Phytoplasma diseases of papaya (*Carica papaya* L.) in Australia: phytoplasma classification, pathology and transmission

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ABSTRACT

In Australia, phytoplasmas have consistently been associated with the papaya (*Carica papaya* L.) diseases known as papaya dieback (PpDB), yellow crinkle (PpYC) and mosaic (PpM). PpDB is the most economically important of these diseases, followed by PpYC. The investigations presented in this thesis have therefore focused primarily on PpDB.

Analysis of the DNA sequences of the 16S rRNA gene and the 16S-23S rRNA intergenic spacer region (SR) of the PpDB, PpYC and PpM phytoplasmas showed that the PpYC and PpM phytoplasma DNA sequences were identical to each other, but were distinctly different to that of the PpDB phytoplasma. A phylogenetic tree based on 16S rDNA sequences revealed that PpDB is most closely related to the Australian grapevine yellows (AGY) phytoplasma and the *Phormium* yellow leaf (PYL) phytoplasma from New Zealand, forming a distinct group within subclade xii. PpYC and PpM phytoplasmas are most closely related to the tomato big bud (TBB) phytoplasma from Australia, within subclade iii. It was proposed that the PpDB phytoplasma be included in the taxon "*Candidatus* Phytoplasma australiense", and that the PpYC and PpM phytoplasmas be assigned to a new taxon, "*Candidatus* P. australasiense".

Histological studies and mapping of phytoplasma distribution using PCR revealed that it is likely that phytoplasma cells are present in very low titre and that, while the plant appears to limit proliferation of the PpDB phytoplasma, this defence response is associated with a rapid decline of the papaya plant. Immature leaf material was sampled weekly for eight months from 60 plants in a commercial papaya plantation, to estimate the minimum time between inoculation and symptom expression of PpDB,

PpYC and PpM. The PpDB phytoplasma was detected by PCR one week prior to, or the same week as, external symptoms were first observed, while phytoplasma DNA was detected between three and eleven weeks prior to expression of PpM symptoms. Examination of lateral shoot regrowth on papaya plants that had recovered from PpDB or were cut back (ratooned) when they initially exhibited PpDB, PpYC or PpM symptoms, revealed that the PpDB phytoplasma did not persist in plants after the initial expression of symptoms. In contrast, the PpYC and PpM phytoplasmas usually persisted in the lower parts of the plant, and then infected the new lateral shoots as they developed.

Dodder (*Cuscuta australis* R. Brown) was used as a phloem bridge between papaya plants affected by PpDB, PpYC and PpM, and periwinkle (*Catharanthus roseus* G. Don) plants. "*Candidatus* P. australasiense", but not the PpDB phytoplasma, was transmitted to periwinkle. The inability to transmit the PpDB phytoplasma corresponds with the view that in papaya, this phytoplasma is likely to be present at low titre, is a highly virulent pathogen, and disrupts phloem function before external disease symptoms are observed.

Based on the results of this study it is recommended that ratooning of PpDB-affected plants and removal of PpYC- and PpM-affected plants are the best strategies currently available for the management of these diseases. Suggestions for future research and disease control strategies are discussed.

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DECLARATION

I declare that the work presented in this thesis, to the best of my knowledge and belief, is original and my own, with the exception of work presented in Chapters 3 and 4. Chapters 3 and 4 are the results of team efforts of the authors listed in the corresponding publications, of which I estimate my contribution to be approximately 25% and 33%, respectively. Chapters 2, 3 and 4 have been published respectively as the following refereed journal articles:

White, D. T., Blackall, L. L., Scott, P. T. & Walsh, K. B. (1998). Phylogenetic positions of phytoplasmas associated with dieback, yellow crinkle and mosaic diseases of papaya, and their proposed inclusion in "*Candidatus* Phytoplasma australiense" and a new taxon, "*Candidatus* Phytoplasma australasia". *International Journal of Systematic Bacteriology* **48**, 941-951.

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The material presented in this thesis has not previously been submitted in any form for a degree or diploma at this or any other university or institution of tertiary education.

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[The name "*Candidatus* Phytoplasma australasiense" as presented in this thesis is the etymologically correct form of the name "*Candidatus* Phytoplasma australasia" that was proposed in the journal article]

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To Jo, my wife and best friend…

…Thank you for being there.

CHAPTER 1

Introduction

1.1 Literature review

1.1.1 Papaya

Papaya (*Carica papaya* L.), also known in Australia as papaw or pawpaw, is produced commercially as a perennial fruit crop throughout tropical and subtropical regions of the world. It is thought to have originated in the tropical lowlands of central America, which is characterised by a warm climate with high humidity all year round (Glennie & Chapman, 1976; Purseglove, 1968; Theakston, 1976).

The papaya plant is a short-lived, fast-growing, soft-wooded dicotyledon, palm-like in appearance, reaching a height at maturity of 2-10 m (Purseglove, 1968). Usually, the mature plant consists of a single, straight, unbranched stem, 10-30 cm in diameter, and hollow in the centre. Branching of the stem can be induced by damage to the apical meristem or by cutting back the main stem. The leaves are clustered towards the apex of the stem and arranged spirally. The mature leaves are large and consist of a hollow petiole, 25-100 cm long, and a lamina, 25-75 cm across, and palmately and deeply lobed. Papaya is also characterised by the presence of laticifers (latex vessels) throughout all parts of the plant. Latex of these vessels contains the proteolytic enzymes papain and chymopapain (Purseglove, 1968).

1.1.2 Papaya in Queensland

Papaya was introduced into Queensland more than a century ago and commercial production began in Queensland more than 85 years ago (Glennie & Chapman, 1976;

MacLeod, 1995). The majority of Australian papaya production occurs in coastal districts of Queensland, with relatively minor production in northern New South Wales, the Northern Territory and Western Australia (MacLeod, 1995). There has been a notable increase in production in northern Western Australia in recent years (Richards, 2000).

Prior to the mid 1980s, as much as 90% of Queensland papaya production occurred in subtropical central and southeastern Queensland (Glennie & Chapman, 1976; MacLeod, 1995). Since the mid 1980s, there has been a major shift to increased production in northern Queensland and decreased production in central and southern Queensland, in terms of absolute output and proportions of the state total (MacLeod, 1995), such that more than 85% of Queensland production now occurs in northern Queensland (Production trends, 1999; Queensland papaya production statistics - 1997, 1998). Central Queensland papaya production is based mainly around Yarwun, the oldest established commercial production district in Queensland, and has decreased from 40% of the state total in the mid 1980s (MacLeod, 1995) to around 5% (Production trends, 1999; Queensland papaya production statistics - 1997, 1998).

The decreased production in central Queensland has been attributed to a number of factors, including a series of dry seasons coupled with inadequate supply of good quality irrigation water, and the occurrence of three diseases, known as Australian papaya dieback, yellow crinkle, and mosaic (Drew & Considine, 1995; Elder *et al.*, accepted 2001; Glennie & Chapman, 1976; MacLeod, 1995). Collectively, these diseases also have a limiting effect on production in southern Queensland (Da Costa, 1944; Drew & Considine, 1995; Glennie & Chapman, 1976; McKnight, 1949).

Dieback, yellow crinkle and mosaic have also been recorded in the Northern Territory (Condé *et al.*, 1996).

1.1.2.1 Dieback

The occurrence of papaya dieback appears to be the major factor that has limited the success and expansion of the papaya industry in central and southern Queensland (Drew & Considine, 1995; Glennie & Chapman, 1976; MacLeod, 1995). In 1976, Glennie and Chapman noted that the occurrence of dieback in tropical northern Queensland was rare and suggested that this reflected the effect of environmental conditions. Since the warm wet climate of tropical northern Queensland is similar to that of the region of origin of the papaya in central America, papaya plants in northern Queensland normally experience optimal growth and health. In contrast, the climate in subtropical central and southern Queensland is generally cooler and drier than in northern Queensland, and papaya is therefore outside of it's optimal climatic range, and thus more susceptible to poor health and disease. In recognition of more suitable growing conditions, it was suggested by Glennie and Chapman (1976) that the papaya industry should be encouraged to further develop in the wet tropics of northern Queensland. However, there have been reports in subsequent years of major outbreaks of dieback in northern Queensland (Drew & Considine, 1995). This may be a reflection of increased papaya cultivation in northern Queensland or it may indicate a progressive spread of the disease into more northern regions.

Dieback was first recorded in 1922 (Glennie & Chapman, 1976) and is the most serious papaya disease in Queensland. In the Northern Territory, papaya dieback was first recorded in 1984 (Condé *et al.*, 1996). Plant losses of up to 10% occur in all years in plantations in coastal areas of central and southern Queensland, while severe epidemics

have resulted in losses of up to 100% of trees in some plantations (Glennie & Chapman, 1976). The incidence of dieback is generally low throughout most of the year with increased incidence and outbreaks usually occurring during October and November in spring in hot dry periods following heavy rain, and during the cooler drier period from February to May in autumn (Aleemullah & Walsh, 1996; Elder *et al.*, accepted 2001; Glennie & Chapman, 1976).

Dieback is a rapid decline disease. Typical external symptoms of dieback include bunching of the inner crown leaves due to reduced petiole and internodal elongation, bending of the apical growing point to one side, necrosis of stem tissue below the growing point on the side to which the growing point bends, chlorosis and necrosis of crown leaves, followed by basipetal progression of chlorosis and necrosis of mature leaves and necrosis of the stem tissues (Aleemullah & Walsh, 1996; Glennie & Chapman, 1976; Harding, 1989). Fruit that are present either abscise while green or ripen abnormally and rot. Other characteristic symptoms include reduced latex flow (Harding & Teakle, 1988) and brown discolouration of the phloem tissues (Glennie & Chapman, 1976). The vascular discolouration is present before external symptoms appear, and begins on one side of the plant in the upper stem, but below the apex (Aleemullah & Walsh, 1996). As the disease progresses, the vascular discolouration extends acropetally toward the apex and basipetally into the roots, then around the rest of the stem (Aleemullah & Walsh, 1996; Glennie & Chapman, 1976). The time from first observed external symptoms to death of the growing point can be as little as one to four weeks, although this varies between individual plants and varies under seasonal conditions (Glennie & Chapman, 1976). An indication of the rapid progression of the disease is that the abscission process of the mature leaves is not completed, such that leaves characteristically remain attached and hanging from the stem for weeks after the

death of the upper stem. Plants can recover at any stage of symptom development, and even after death of the upper stem, growth of lateral shoots on the lower stem can allow recovery of the plant to continue viable fruit production (Aleemullah & Walsh, 1996).

1.1.2.2 Yellow crinkle

Yellow crinkle was first recorded in Queensland in 1927 (Greber, 1966). The occurrence of yellow crinkle is widespread in southeastern Queensland and sporadic in distribution (Simmonds, 1965). While incidence varies within districts, losses of up to 10% are common in southeastern Queensland, and losses of up to 30% during epidemics have been recorded in central and northern Queensland (McKnight, 1949). Da Costa (1944) noted that yellow crinkle constitutes a limiting factor in the commercial life of plantations in southeastern Queensland. Yellow crinkle is prevalent in the summer months, often observed from November though to March (Elder *et al.*, accepted 2001; Simmonds, 1965), and epidemics appear to follow periods of hot dry weather (Da Costa, 1944; McKnight, 1949; Peterson *et al.*, 1993; Simmonds, 1965).

