forward / reverse

Sequences:

Rohde forward 5'-GAG TAC TAA GTG TCG GGG CAA-3'

Rohde reverse 5'-AAA AAC TCG CGT TTC AGC TAC-3'

Melting temperature (T_m) is calculated by allocating 4^0 to every C and G, and 2^0 to very A.

The annealing temperature is obtained by subtracting 5^0C from T_m.

Thus, T_m is:

Rohde forward $4(11) + 2(10) = 44 + 20 = 64^0$C

Rohde revrse $4(9) + 2(12) = 36 + 24 = 60^0$C

Annealing temperature:

Forward primer: $64-5 = 59^0$C

Reverse primer: $60-5 = 55^0$C

To obtain the optimum annealing temperature for this primer pair, temperatures between 55^0C and 59^0C were tested and the optimum judged depending on the PCR products amplified to be 57^0C.

2). Primer pair LD 16-1 / LD SR

Sequences:

LD 16-1 5'-CGG AAA CCT TCG GGT TTT AG-3'

LD SR 5'-GGT GCC ATA TAT ATT AGA TTG-3'

Melting temperature (T_m):

LD 16-1 $4(10) + 2(10) = 36 + 20 = 60^0C$

LD SR $4(7) + 2(14) = 24 + 24 = 56^0C$

Annealing temperature:

LD 16-1 $60-5 = 55^0C$

LD SR $56-5 = 51^0C$

Thus to obtain the optimum annealing temperature for this primer pair, reactions were set up at 52^0C, 53^0C, and 54^0C , and the optimum was 53^0C.

3) Primer pair, P1 / Rohde reverse

Sequences:

P1 5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3'

Rohde rev. 5'-AAA AAC TCG CGT TTC AGC TAC-3'

T_m:

P1 $4(11) + 2(14) = 44 + 28 = 72^0C$

Rohde rev. $4(9) + 2(12) = 36 + 24 = 60^0C$

Annealing temperature:

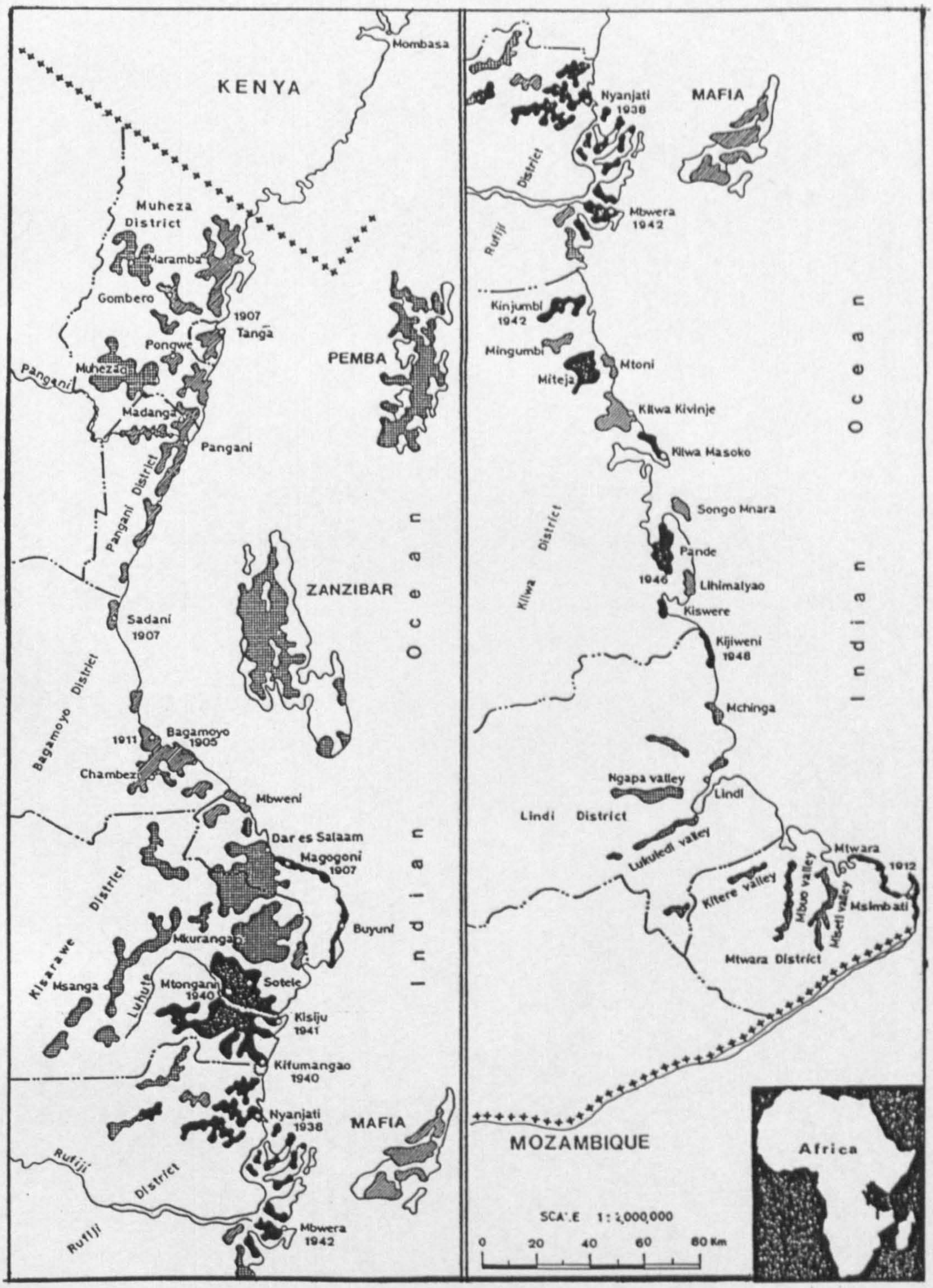
P1 $72 -5 = 67^0C$

Rohde rev. $60-5 = 55^0C$

The temperatures tested must be below the melting point of the primer with lower melting point, thus temperatures tested were 57^0C and 58^0C . The optimum annealing temperature was 58^0C.

Appendix 3

Coconut growing areas and distribution of lethal disease in the coastal districts of mainland Tanzania and the adjacent islands of Pemba, Zanzibar and Mafia



++++++ INTERNATIONAL BOUNDARIES

.. —— .. DISTRICT BOUNDARIES

RIVERS

AREAS FREE OF LETHAL DISEASE

AREAS LIGHTLY AFFECTED BY LETHAL DISEASE

AREAS HEAVILY AFFECTED BY LETHAL DISEASE

1905 YEAR OF EARLY OUTBREAK