The first noticeable symptom is usually the yellowing of mature leaves, the petioles of which bend slightly near the plant stem. Translucent areas develop near the margins and in the intervein areas of the laminas of crown leaves. As the crown leaves expand, the weakened translucent areas break apart giving a ragged, "crinkle" appearance to the leaves. The developing young crown leaves are stunted, with a reduced interveinal lamina, and the main veins recurve to give the leaves a claw-like appearance. New flowers, if produced, develop marked virescence (green colouring of floral parts) and phyllody (production of leaf-like structures instead of normal floral parts) (McKnight & Everist, 1948). Older leaves eventually abscise, leaving only stunted deformed leaves

clustered at the top of the stem. The plant may remain in this condition for months or years (Peterson *et al.*, 1993; Simmonds, 1965).

1.1.2.3 Mosaic

Mosaic is sporadic in occurrence and distribution, and since it rarely reaches epidemic proportions it is economically much less important than dieback and yellow crinkle (Peterson *et al.*, 1993; Simmonds, 1965). In the early stages of the disease, young leaves become chlorotic and stunted, and develop translucent areas around the margins. In field-grown papaya plants, it can be difficult to differentiate between mosaic and yellow crinkle in early stages of the respective diseases. However, distinguishing symptoms of mosaic are the presence of narrow, dark green water-soaked streak marks on the petioles and upper stem and reduced or absent latex flow (Simmonds, 1965). Phyllody, which is a typical symptom of yellow crinkle is not observed with mosaic-affected plants (Peterson *et al.*, 1993; Simmonds, 1965). Immature fruit on plants that have been affected by mosaic for some time, exhibit light green areas, distinct from the normal darker green, and in contrast to the adjacent darker green tissue there is a reduction in latex (Peterson *et al.*, 1993). Mosaic-affected plants also have a tendency to be stunted and produce multiple stunted side shoots (Simmonds, 1965).

1.1.3 Phytoplasmas

Phytoplasmas are phytopathogenic procaryotes that belong to the class *Mollicutes*. The members of the class *Mollicutes* are characterised by the lack of a cell wall, small cell size (approximately 60-1100 nm), small genomes (580-2200 kb) with low relative guanine plus cytosine content (23-40% G+C) (Razin *et al.*, 1998), and are trivially known as "mycoplasmas". Previously known as mycoplasma-like organisms (MLOs), due to their morphological resemblance to the animal- and human-infecting

mycoplasmas (Lee *et al.*, 2000), phytoplasmas were first reported as plant pathogens in 1967 after transmission electron microscopy (TEM) of tissues of plants suffering from the "yellows" diseases mulberry dwarf, potato witches' broom, aster yellows and paulownia witches' broom (Doi *et al.*, 1967). Prior to this, "yellows" diseases were presumed to be of viral etiology. Phytoplasmas have since been shown to be associated with diseases of several hundred plant species worldwide (McCoy *et al.*, 1989; Seemüller *et al.*, 1998a), many of which are economically important (Lee *et al.*, 2000).

Plants infected by phytoplasmas display one or more of the following external symptoms: virescence (development of green flower petals); phyllody (development of floral parts into leaf-like structures); stunting of leaves ("little-leaf") and flowers; floral gigantism ("big bud"); shortening or elongation of internodes; proliferation of axillary shoots ("witches' broom"); abnormal discolouration of leaves or shoots; and generalised decline, including stunting, yellowing of leaves and die-back of branches or stems (Lee *et al.*, 2000; McCoy *et al.*, 1989). The symptoms observed vary depending on plant species, phytoplasma species or strain, and stage of infection.

In plants, phytoplasmas are found in the sieve elements of the phloem, and are transmitted by phloem-feeding leafhoppers (Cicadellidae), planthoppers (Fulgoroidea) and psyllids (Psyllidae) (Maramorosch & Harris, 1979; Tsai, 1979). Phytoplasmas can also be spread through grafting and vegetative propagation of plant host organs that have intact phloem tissue (Lee & Davis, 1992).

1.1.3.1 Phytoplasma classification

To date, extensive attempts to isolate phytoplasma cells from host plants and insects, and culture them *in vitro*, have failed (Hayflick & Arai, 1973; Jacoli, 1981; Lee &

Davis, 1986). The inability to culture phytoplasmas *in vitro* has made their classification based on phenotypic characteristics difficult, and as well has limited reliable detection and identification, and limited pathology, pathogenicity and epidemiology investigations. Initially, diagnosis of phytoplasma infections and differentiation of phytoplasma types depended on the observation of characteristic symptoms in diseased plants, TEM of plant tissues, host range, vector specificity and transmissibility (Chen *et al.*, 1989; Kirkpatrick, 1992; Lee & Davis, 1992; Maramorosch *et al.*, 1970; McCoy *et al.*, 1989; Nienhaus & Sikora, 1979; Ploaie, 1981).

The development of serological, DNA hybridisation and polymerase chain reaction (PCR) techniques has greatly enhanced detection of phytoplasma infections as well as enabled more accurate and reliable differentiation, identification and classification of phytoplasmas (Chen *et al.*, 1989; Davis & Sinclair, 1998; Lee & Davis, 1992; Lee *et al.*, 2000; Lee *et al.*, 1998a; McCoy *et al.*, 1989; Seemüller *et al.*, 1998a). To date, the most reliable classification systems for phytoplasmas have been based on restriction fragment length polymorphism (RFLP) and phylogenetic DNA sequence analyses, of conserved genes amplified by PCR from host plant tissue. In particular, the 16S rRNA gene and adjacent 16S-23S rDNA intergenic spacer region (SR), ribosomal protein genes and the elongation factor Tu (*tuf*) gene have been characterised and analysed for the classification of many phytoplasmas (Table 1.1).

The routine method of choice for the differentiation and classification of phytoplasmas is RFLP analysis of a PCR-amplified rDNA fragment consisting of the 16S rRNA gene and in some cases including the 16S-23S rDNA SR (Seemüller *et al.*, 1998a), since the procedures are simpler and more practical than phylogenetic sequence analysis when differentiating large numbers of phytoplasmas. The classification schemes of Lee *et al.*

Table 1.1. Some key references for genotype-based classifications of phytoplasmas using dot blot (DBlot), Southern hybridisation (SHyb), RFLP, non-phylogenetic DNA sequence analysis (SeqA) or phylogenetic DNA sequence analysis (PhylA) methods.

Reference	Target gene(s)					
	Whole genome	**16S rRNA gene**	**16S-23S rRNA gene SR**	**23S rRNA gene**	**Ribosomal protein genes**	***tuf* gene**
Kuske *et al.* (1991b)	SHyb					
Lee *et al.* (1992)	SHyb					
Lim & Sears (1992)					PhylA	
Lee *et al.* (1993)		RFLP				
Namba *et al.* (1993)		PhylA				
Prince *et al.* (1993)		RFLP				
Schneider *et al.* (1993)		RFLP, SeqA				
Gundersen *et al.* (1994)		PhylA			PhylA	
Kison *et al.* (1994)	SHyb					
Schneider & Seemüller (1994)	SHyb					
Seemüller *et al.* (1994)		PhylA				
Toth *et al.* (1994)					PhylA	
Schneider *et al.* (1995a)		RFLP, PhylA	RFLP			
Schneider *et al.* (1995b)		PhylA	PhylA			
Gundersen *et al.* (1996)	SHyb	RFLP			RFLP	
R. I. Davis *et al.* (1997)	SHyb	RFLP	RFLP			
Kison *et al.* (1997)	SHyb	RFLP	RFLP			
Schneider *et al.* (1997a)						RFLP, SeqA
Tymon *et al.* (1997)	DBlot	RFLP				
Jomantiene *et al.* (1998)		RFLP, SeqA			RFLP, SeqA	
Lee *et al.* (Lee *et al.*, 1998b)		RFLP			RFLP	
Tymon *et al.* (1998)		PhylA				
Berg & Seemüller (1999)						SeqA
Schneider *et al.* (1999)		RFLP, PhylA	RFLP, SeqA			
Tran-Nguyen *et al.* (1999)		RFLP, SeqA	RFLP, PhylA			
Guo *et al.* (2000)				RFLP		
Marcone *et al.* (2000)		RFLP	RFLP			RFLP, PhylA

(1993) and Schneider *et al.* (1993), based on RFLP analyses of PCR-amplified 16S rDNA, have been expanded using results of RFLP analyses of PCR-amplified ribosomal protein genes and *tuf* gene fragments, such that 14 groups and a total of at least 46 subgroups have been recognised (Jomantiene *et al.*, 1998; Lee *et al.*, 1998b; Marcone *et al.*, 2000). Further distinctions between closely grouped phytoplasmas have been discovered by Southern hybridisation analyses of whole genomic DNA using cloned chromosomal and extrachromosomal DNA probes (Table 1.1; Kuske *et al.*, 1991a; Schneider *et al.*, 1992). These genotype-based analyses have allowed the provisional classification of phytoplasmas from Europe, North America, Asia, Australia and Africa.

Phylogenetic analysis of DNA sequences of highly conserved genes, such as the 16S rRNA gene, has become an important method to complement phenotypic characterisation for the classification and taxonomy of procaryotes (Maniloff, 1992; Weisburg *et al.*, 1989; Woese, 1987; Woese *et al.*, 1990). With the absence of reliable and extensive phenotypic characterisation of phytoplasmas, phylogenetic sequence analyses of phytoplasma genes has provided the most reliable basis for the classification and provisional taxonomy of phytoplasmas. Based on DNA sequences of the 16S rRNA genes and ribosomal protein genes, the phytoplasmas are phylogenetically distinct from the other members of the class *Mollicutes*. Phytoplasmas form a monophyletic clade, with the closest relatives belonging to the genus *Acholeplasma* (Gundersen *et al.*, 1994; Kuske & Kirkpatrick, 1992b; Namba *et al.*, 1993; Seemüller *et al.*, 1994). Among the phytoplasmas, Seemüller *et al.* (1994) identified five phylogenetic strain clusters, three of which were divided into subgroups. Schneider *et al.* (1995a) later distinguished one of these subgroups as a sixth major strain cluster. In an alternative classification, Gundersen *et al.* (1994) identified eleven subclades within five major phylogenetic groups. Two additional subclades were proposed by R. E.

Davis *et al.* (1997) and (Lee *et al.*, 1998b) within the classification scheme of Gundersen *et al.* (1994). The classification schemes of Seemüller *et al.* (1994) and Gundersen *et al.* (1994) are both commonly cited, and can be compared with each other by including representative phytoplasma strains used to establish both systems. Seemüller *et al.* (1998a) have since recognised 20 major phylogenetic groups or phytoplasma subclades. The groups identified by RFLP analyses of the 16S rRNA and ribosomal protein genes mostly correspond with the phylogenetic subclades (Lee *et al.*, 2000; Lee *et al.*, 1998b). Minor differences reflect the fact that complete sequence analysis of genes is more accurate than restriction site analysis (Seemüller *et al.*, 1998a)

Traditionally, phytoplasma strains have been named according to the plant disease with which they are associated. In light of phylogenetic analyses, Gundersen *et al.* (1994) proposed that each of the phytoplasma subclades should tentatively represent species within a phytoplasma genus. The International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of *Mollicutes* (1993; 1997) adopted the policy of basing phytoplasma taxonomy on phylogeny. Based on the guidelines of Murray and Schleifer (1994) for defining taxa of uncultivated procaryotes, the *Candidatus* Phytoplasma species "*Candidatus* P. aurantifolia" (Zreik *et al.*, 1995), "*Candidatus* P. australiense" (R. E. Davis *et al.*, 1997), "*Candidatus* P. japonicum" (Sawayanagi *et al.*, 1999) and "*Candidatus* P. fraxini" (Griffiths *et al.*, 1999) have been described.

1.1.3.2 Histopathology and within-plant distribution of phytoplasmas

Douglas (1993) noted that information on the pathological anatomy of phytoplasma diseases of plants was limited. However, general histopathological characteristics were known to include sieve tube necrosis, hyperactivity of the cambium, callose and/or starch accumulation, formation of replacement phloem and in some cases hyperplasia

and hypertrophy of phloem parenchyma cells. In many diseases of phytoplasmal etiology, such as X-disease of peach and chokecherry (Douglas, 1986) yellow sorghum stunt (Bradfute *et al.*, 1979), legume little leaf (Bowyer & Atherton, 1970) and tomato big bud (Bowyer *et al.*, 1969), phytoplasma cells are present in high numbers within sieve elements of plant tissues with visible external symptoms. Plant metabolism is evidently disturbed in the near vicinity of phytoplasma cells. Curiously, the sieve elements containing phytoplasma cells often appear normal in structure, while nearby sieve elements that lack phytoplasma cells may be degenerate (Credi, 1994; Douglas, 1993; Schneider, 1977). In other diseases involving phytoplasmas, the pathogen is present in very low titre or may not be present within tissues that display external symptoms. For example, phytoplasma cells were observed by TEM in young inflorescences of coconut palms, but not in mature inflorescences, leaves, or stems with symptoms of the lethal yellows disease (Parthasarathy, 1974). These observations of cell damage at locations distant from the site of the phytoplasma indicate the likely involvement of pathogen-produced or -induced toxins.

To better understand the interaction between phytoplasmas and their hosts, and the spatial relationship between location of phytoplasma cells and sites of disease symptom expression, various molecular techniques, such as *in situ* DNA hybridisation (Deng & Hiruki, 1991b; Lherminier *et al.*, 1999; Webb *et al.*, 1999) and *in situ* immunolabelling (Cousin *et al.*, 1989; Lherminier *et al.*, 1994; Lherminier *et al.*, 1990), have been used to specifically locate phytoplasma cells in tissue sections of plant and insect hosts. PCR (Jarausch *et al.*, 1999), DNA hybridisation (Kuske & Kirkpatrick, 1992a; Nakashima & Hayashi, 1995) and enzyme-linked immunosorbent assay (ELISA) (Lefol *et al.*, 1994; Lherminier *et al.*, 1994) analyses of tissue extracts have been used to map phytoplasma distribution and spread within plants, in relation to disease symptoms. This has

implications for tissue selection in sampling for continuous in-field monitoring for pathology and epidemiology studies (Jarausch *et al.*, 1999).

1.1.3.3 Phytoplasma epidemiology

Understanding the epidemiology of phytoplasma diseases is an important step to the development of management or control strategies. Phytoplasmas are primarily spread by phloem-feeding leafhoppers (Cicadellidae), planthoppers (Fulgoroidea) and psyllids (Psyllidae) (Tsai, 1979). For successful transmission, phytoplasma cells that are ingested by the vector insect must penetrate the midgut wall into the haemocoel, where they multiply, then cross into the salivary glands and undergo further multiplication until they reach high enough numbers in the saliva such that subsequent feeding will introduce an infective dose to the recipient plant (Fletcher *et al.*, 1998; Kirkpatrick, 1992). Insect vector transmission of phytoplasmas therefore consists of three phases: acquisition, latency or incubation, and inoculation (Purcell, 1982). It follows that the spread of phytoplasma diseases is therefore strongly linked to the feeding habits and biology of the insect vector, the condition of the host plant, the presence of alternative host plant species of the phytoplasma, and environmental conditions (Maramorosch & Harris, 1979).

If the vector is known, then knowledge of its feeding patterns and population biology may enable a certain degree of disease control by using insecticides or netting for plant protection during times of peak vector activity. The prospects for control of a particular phytoplasma disease are therefore limited if the insect vector or vectors are unknown. In such cases, the search for potential vectors, and better understanding of the epidemiology in general, would be assisted if the time of inoculation of the plant and the lag time for symptom appearance were known.

Reports of the lag or "incubation" period between inoculation, first detection of phytoplasma within the plant, and onset of visible symptoms vary according to the plant host and phytoplasma. Also, methods used to detect phytoplasma, which include the DNA stain 4,6-diamidino-2-phenylindole (DAPI) (Douglas, 1986; Schaper & Converse, 1985; Sinclair & Griffiths, 1995), DNA probes and hybridisation (Kuske & Kirkpatrick, 1992a; Nakashima & Hayashi, 1995), indirect ELISA, or immunohistochemistry (Lherminier *et al.*, 1994) are not equally sensitive, and can be expected to give different estimates of the time of first detection of phytoplasma within the plant. However, in all the above cases, phytoplasma are generally detected within the plant less than three weeks after experimental inoculation, and visible symptoms are apparent between two and eight weeks after inoculation (Chen & Lin, 1997; Douglas, 1986; Kuske & Kirkpatrick, 1992a; Lherminier *et al.*, 1994; Nakashima & Hayashi, 1995; Sinclair & Griffiths, 1995). For example, following infection of *Vicia faba* shoots by the flavescence dorée (FD) phytoplasma via a leafhopper vector, phytoplasma was first detected (by ELISA) in the roots, not the stem, 17 days after inoculation (Lherminier *et al.*, 1994). Phytoplasma cells then multiplied within the roots, and were subsequently detected in the shoot. Symptom expression in the shoot was evident at 24 to 28 days, with strong expression of symptoms evident 35 days after inoculation. This lag period was attributed to a period of translocation and multiplication of the phytoplasma within the plant (Douglas, 1986). In contrast, detection of visible symptoms of ash yellows, a phytoplasma disease causing slow decline, lagged between one and two, and up to four years behind detection of phytoplasma DNA by DAPI (Sinclair & Griffiths, 1995).

Another important epidemiological aspect is the survival or persistence of phytoplasmas in plant hosts. In apple proliferation- and pear decline-diseased trees, phytoplasma

populations of the stem and branches are eliminated during winter, due to natural degeneration of the sieve tubes, overwinter in the roots where the phloem remains functional, and reinfect the stem in spring when new sieve tubes develop (Schaper & Seemüller, 1984; Seemüller *et al.*, 1984). In contrast, when stone fruit trees (*Prunus* spp.) were experimentally infected with European stone fruit yellows (ESFY) phytoplasma, viable phytoplasma were found to persist in both the stem and roots throughout winter (Jarausch *et al.*, 1999; Seemüller *et al.*, 1998b).

1.1.3.4 Experimental transmission of phytoplasmas

Since phytoplasmas are currently unable to be cultured *in vitro*, to maintain cultures and ready sources of phytoplasmas, it is a common practice to transmit the phytoplasma of interest from the naturally infected plant host to an herbaceous experimental host (Firrao *et al.*, 1996; Kaminska & Korbin, 1999; Lherminier *et al.*, 1999; Marcone *et al.*, 1997; McCoy *et al.*, 1989) such as *Catharanthus roseus* (L.) G. Don (periwinkle). Periwinkle plants are hardy, easily maintained, of a manageable size, and cuttings are easily grafted to other periwinkle plants.

The three methods for transmitting phytoplasmas are, (i) insect vectors, typically leafhoppers or planthoppers (Bowyer *et al.*, 1969; Firrao *et al.*, 1996); (ii) dodder (*Cuscuta spp.*), a holoparasitic plant that acts as a phloem bridge through which the phytoplasmas can be transferred from one plant to another (Alivizatos, 1989; Bowyer & Atherton, 1971; Carraro *et al.*, 1991; Credi & Santucci, 1992; Dafalla & Cousin, 1988; Heintz, 1989; Marcone *et al.*, 1997); and (iii) grafting naturally infected plant stems to the experimental host (Bowyer & Atherton, 1970; Jarausch *et al.*, 1999; Kaminska & Korbin, 1999). When the natural insect vector or vectors of the phytoplasma are known, or a suitable alternative experimental insect vector species is available, then

transmission to periwinkle using an insect vector is often the preferred method (McCoy *et al.*, 1989). When the natural insect vector is unknown or an alternative vector is not available, then transmission using one or more dodder species is commonly attempted. The use of dodder species is particularly advantageous when attempting to transfer phytoplasmas between two graft-incompatible hosts, for example, between a tree species and an herbaceous species (Noordam, 1973). If the two plant species are graft-compatible, then phytoplasmas may be transmitted by grafting naturally infected plant host scions to periwinkle, or vice versa. Grafting is also most often employed for transmitting phytoplasmas to recipient plants of the same species as the donor plant (Kaminska & Korbin, 1999). Phytoplasma cultures in periwinkles are usually perpetuated by grafting infected branches to other healthy periwinkle plants.

1.1.4 The etiologies of Australian papaya dieback, yellow crinkle and mosaic

Out of papaya dieback, yellow crinkle and mosaic, dieback is the most economically important disease, particularly in central and southern Queensland (Drew & Considine, 1995; Elder *et al.*, accepted 2001; Glennie & Chapman, 1976). Since the first report of dieback in 1922, attempts to determine the etiology of dieback have included investigations focusing on nutritional and physiological disorders, and potential pathogenic organisms (Aleemullah & Walsh, 1996; Catesby, 1994; Drew & Considine, 1995; Harding, 1989; Harding & Teakle, 1993; Harding *et al.*, 1991). Alleemullah and Walsh (1996) and Harding and Teakle (1988) speculated that the vascular browning and laticifer autofluorescence symptoms might result from infection by a pathogen such as a virus or phytoplasma, even though a potential pathogen had not been observed by microscopy.

Research during 1995 focused on the search for phytoplasmas in tissues of dieback-affected plants, and using the PCR with phytoplasma-specific primers, phytoplasma DNA was consistently detected in papaya plants exhibiting dieback symptoms (Davis & Teakle, 1995; Gibb *et al.*, 1996; Liu *et al.*, 1996; White, 1995; White *et al.*, 1997). These results were the first to consistently link dieback to a pathogenic organism. This discovery has allowed more specific and directed dieback research, using phytoplasma-specific PCR as an essential detection tool.

Using dodder (*C. australis*), as a transmission vector, Greber (1966) identified the papaya yellow crinkle agent as that causing tomato big bud disease. Originally believed to be caused by viruses (Greber, 1966; McKnight, 1949; Simmonds, 1937), both tomato big bud and papaya yellow crinkle have been shown to be associated with phytoplasmas by TEM of tissues of diseased plants (Bowyer *et al.*, 1969; Gowanlock *et al.*, 1976), and by PCR detection of phytoplasma DNA (Davis & Teakle, 1995; Gibb *et al.*, 1996; Liu *et al.*, 1996; White, 1995; White *et al.*, 1997).

Australian papaya mosaic was originally thought to be caused by a virus (Simmonds, 1965); however, consistent PCR detection of phytoplasma DNA in tissues of mosaic-affected plants implicated a phytoplasma as the primary pathological agent (Davis & Teakle, 1995; Gibb *et al.*, 1996; Liu *et al.*, 1996; White, 1995; White *et al.*, 1997).

1.2 Aims and objectives of this thesis

The discovery of phytoplasmas associated with papaya dieback, yellow crinkle and mosaic has been the impetus for a range of detailed pathology and epidemiology based studies of these diseases. The work in this thesis addressed the following aspects of papaya dieback, yellow crinkle and mosaic in central Queensland:

1. The phylogenetic relationships of phytoplasmas associated with the three diseases, to each other and to other known phytoplasmas;
2. The histopathology of papaya dieback and within-plant distribution of phytoplasma cells during disease progression;
3. An estimation of the time of natural inoculation of papaya plants with phytoplasmas and the incubation or lag period to appearance of disease symptoms;
4. The survival or persistence of phytoplasmas in infected plants;
5. The transmission of phytoplasmas from diseased papaya plants to an experimental plant host for *in situ* culture.

Determination of the phylogenetic positions of phytoplasmas is important for their classification. Characterisation of the histopathology and within-plant distribution of phytoplasmas allows a better understanding of the disease mechanisms. Knowledge of the approximate time of inoculation, the lag period, and persistence of phytoplasma infections is useful for disease management, and for focusing the search for insect vectors. The ability to experimentally transmit phytoplasmas from papaya to another experimental host plant species and then to healthy papaya plants would allow confirmation of phytoplasmas as the causal organisms of the diseases, and allow more controlled and detailed investigations of the phytoplasma-plant interaction. Ultimately, the aim of the investigations in this thesis was to provide fundamental information about the diseases so that effective disease prevention, control or management practices can be developed in the future.

CHAPTER 2

Phylogenetic classification of the phytoplasmas associated with papaya dieback, yellow crinkle and mosaic

2.1 Introduction

The association of phytoplasmas with dieback and mosaic diseases of papaya has been based solely on PCR amplification of the 16S rDNA and 16S-23S SR DNA using phytoplasma-specific primers (Davis *et al.*, 1996; Gibb *et al.*, 1996; Liu *et al.*, 1996), while in the case of yellow crinkle PCR detection of phytoplasma DNA confirmed the earlier TEM observations of phytoplasma cells in yellow crinkle-affected papaya tissues (Gowanlock *et al.*, 1976). The phytoplasmal origin of PCR products from dieback-, yellow crinkle- and mosaic-affected papaya has been confirmed by RFLP and DNA sequence analyses (Gibb *et al.*, 1996; White *et al.*, 1997). The papaya yellow crinkle (PpYC) and papaya mosaic (PpM) phytoplasmas were indistinguishable from each other by RFLP analysis of the 16S rDNA-plus-SR DNA fragment (Gibb *et al.*, 1996) and by DNA sequence analysis of the 16S-23S SR and a 500 bp fragment of the 16S rRNA gene (White *et al.*, 1997). Based on these analyses, the papaya dieback (PpDB) phytoplasma was distinctly different from the PpYC and PpM phytoplasmas.

The PpYC and PpM phytoplasmas were found to have identical 16S rDNA RFLP profiles to that of the Australian tomato big bud (TBB) phytoplasma and similar to the RFLP profiles of the sweet potato little leaf (SPLL) phytoplasma (R. I. Davis *et al.*, 1997). These phytoplasmas belong to the peanut witches' broom phytoplasma group (Lee *et al.*, 1998b). The PpDB phytoplasma was found to have identical 16S rDNA

RFLP profiles to that of the Australian grapevine yellows (AGY) phytoplasma (R. I. Davis *et al.*, 1997), which belongs to the stolbur phytoplasma group (Lee *et al.*, 1998b).

In this chapter, analysis of the DNA sequences of the 16S rRNA gene and 16S-23S SR of the PpDB, PpYC and PpM phytoplasmas is reported, as are the positions of these strains in the current 16S rDNA phylogenetic classification schemes.

2.2 Materials and Methods

2.2.1 Extraction of phytoplasma DNA

Papaya plants exhibiting characteristic symptoms of PpDB, PpYC or PpM (Glennie & Chapman, 1976; Peterson *et al.*, 1993; Simmonds, 1965) were collected from a commercial plantation at Yarwun, central Queensland. Nucleic acids were extracted from the midribs of fresh, symptomatic leaves as previously described (Liu *et al.*, 1996). The dried DNA pellets were resuspended in 50 µL of sterile, Millipore®-filtered, distilled water and stored at -20°C. One extract was prepared from each of a dieback, yellow crinkle, and mosaic affected papaya plant.

2.2.2 PCR amplification

Phytoplasma-specific PCR primers P1 and P7 (Table 2.1) were used to amplify a region approximately 1800 bp in length, consisting of the 16S rRNA gene, the 16S-23S rRNA SR, and approximately 50 bp of the 5′ end of the 23S rRNA gene. Total PCR volumes were 100 µL, and contained 200 µM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim GmbH, Mannheim, Germany), 0.4 µM of each primer, 1× DNA polymerase reaction buffer (Boehringer Mannheim GmbH), 1 U *Taq* DNA polymerase (Boehringer Mannheim GmbH) and 50 to 500 ng of template DNA. Each reaction

Table 2.1. PCR amplification and sequencing primers.

Primer	Nucleotide sequence* (5′ → 3′)	Reference
P1	AAGAGTTTGATCCTGGCTCAGGATT	Deng & Hiruki (1991a)
27f	GAGTTTGATCCTGGCTCAG	Dorsch & Stackebrandt (1992)
342r	CTGCTGCSYCCCGTAG	Lane (1991)
357f	CTCCTACGGGAGGCAGCAG	Lane (1991)
519r	GWATTACCGCGGCKGCTG	Lane (1991)
530f	GTGCCAGCMGCCGCGG	Lane (1991)
787r	CTACCAGGGTATCTAAT	Stackebrandt & Charfreitag (1990)
803f	ATTAGATACCCTGGTAG	Stackebrandt & Charfreitag (1990)
907r	CCGTCAATTCMTTTRAGTTT	Lane (1991)
926f	AAACTYAAAKGAATTGACGG	Lane (1991)
1100r	GGGTTGCGCTCGTTG	Lane (1991)
1114f	GCAACGAGCGCAACCC	Lane (1991)
1392r	ACGGGCGGTGTGTRC	Lane (1991)
1492r	TACGGYTACCTTGTTACGACTT	Lane (1991)
P3	GGATGGATCACCTCCTT	Schneider *et al.* (1995b)
P7	CGTCCTTCATCGGCTCTT	Schneider *et al.* (1995b)

* M = C:A, Y = C:T, K = G:T, R = A:G, S = G:C, W = A:T; all 1:1.

mixture was covered with 50 µL of sterile mineral oil (Sigma Chemical Co., St Louis, USA). Reactions were performed in a Minicycler (M.J. Research, Watertown, MA, USA) with initial denaturation at 94°C for 2 min, followed by 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 52°C for 30 s, and extension at 72°C for 30 s, with extension in the final cycle for 2 min. Five microlitres of each PCR product was subjected to electrophoresis in a 1.0% (w/v) agarose gel, stained with ethidium bromide and observed under UV illumination. A total of four PCRs were performed for each DNA extract and the amplification products were pooled for each disease.

2.2.3 DNA sequencing

The PCR products were purified for sequencing using the Wizard™ PCR Preps spin column purification system (Promega, Madison, USA), according to the manufacturer's instructions for PCR product purification without a vacuum manifold. For each of the DNA extracts, the pooled PCR products were eluted from the mini columns with 100 µ L of sterile, Millipore®-filtered, distilled water.

Overlapping regions of both strands of the amplimers were sequenced using thirteen primers typically used for sequencing bacterial 16S rRNA genes (Table 2.1; Blackall *et al.*, 1994; Bradford *et al.*, 1996). The forward primer P3 (Table 2.1) was used in conjunction with P7 to sequence the 16S-23S rRNA SR. Direct cycle sequencing reactions were performed using the PRISM™ Ready Reaction Dyedeoxy terminator sequencing kit (Applied Biosystems, Foster City, CA, USA). Three to five microlitres of purified PCR product was used as template for each of the sequencing reactions. Reactions were performed in a Perkin Elmer model 480 thermal cycler and the thermal cycling profile was initial denaturation at 96°C for 2 min, followed by 25 cycles consisting of denaturation at 96°C for 30 s, annealing at 50°C for 15 s and extension at

60°C for 4 min. Reaction products were purified by the chloroform method described in the manufacturer's instructions for the sequencing kit, and were electrophoresed and detected using an Applied Biosystems model 373A automated DNA sequencer. The compiled 16S rDNA sequences for each phytoplasma strain were submitted to the EMBL nucleic acids database. The accession numbers for the sequences are Y10095 (PpDB), Y10097 (PpYC) and Y10096 (PpM).

2.2.4 Comparative sequence analysis

Initial sequence alignment and editing was done using the computer program SeqEd (Applied Biosystems). The overlapping sequence fragments were manually aligned against the *Escherichia coli* 16S rDNA sequences according to secondary structure (Lane, 1991), and were compiled to give the full 16S rDNA plus 16S-23S SR DNA sequence of the PCR amplimer from each of the three papaya disease DNA extracts.

Further analyses were conducted using programs available via the Australian National Genomic Information Service (ANGIS). The full 16S rDNA plus 16S-23S SR sequences were subjected to Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) analyses to search for similar sequences in the nucleic acid databases. All phytoplasma 16S rDNA sequences available in the nucleic acid databases, with a length of at least 1300 nucleotides, were used in the phylogenetic analyses (Table 2.2). *Acholeplasma palmae*, a closely related non-phytoplasma mollicute (Gundersen *et al.*, 1994; Lee *et al.*, 1998b), was used as the outgroup. The reference sequences were aligned with the PpDB, PpYC and PpM sequences using CLUSTAL W (Thompson *et al.*, 1994) and the AE2 editor (Larsen *et al.*, 1993).

Table 2.2. Phytoplasma strains, the associated diseases and accession numbers of 16S rDNA and 16S-23S spacer region DNA sequence used in this study.

Strain[a]	Associated plant disease & origin	Access. No.	Sequence[b]	Reference
PpDB	Papaya dieback - Queensland, Australia	Y10095	16S, SR	this study
AGY	Grapevine yellows - South Australia, Australia	X95706	16S, SR	Padovan *et al.* (1996)
PYL	*Phormium* yellow leaf (*rrn*B operon) - New Zealand	U43570	16S	Liefting *et al.* (1996)
PYL	*Phormium* yellow leaf - New Zealand	U43571	SR	Liefting *et al.* (1996)
STOL	Stolbur of pepper - Serbia	X76427	16S	Seemüller *et al.* (1994)
VK	Vergilbungskrankheit (grapevine yellows) – Germany	X76428	16S	Seemüller *et al.* (1994)
AAY	American aster yellows - Florida, USA	X68373	16S	Schneider *et al.* (1993)
SAY	Severe western aster yellows - California, USA	M86340	16S, SR	Kuske & Kirkpatrick (1992b)
AY1	Maryland aster yellows – Maryland, USA	L33767	16S	Gundersen *et al.* (1994)
OY	Onion yellows – Japan	D12569	16S	Namba *et al.* (1993)
RpPh	Winter oilseed rape phyllody - Czech Republic	U89378	16S, SR	Bertaccini (1998)
OAY	*Oenothera* (Michigan) aster yellows - Michigan, USA	M30790	16S	Lim & Sears (1989)
OAY	*Oenothera* (Michigan) aster yellows - Michigan, USA	-	SR	Lim & Sears (1989)
BB	Tomato big bud - Arkansas, USA	L33760	16S	Gundersen *et al.* (1994)
CCPh	Clover phyllody - Ontario, Canada	L33762	16S	Gundersen *et al.* (1994)
KV	Clover phyllody - Germany	X83870	16S	Schneider *et al.* (1997b)
ACLR	Apricot chlorotic leaf roll - Spain	X68338	16S	Schneider *et al.* (1993)
PPER	European stone fruit yellows of peach - Germany	X68374	16S	Schneider *et al.* (1993)
ESF-PCH	European stone fruit yellows of peach - Germany	U54988	SR	Smart *et al.* (1996)
ESFY	European stone fruit yellows of apricot - Czech Republic	Y11933	16S, SR	--[d]

Table 2.2 (continued)

AT	Apple proliferation - Germany	X68375	16S	Schneider *et al.* (1993)
AT	Apple proliferation - Germany	U54985	SR	Smart *et al.* (1996)
PD	Pear decline - Germany	X76425	16S	Seemüller *et al.* (1994)
PD-308	Pear decline - Germany	U54989	SR	Smart *et al.* (1996)
PYLR2	Peach yellow leaf roll - California, USA	U54990	SR	Smart *et al.* (1996)
APS	Apple proliferation - Spain	X76426	16S	Seemüller *et al.* (1994)
SPAR	Spartium witches' broom - Italy	X92869	16S, SR	Marcone *et al* (1996)
BAWB	Black alder witches' broom - Germany	X76431	16S	Seemüller *et al.* (1994)
PpYC	Papaya yellow crinkle - Queensland, Australia	Y10097	16S, SR	this study
PpM	Papaya mosaic - Queensland, Australia	Y10096	16S, SR	this study
TBB	Tomato big bud - South Aust., Australia	Y08173	16S, SR	Gibb *et al.* (1998)
PnWB	Peanut witches' broom - Taiwan	L33765	16S	Gundersen *et al.* (1994)
SPWB	Sweet potato witches' broom - Taiwan	L33770	16S	Gundersen *et al.* (1994)
SUNHP	Sunn hemp witches' broom - Thailand	X76433	16S	Seemüller *et al.* (1994)
SPLL	Sweet potato little leaf - Northern Territory, Australia	X90591	16S, SR	Padovan *et al.* (1996)
WBDL	Witches' broom disease of lime - Oman	U15442	16S, SR	Zreik *et al.* (1995)
FBP	Faba bean phyllody - Sudan	X83432	16S, SR	Schneider *et al.* (1995a)
WX	Western X-disease - California, USA	L04682	16S	Schneider *et al.* (1993)
WX	Western X-disease - California, USA	U54992	SR	Smart *et al.* (1996)
CX	Canadian peach X-disease - Ontario, Canada	L33733	16S	Gundersen *et al.* (1994)
VAC	Vaccinium witches' broom - Germany	X76430	16S	Seemüller *et al.* (1994)
TWB	Tsuwabuki witches' broom - Japan	D12580	16S	Namba *et al.* (1993)
CYE	Clover yellow edge, Ontario - Canada	L33766	16S	Gundersen *et al.* (1994)
ICPh	Clover phyllody - Italy	X77482	16S	Firrao *et al.* (1996)

Table 2.2 (continued)

SCWL	Sugar cane white leaf - Thailand	X76432	16S	Seemüller *et al.* (1994)
RYD	Rice yellow dwarf - Japan	D12581	16S	Namba *et al.* (1993)
BVK	Blütenverkleinerung (from leafhopper *Psammotettix cephalotes*) - Germany	X76429	16S	Seemüller *et al.* (1994)
CIRP	Cirsium phyllody – Germany	X83438	16S, SR	Schneider *et al.* (1997b)
PPWB	Pigeon pea witches' broom - Florida, USA	L33735	16S	Gundersen *et al.* (1994)
CPPWB	Caribbean pigeon pea witches' broom[c]	U18763	16S	--[d]
LY	Coconut lethal yellowing - Florida, USA	U18747	16S	Gundersen *et al.* (1994)
LDY	Yucatan coconut lethal decline - Mexico	U18753	16S	Tymon *et al.* (1998)
LDT	Coconut lethal disease - Tanzania	X80117	16S	Tymon *et al.* (1998)
ASHY	Ash yellows - New York, USA	X68339	16S	Schneider *et al.* (1993)
ASHY	Ash yellows - New York, USA	U54986	SR	Smart *et al.* (1996)
CP	Clover proliferation - Alberta, Canada	L33761	16S	Gundersen *et al.* (1994)
BLL	Eggplant (brinjal) little leaf - India	X83431	16S, SR	Schneider *et al.* (1995a)
BLTVA	Beet-leafhopper-transmitted virescence agent - California, USA	U54987	SR	Smart *et al.* (1996)
EY	Elm yellows - New York, USA	L33763	16S	Gundersen *et al.* (1994)
ULW	Elm yellows - France	X68376	16S	Schneider *et al.* (1993)
ULW	Elm yellows - France	U54991	SR	Smart *et al.* (1996)
FD	Flavescence dorée of grapevine - France	X76560	16S, SR	Seemüller *et al.* (1994)
LfWB	Loofah witches' broom, - Taiwan	L33764	16S	Gundersen *et al.* (1994)
A. palmae		L33734	16S	Gundersen *et al.* (1994)

[a] Strains are presented in vertical order as they appear in Fig. 2.1, or for strains listed with only SR sequences, presented under phylogenetically similar strains.

[b] 16S: 16S rRNA gene; SR: 16S-23S rRNA spacer region

[c] Geographical origin could not be determined from database record.

[d] Database record was the only available reference at the time of analysis.

Phylogenetic trees were constructed using distance (DNADIST) and maximum parsimony (DNAPARS) method programs in PHYLIP version 3.5 (Felsenstein, 1993). Nucleotide positions at which a gap occurred in any of the aligned sequences were excluded from the analysis. An evolutionary distance matrix was calculated using the Jukes and Cantor-parameter model in DNADIST, and trees were constructed using the neighbour-joining method (NEIGHBOR). To quantify relative support for branches inferred from genetic distance analyses and parsimony, "bootstrap" resampling (100 resamplings) was employed. A significance level of 95% was adopted for testing hypotheses proposed *a priori* (Felsenstein, 1985).

Evolutionary distance trees were calculated from two data sets of 16S rDNA sequences. One set included the Japanese phytoplasma strains OY, TWB, and RYD (Table 2.2), to give a total of 52 phytoplasma strains in the analyses and the comparison of 1251 nucleotide positions. A second data set excluded the Japanese phytoplasmas allowing the analysis of only 49 phytoplasma strains, but enabling the comparison of 1353 nucleotide positions. The phylogenetic tree generated from the second data set is presented in the results since it is based on more sequence information than the tree based on the first data set. Using the AE2 editor, a similarity matrix was constructed by direct pairwise comparison of all phytoplasma 16S rDNA sequences used for phylogenetic inferences. The PpDB, PpYC, and PpM 16S-23S SR DNA sequences were aligned and compared with 22 other available phytoplasma spacer region sequences (Table 2.2) using AE2. A similarity matrix was constructed as for the 16S rDNA sequences.

2.3 Results

2.3.1 DNA sequences

Almost the entire P1/P7 PCR amplimer was sequenced for each of the PpDB (1761 bp), PpYC (1799 bp), and PpM (1797 bp) phytoplasmas. Near-complete 16S rDNA sequences were obtained, except for up to 12 bp at the 5′ end of the 16S rRNA gene of all three amplimers. The 16S-23S SR DNA sequences were 208 bp in length for PpDB, and 222 bp in length for PpYC and PpM. Thirty-two base pairs of the 5′ end of the 23S rRNA gene were determined for PpDB, and 56 bp were determined for PpYC and PpM. The PpYC and PpM sequences were identical to each other.

2.3.2 Phylogenetic analysis of 16S rDNA sequences

An evolutionary distance tree was calculated from a data set that excluded the strains OY, TWB, and RYD (Fig. 2.1). This tree and that calculated from a data set that included strains OY, TWB, and RYD both exhibited branching orders similar to previously published trees (Gundersen *et al.*, 1994; Liefting *et al.*, 1996; Marcone *et al.*, 1996; Schneider *et al.*, 1995a). High bootstrap values (Fig. 2.1) supported the same major phylogenetic clusters identified by Seemüller *et al.* (1994) and Schneider *et al.* (1995a), and the phylogenetic subclades identified by Gundersen *et al.* (1994) and Lee *et al.* (1998b).

In this phylogenetic analysis (Fig. 2.1) papaya dieback phytoplasma (PpDB) was most closely related to the Australian grapevine yellows strain AGY and the New Zealand *Phormium* yellow leaf strain PYL phytoplasmas within subclade xii (R. E. Davis *et al.*, 1997), which corresponds to the stolbur subgroup of the aster yellows strain cluster, cluster I (Seemüller *et al.*, 1994). Bootstrap values of 100% (Fig. 2.1) support the PpDB/AGY/PYL subgroup as being distinct from the STOL/VK subgroup. Direct

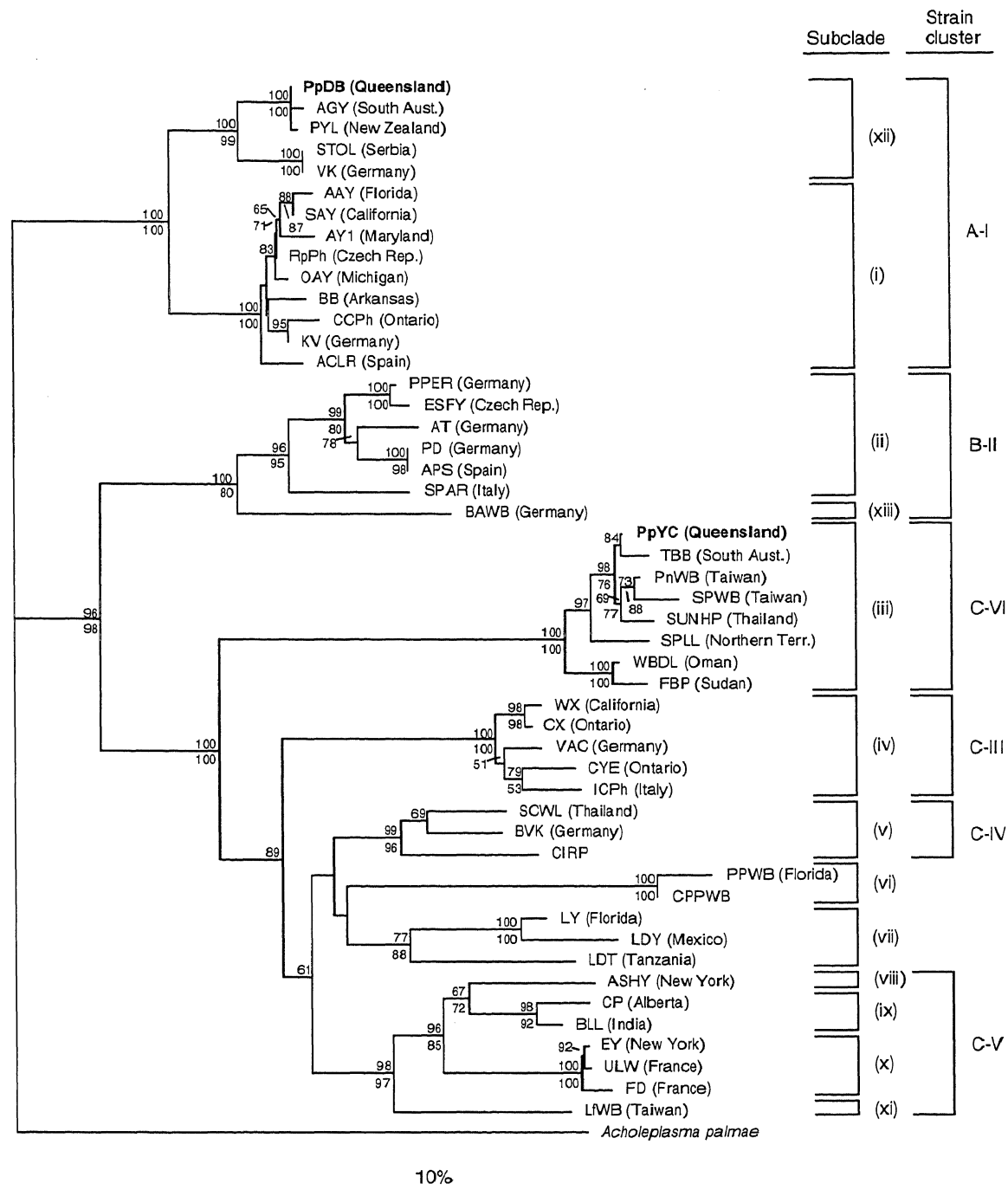


Figure 2.1. Phylogenetic distance tree of PpDB, PpYC and other phytoplasmas based on the comparative analysis of 1353 nucleotide positions of 16S rRNA gene sequences, with two phylogenetic group classification systems presented [subclades of Gundersen *et al.*, (1994) and Lee *et al.* (1998b), and strain clusters of Seemüller *et al.* (1994) and Schneider *et al.* (1995a)]. Bar represents phylogenetic distance of 10%. Names of state or country of origin are in parentheses after strain name abbreviations (as presented in Table 2.2). Bootstrap values greater than 50% (100 bootstrap resamplings) from distance (upper) and parsimony (lower) analyses are presented at the nodes. *Acholeplasma palmae* was used as the outgroup in the analyses.

pairwise comparison of the 16S rDNA sequences (Table 2.3) showed that the PpDB sequence was most similar to those of PYL (99.9%), AGY (99.7%), STOL (98.3%) from Serbia, and VK (98.3%) from Germany. Similarity of PpDB 16S rDNA sequence with those of the aster yellows strains ranged from 95.9% (AAY) to 97.3% (KV).

The PpYC and PpM phytoplasma 16S rDNA sequences were identical to each other. In the phylogenetic analysis (Fig. 2.1), PpYC was most closely related to tomato big bud strain TBB from Australia, within the peanut witches' broom subclade (subclade iii) described by Gundersen *et al.* (1994), which corresponds to the faba bean phyllody strain cluster, cluster VI (Schneider *et al.*, 1995a). PpYC, together with TBB, peanut witches' broom strain PnWB from Taiwan, sweet potato witches' broom strain SPWB from Taiwan, sunn hemp witches' broom strain SUNHP from Thailand, and sweet potato little-leaf strain SPLL from Australia, form a subgroup (distance bootstrap value: 97%) distinct from lime witches' broom disease strain WBDL from Oman and faba bean phyllody strain FBP from Sudan (bootstrap value: 100%, Fig. 2.1). Direct pairwise comparisons of sequences (Table 2.3) showed that the PpYC 16S rDNA sequence was most similar to those of TBB (99.7%), PnWB (99.7%), SUNHP (99.4%), SPWB (99.1%), and SPLL (99.1%). The PpYC sequence was 98.8% similar to the WBDL sequence and 98.6% similar to the FBP sequence, while only 92.3% similar to the sequence of strain WX in subclade iv. Direct sequence comparison also showed that the PpYC 16S rDNA sequence was 90.3% similar to the PpDB sequence.

2.3.3 16S rRNA signature sequences

Davis *et al.* (1997) described three 16S rRNA signature sequences that are unique to subclade xii phytoplasma strains and two signature sequences that are unique to the Australian grapevine yellows strain AUSGY. Although the available AUSGY 16S

Table 2.3. Matrix of direct pairwise similarities between the 16S rDNA sequences of members of phytoplasma subclades (xii), (i), (iii), and VAC from subclade (iv).

Strain	Percentage sequence similarity with strain number:																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. PpDB																							
2. PYL	99.9																						
3. AGY	99.7	99.7																					
4. STOL	98.3	98.2	98.0																				
5. VK	98.3	98.1	98.0	99.9																			
6. KV	97.3	97.1	97.0	96.9	96.8																		
7. RPH	97.1	96.9	97.0	97.0	96.9	99.6																	
8. OY	97.1	97.0	96.8	96.9	96.9	99.6	100																
9. OAY	97.1	97.0	96.8	96.8	96.8	99.5	99.9	99.9															
10. SAY	97.0	96.8	96.7	96.7	96.7	99.4	99.8	99.9	99.7														
11. CPH	96.8	96.6	96.6	96.5	96.5	99.6	99.2	99.1	99.0	98.9													
12. AY1	96.6	96.5	96.4	96.5	96.5	99.0	99.5	99.6	99.3	99.4	98.9												
13. BB	96.7	96.6	96.6	96.5	96.4	99.3	99.4	99.4	99.3	99.2	98.8	99.0											
14. ACLR	96.6	96.6	96.3	96.4	96.3	99.0	99.2	99.5	99.1	99.1	98.6	98.9	98.8										
15. AAY	95.9	96.6	95.5	95.6	95.5	98.3	98.7	99.4	98.4	98.8	97.8	98.4	98.1	98.9									
16. PpYC	90.3	89.9	89.8	90.0	90.0	90.4	90.1	90.2	90.3	90.2	89.6	89.4	90.1	90.2	89.1								
17. TBB	89.9	89.5	89.6	89.7	89.7	90.1	89.8	89.7	89.8	89.7	89.3	89.1	89.9	89.8	88.6	99.7							
18. PnWB	89.6	89.3	89.4	89.6	89.5	90.0	89.8	89.6	89.6	89.6	89.3	89.1	89.8	89.7	88.6	99.7	99.4						
19. SUNHP	89.4	89.0	89.3	89.3	89.2	89.8	89.5	89.3	89.4	89.4	89.0	88.9	89.5	89.4	88.3	99.4	99.1	99.3					
20. SPWB	89.3	89.0	89.1	89.1	89.1	89.5	89.2	89.1	89.1	89.1	89.1	88.8	89.3	89.3	88.0	99.1	98.9	99.4	98.8				
21. SPLL	90.0	89.6	89.8	89.9	89.9	90.2	90.0	89.8	89.9	89.8	89.5	89.4	90.0	89.9	88.7	99.1	98.9	98.8	98.6	98.4			
22. WBDL	90.4	90.0	90.0	90.2	90.1	90.5	90.3	90.3	90.3	90.3	89.7	89.6	90.3	90.2	89.2	98.8	98.6	98.5	98.2	97.9	98.4		
23. FBP	90.1	89.7	89.9	90.0	89.9	90.2	90.0	89.9	89.9	89.8	89.4	89.3	90.0	89.7	88.7	98.6	98.3	98.4	98.0	97.8	98.2	99.5	
24. VAC	91.1	90.8	90.7	90.4	90.4	91.3	91.0	90.7	91.2	91.0	90.6	90.4	90.9	90.9	89.9	92.5	92.1	91.9	91.9	91.5	92.1	92.2	91.6

rRNA sequence is shorter than that of Australian grapevine yellows strain AGY (Padovan *et al.*, 1996), the two sequences are identical within the equivalent regions. Since the AGY sequence is longer, it was used in the phylogenetic analysis of this study. The PpDB and PYL phytoplasma 16S rRNA sequences (this study, Liefting *et al.*, 1996) have the same signature sequences described for AUSGY, thus distinguishing PpDB, PYL and AGY from STOL and VK, within subclade xii. Gundersen *et al.* (1994) reported two 16S rRNA signature sequences that are unique to subclade iii. These signature sequences occur only in PpYC, PpM and the other strains in subclade iii (Fig. 2.1). Additionally, the following two unique sequences that distinguish PpYC, PpM and TBB from other phytoplasma strains were found: 5′-TAAAAGGCATCTTTTATC-3′ at positions 178 to 195 (numbering corresponding to 16S rRNA gene sequence of OAY, Lim & Sears, 1989) and 5′-CAAGGAAGAAAAGCAAATGGCGAACCATTTGTTT-3′ at positions 444 to 477. PnWB, SPWB and SUNHP differ from the first unique sequence by having the same single nucleotide substitution, 5′-TAAAAGGCATCTT**G**TATC-3′, and SPLL differs by an additional nucleotide substitution, 5′-TA**G**AAGGCATCTT**G**TATC-3′. SPLL contains the second unique oligonucleotide sequence, whereas PnWB, SPWB and SUNHP differ by the same single nucleotide substitution, 5′-C**G**AGG(25 nucleotides, see above)GTTT-3′.

2.3.4 Analysis of 16S-23S spacer region DNA sequences

All 25 phytoplasma sequences that were compared had a $tRNA^{Ile}$ (GAT anticodon) gene 77 bp in length. The $tRNA^{Ile}$ sequences of PpDB, PpYC, and PpM were identical to that of the OAY phytoplasma (Lim & Sears, 1989). The similarity of sequences external to the $tRNA^{Ile}$ gene in different phytoplasma strains reflected the same grouping observed in the phylogenetic tree based on the 16S rDNA sequence (data not

shown). Direct pairwise comparisons of whole spacer region sequences (Table 2.4) showed PpDB to be most similar to PYL (100%) and AGY (99.6%), while showing only about 95% identity with subclade i strains SAY, RpPh and OAY. The 16S-23S SR DNA sequences of the PpYC and PpM phytoplasmas were identical to each other. PpYC and PpM were most similar to TBB (99.6%) and SPLL (99.6%), while showing 98.9% and 98.4% identity with WBDL and FBP, respectively, and only 83.0% identity with the subclade iv strain WX. The PpYC spacer region sequence was 87.8% similar to the PpDB sequence.

2.4 Discussion

2.4.1 Phytoplasma phylogenetic classification

The phylogenetic tree presented in this chapter (Fig. 2.1) was based on all near-complete phytoplasma 16S rDNA sequences that were available on public nucleic acid databases at the time of analysis. This represents an advance on earlier phylogenetic classifications, since previous studies (R. E. Davis *et al.*, 1997; Liefting *et al.*, 1996; Marcone *et al.*, 1996; Padovan *et al.*, 1996; Schneider *et al.*, 1995a; Zreik *et al.*, 1995) included only representative strains from the subclades and strain clusters defined by Gundersen *et al.* (1994) and Seemüller *et al.* (1994). By including all available phytoplasma 16S rDNA sequences in a single evolutionary distance tree, the relationships of all strains to each other are clear, and the two current classification schemes can be directly compared (Fig. 2.1). Although the original phylogenetic trees published by Gundersen *et al.* (1994) and Seemüller *et al.* (1994) included only some strains from each of the distinct clades, the clustering of strains is sufficient to identify corresponding clades between the two systems.

Based on near-complete 16S rDNA sequences of 21 phytoplasma strains, Seemüller *et*

Table 2.4. Matrix of direct pairwise similarities between the 16S-23S spacer region DNA sequences of members of phytoplasma subclades (xii), (i), (iii), and WX from subclade (iv).

Strain	Percentage sequence similarity with strain number:										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1. PpDB											
2. PYL	100										
3. AGY	99.6	99.6									
4. SAY	95.1	94.5	94.5								
5. RPh	94.6	94.5	94.1	99.6							
6. OAY	95.0	95.0	94.5	99.6	100						
7. PpYC	87.8	87.3	87.2	85.0	83.8	83.3					
8. TBB	86.8	87.3	87.2	83.3	83.8	83.3	99.6				
9. SPLL	86.8	87.3	87.2	83.3	83.8	83.3	99.6	100			
10. WBDL	87.3	86.7	86.7	84.5	83.3	82.8	98.9	98.4	98.4		
11. FBP	86.5	86.8	86.7	83.0	83.3	82.9	98.4	98.0	98.0	99.6	
12. WX	88.3	88.2	87.7	82.7	82.8	82.8	83.0	82.5	82.4	83.4	83.0

al. (1994) originally identified the following five primary clusters: I, the aster yellows strain cluster; II, the apple proliferation strain cluster; III, the western X-disease strain cluster; IV, the sugar cane white leaf strain cluster; and V, the elm yellows strain cluster. Schneider *et al.* (1995a) later distinguished the faba bean phyllody strain cluster, cluster VI that includes strain SUNHP, previously included in cluster III (Seemüller *et al.*, 1994). Based on near-complete 16S rDNA sequences of 19 phytoplasma strains, Gundersen *et al.* (1994) also recognised five major phylogenetic groups, however, two of these groups were different to those identified by Seemüller *et al.* (1994) due to the analysis of different phytoplasma strains. The five main groups distinguished by Gundersen *et al.* (1994) were further refined into the following eleven subclades by analysis of partial 16S rDNA sequences of 30 phytoplasma strains: i, aster yellows strains; ii, apple proliferation strains; iii, peanut witches' broom strains; iv, X-disease strains; v, strain RYD; vi, strain PPWB; vii, strain LY; viii, strain ASHY; ix, clover proliferation strains; x, elm yellows strains; and xi, strain LfWB. Davis *et al.* (1997) later added strains STOL, VK, and AUSGY which formed a distinct subclade, designated subclade xii, most closely related to subclade i, the aster yellows strains. Lee *et al.* (1998b) then added strain BAWB, which could be distinguished as a new subclade, designated subclade xiii. With the addition of new strains, and further division of some of the previously defined subclades, Seemüller *et al.* (1998a) recognised a total of 20 subclades.

2.4.2 PpDB and related strains

The PpDB phytoplasma is clearly related to the AGY and PYL phytoplasmas within subclade xii. Previously, restriction endonuclease analysis of the P1/P7 amplimer from papaya dieback nucleic acid extracts revealed the similarities with AGY, STOLF (stolbur of tomato from France), and AAY (R. E. Davis *et al.*, 1997; Gibb *et al.*, 1996).

Further, sequence analysis of a 500 bp region of the 16S rRNA gene and the 16S-23S SR also revealed that the PpDB phytoplasma was closely related to STOL, VK and SAY (White *et al.*, 1997). Restriction endonuclease analysis of the PCR-amplified *tuf* gene further supported the close genetic relationship between the PpDB and AGY phytoplasmas (Padovan *et al.*, 1996). These studies also indicated that the PpDB/AGY strains are distinct from the STOL/VK strains. The results of 16S rDNA and 16S-23S SR DNA sequence analyses presented in this chapter confirm the close genetic relatedness of PpDB to AGY, and clearly demonstrate the close relationship of these phytoplasma to the PYL phytoplasma from New Zealand. Within subclade xii, PpDB, AGY and PYL form a subgroup distinct from the European strains STOL and VK (Fig. 2.1). Based on the 16S rDNA sequence data, PpDB and PYL can also be included in the 16S rDNA RFLP subgroup 16SrXII-B, with AUSGY and AGY (Lee *et al.*, 1998b).

2.4.3 PpYC, PpM and related strains

Previous restriction endonuclease analysis (Gibb *et al.*, 1996) and sequence analysis (White *et al.*, 1997) of amplified 16S rDNA and 16S-23S SR DNA revealed identity between the PpYC and PpM phytoplasmas. Gibb *et al.* (1996) and White *et al.* (1997) speculated that the same strain, or very similar strains of phytoplasmas were responsible for PpYC and PpM, and that the differences in disease symptoms may be due to differences in plant physiological and/or other pathological factors. De La Rue *et al.* (1999) employed genomic Southern blot analyses using randomly cloned DNA fragments from sweet potato little leaf variant (SPLL-V4) phytoplasma to attempt differentiation of the PpYC and PpM phytoplasmas. However, they failed to find a genetically distinct phytoplasma in exclusive association with either disease. The PpYC and PpM phytoplasmas were previously found to be similar to TBB and SPLL from Australia, and SUNHP, SEPT (sesame phyllody), CLP (*Cleome viscosa* phyllody) and

CROP (crotalaria phyllody) from Thailand, by RFLP analysis of P1-P7 amplimers (R. E. Davis *et al.*, 1997; Gibb *et al.*, 1996), and similar to PnWB, SUNHP and WBDL by sequence analysis (White *et al.*, 1997). Greber (1966) had previously demonstrated the close relationship between the PpYC and TBB agents by dodder transmission experiments.

The results presented in this chapter confirm that the PpYC and PpM phytoplasmas are most closely related to the Australian TBB and SPLL phytoplasmas, as well as the southeast Asian SUNHP, PnWB and SPWB phytoplasmas, which belong to subclade iii (Gundersen *et al.*, 1994) or strain cluster VI (Schneider *et al.*, 1995a). Within subclade iii are the FBP and WBDL phytoplasmas from Sudan and Oman, respectively. Previously, strains FBP and WBDL were each grouped only with SUNHP in separate publications (Schneider *et al.*, 1995a; Zreik *et al.*, 1995). The phylogenetic tree presented in Figure 2.1, along with pairwise comparisons of the 16S rDNA and 16S-23S SR sequences (99.5% similarity in 16S rDNA sequence and 99.6% similarity in the 16S-23S SR sequence), clearly show that strains FBP and WBDL are more closely related to each other than to the other subclade iii strains.

2.4.4 Origins of Australian phytoplasma strains

Due to the ubiquity of the TBB phytoplasma and the close relationship of TBB and SPLL phytoplasmas to other subclade iii strains occurring only in southern Asia, Davis *et al.* (1997) hypothesised an Australasian origin of TBB and SPLL strains. WBDL and the strains represented by FBP (Schneider *et al.*, 1995a) form a distinct subgroup within subclade iii, and have a recorded geographical distribution extending from Thailand in southeast Asia to Sudan in northeast Africa. Based on this distinct geographical distribution, it seems very likely that the subclade iii phytoplasmas originated and

evolved in southern Asia, with an apparent evolutionary and geographical divergence to form the southwest Asian strains (FBP and WBDL) and the southeast Asian/Australasian strains.

In Australia, the AGY-type phytoplasma has been detected in grapevines (*Vitis vinifera*), papaya, strawberry plants (*Frageria* × *ananassa*) (Padovan *et al.*, 2000), and a garden bean (*Phaseolus vulgaris*) in Western Australia (Schneider *et al.*, 1999). In New Zealand, the closely related PYL–type phytoplasma has been detected in New Zealand flax (*Phormium tenax*) (M. T. Andersen *et al.*, 1998), strawberry plants (M.T. Andersen *et al.*, 1998), and New Zealand cabbage tree (*Cordyline australis*) (Andersen *et al.*, 2001). While PYL is almost indistinguishable from the Australian AGY-type strains based on 16S rRNA gene analysis, PYL can be differentiated from the AGY-type strains using analysis of *tuf* gene sequences (Liefting *et al.*, 1998; Padovan *et al.*, 2000). The next most closely related strains, the STOL and VK, have only been reported in Europe. Although there is obviously a close evolutionary and geographic relationship between the Australian and New Zealand subclade xii phytoplasmas, currently, there is no explanation for the apparent close relatedness yet distinct geographical separation of the AGY-type and STOL-type strains. Although, at present, it seems that the AGY-type and PYL-type strains are endemic to Australia and New Zealand, their actual origin, and evolutionary relationship with the STOL-type strains, can only be speculated until they are detected in more plant hosts and or insect vector species.

2.4.5 Phytoplasma taxa

Since the proposal to use the name "phytoplasma" for the plant-pathogenic mycoplasma-like organisms (International Committee on Systematic Bacteriology, 1993; Sears & Kirkpatrick, 1994), there has been increasing support for recognising the

phytoplasmas as a distinct genus (R. E. Davis *et al.*, 1997; Gundersen *et al.*, 1994; Zreik *et al.*, 1995). Also, Gundersen *et al.* (1994) proposed that each phylogenetically distinct subclade should represent at least distinct species.

Davis *et al.* (1997) defined the provisional taxon "*Candidatus* Phytoplasma australiense" based on 16S rRNA signature sequences of AUSGY, which have since been found in strains PpDB, AGY and PYL, thus supporting a distinct group of closely related strains. Due to their close genetic relationship, revealed in the work presented in this thesis, and distinct geographical range, it is proposed that strains PpDB, AGY and PYL be included in the taxon "*Candidatus* Phytoplasma australiense". After independent investigations, Liefting *et al.* (1998) also supported this grouping.

Zreik *et al.* (1995) proposed the taxon "*Candidatus* Phytoplasma aurantifolia" based on the WBDL strain 16S rDNA sequence. Phylogenetic analysis and direct pairwise sequence comparison in the present study has shown that strain FBP and strain WBDL are more closely related to each other than to any other characterised strains. The 16S rRNA gene oligonucleotide sequence listed by Zreik *et al.* (1995) to define strain WBDL as "*Candidatus* Phytoplasma aurantifolia" differs from the corresponding sequence of other subclade (iii) phytoplasma strains by two nucleotide substitutions and differs from that of FBP by a single nucleotide substitution. Despite this single nucleotide difference in the definitive oligonucleotide, the current phylogenetic study statistically supports (100% bootstrap, Fig. 2.1) the inclusion of FBP and WBDL in a taxon distinct from the other subclade iii strains. Thus, it is suggested that strain FBP is sufficiently similar to WBDL to provisionally be included in the taxon "*Candidatus* Phytoplasma aurantifolia".

Based on the guidelines of Murray and Schleifer (1994), it is proposed that the PpYC, PpM and TBB phytoplasmas be assigned to a new *Candidatus* species with the following description: "*Candidatus* Phytoplasma australasiense" [(*Mollicutes*) NC; NA; O; NAS (EMBL Y10097), oligonucleotide sequences of unique regions of the 16S rRNA gene 5′-TAAAAGGCATCTTTTATC-3′, and 5′-CAAGGAAGAAAAGCAAATGGCGAACCATTTGTTT-3′; P (*Lycopersicon esculentum, Carica papaya*, phloem); M]. Although strains PnWB, SpWB, SUNHP and SPLL from subclade iii have minor variations in sequence regions that are unique to strains in "*Candidatus* Phytoplasma australasiense" (TBB, PpYC and PpM), it is suggested that they be provisionally included in this taxon because of the close phylogenetic relationships of all these strains (Fig. 2.1) and their distinct geographic range from south east Asia to Australia.

As an economically important group of mollicutes, efforts to 'facilitate reference to (each) unique phytoplasma lineage' (R. E. Davis *et al.*, 1997) by describing putative taxa, despite the inability to culture these organisms, should be supported. Future taxonomic definitions that are based primarily on nucleic acid sequence information should ideally be based on more than one conserved gene. Sequence analysis of conserved phytoplasma genes, in addition to the 16S rRNA gene, is likely to reveal more clearly the relationships between those strains that have been provisionally placed in the discussed *Candidatus* species. Geographic and host range should also be considered as important criteria. Fortunately, due to their obligate parasitic nature, it is likely that phytoplasma genetic diversity will reflect the biogeography of their hosts. Finally, for practical reasons, the taxonomic system may be weighted with taxa for which there is a need to refer to distinct pathogens of cultivated plants. For example, two geographically isolated strains of economic importance, with greater than 99%

sequence similarity or significant phylogenetic confidence (bootstrap) values, may be distinguished as separate species, while two co-located strains, or strains of no economic significance and with a lesser degree of sequence similarity, may remain grouped within a single species.

CHAPTER 3

Histopathology of papaya dieback and within-plant distribution of the associated phytoplasma

3.1 Introduction

Little work has been published on the anatomical pathology of the phytoplasma diseases of papaya. The infectious agent involved in the yellow crinkle disease was demonstrated to be phloem mobile using dodder transmission experiments (Greber, 1966), and phytoplasma cells were observed by TEM in minor leaf veins of symptomatic leaves (Gowanlock *et al.*, 1976). In contrast, phytoplasma cells have not been visualised by TEM in mosaic-affected tissues (D. H. Gowanlock, *personal communications*).

Molecular studies (PCR and RFLP) subsequently detected phytoplasma in both yellow crinkle- and mosaic-affected tissues (R. I. Davis *et al.*, 1997; Gibb *et al.*, 1996; Liu *et al.*, 1996; White *et al.*, 1997). These later studies were not able to distinguish between the phytoplasmas associated with these two diseases, although external symptoms and the titre of phytoplasma, as indicated by TEM studies, are different for the two diseases. Although De La Rue *et al.* (1999) failed to find a genetically distinct phytoplasma exclusively associated with either disease, they did find the TBB-type and SPLL-V4-type phytoplasmas associated with different mosaic-affected papaya plants, and the TBB-, SPLL-V4- and cactus witches' broom (CWB) -type phytoplasmas associated with different yellow crinkle-affected papaya plants. This demonstrated that these different strains of phytoplasmas could likely cause the same or very similar symptoms

in papaya. Further, the mechanisms by which plant physiology and metabolism are disturbed have not been elucidated for either PpYC or PpM diseases.

Attempts to visualise phytoplasma cells or any other etiological agent within dieback-affected papaya tissues, using TEM, have been unsuccessful (Harding & Teakle, 1988, D. H. Gowanlock, personal communications). Phytoplasmas have been associated with dieback by phytoplasma-specific PCR and subsequent RFLP, and DNA sequence analyses of the PCR products (R. I. Davis *et al.*, 1997; Gibb *et al.*, 1996; Liu *et al.*, 1996; White *et al.*, 1997). However, without demonstrated transmission of the phytoplasma, proof of a causative role is still lacking.

In addition to the visible external symptoms (Section 1.1.2.1), plants with dieback also exhibit a brown discolouration of the vascular tissues, although not all plants with this internal symptom will develop external symptoms (Aleemullah & Walsh, 1996). The browning is typically most pronounced on the side of the plant that develops the external symptoms first; that is, the side to which the stem apex bends (Aleemullah & Walsh, 1996). This discolouration represents a browning of the laticifer contents, which autofluoresce under blue and ultraviolet excitation (Aleemullah & Walsh, 1996; Harding & Teakle, 1988). Harding and Teakle (1988) also reported the presence of necrotic laticifer and phloem cells in tissue affected by dieback.

In the work from this chapter, the anatomical observations of Harding and Teakle (1988) and Aleemullah and Walsh (1996) on the histopathology of the dieback disease of papaya were extended using bright field microscopy and TEM. PCR was employed to map the distribution of phytoplasmas throughout the plant body at progressive stages of symptom development, with the aim of better understanding the role and affects of

the phytoplasma. The PCR primer AGY2 was designed for the specific detection of the AGY and PpDB phytoplasmas (Gibb *et al.*, 1998), which belong to the taxon "*Candidatus* Phytoplasma australiense", and was used for specific detection of the PpDB phytoplasma in the investigations presented in this chapter.

3.2 Materials and Methods

3.2.1 Plant material: TEM study

Plant materials were collected from a commercial plantation at Yarwun in central Queensland, Australia. The presence of phytoplasmas in plants exhibiting external dieback or yellow crinkle symptoms was confirmed by PCR testing. Vascular tissues from the following plant parts were examined by TEM:

(i) an asymptomatic plant: stem, secondary, and tertiary veins of a mature leaf (2 blocks)

(ii) plants displaying dieback symptoms, and from tissues where an adjacent hand-cut section demonstrated laticifer browning and autofluorescence: petiole (3 blocks), stem (3 blocks), fruit peduncle (3 blocks), fruit mesocarp (3 blocks), and primary, secondary, and tertiary veins within immature (expanding) leaves (11 blocks), mature leaves with general yellowing (20 blocks), and mature leaves with veins having a water-soaked appearance ("X-Y" patterning, as described by Glennie & Chapman, 1976) (19 blocks; tissue samples from 9 plants)

(iii) one of the plants displaying dieback symptoms in (ii): petiole and stem adjacent to tissues free of the symptom of laticifer browning and autofluorescence within hand cut sections (2 blocks)

(iv) plants displaying symptoms of yellow crinkle: primary, secondary, and tertiary veins of mature leaves (9 blocks from 3 plants).

Yellow crinkle tissue was included as a control for recognition of phytoplasma and sieve elements, given the previous report of TEM observation of phytoplasma within sieve elements (Gowanlock *et al.*, 1976).

3.2.2 Plant material: Phytoplasma distribution study

Observations on laticifer browning and autofluorescence and the presence of phytoplasma DNA (as determined by PCR testing) in leaf, petiole, fruit, stem, and root tissues were undertaken of three dieback plants collected from the Yarwun plantation and a small plot in north Rockhampton. The first plant was in a very early stage of dieback, as judged by external symptoms (some chlorosis and minor tip bending). The second plant was in a late stage of dieback, as evidenced by the dead crown region and necrotic leaves. The third plant was recovering from a dieback infection, with new leaves and flower buds growing adjacent to the dead crown region. In a second part of this study, the distribution of phytoplasmas within three dieback-affected plants growing in a small papaya planting in north Rockhampton was examined.

Leaf samples consisted of portions of the ends of leaf lobes with midribs up to 2.5 cm in length. Stem samples were collected using a 0.5 cm-diameter cork borer. Tissues were sampled on the same day from plants that appeared to have a bending of the stem tip but were otherwise asymptomatic. Tissues were sampled again after a further 4 and 26 days, respectively. All samples were assessed for autofluorescence of the laticifers, and were screened for the presence of phytoplasma DNA using a "*Candidatus* P. australiense"-specific PCR assay.

3.2.3 Microscopy

Hand sections of fresh plant material were examined by epifluorescence microscopy to detect laticifer autofluorescence (Aleemullah & Walsh, 1996). Tissue adjacent to that sectioned for epifluorescence microscopy was sliced into 2 mm cubed to 4 mm cubed pieces and fixed overnight at 4°C in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) with 150 mM sucrose and 2 mM calcium chloride. After fixation, tissue was washed with the same buffer three times, then post-fixed in 1% osmium tetroxide in cacodylate buffer overnight. Tissue was then washed three times with the same buffer, passed through an ethanol series, and embedded in Spurr's embedding medium. Thick and thin sections were cut from each sample for light and electron microscopy, respectively. Thick sections were stained with toluidine blue. Thin sections were stained with lead citrate and uranyl acetate before observation under a J.E.O.L. 101 transmission electron microscope.

3.2.4 DNA extraction and PCR

DNA was extracted from papaya leaf and stem tissue samples using the cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1990), scaled down so that, for each preparation, approximately 0.2 g of plant tissue was ground in CTAB isolation buffer in a 1.5 mL microcentrifuge tube using a minipestle.

Total PCR volumes were 25 µl containing 1× DNA polymerase reaction buffer (Boehringer Mannheim GmbH, Mannheim, Germany), 200 µM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim GmbH), 0.4 µM of each primer, 4 ng bovine serum albumin (BSA; Sigma Chemical Co., St Louis), 1 U Red Hot DNA polymerase (Advanced Biotechnologies Ltd., Surrey, UK), and 50 to 100 ng template DNA. The phytoplasma-specific primers P1 and P7 (Table 2.1) were used to amplify an

approximately 1800-bp fragment in a region encompassing the 16S rRNA gene plus the 16S-23S SR. A phytoplasma-specific primer, fU5, (Lorenz *et al.*, 1995) and a primer specific for the "*Candidatus* P. australiense" phytoplasmas, AGY2 (Gibb *et al.*, 1998), were used to amplify a section of the 16S rRNA gene plus the 16S-23S SR, approximately 1300 bp in length. PCRs were performed in a Minicycler (M. J. Research, Watertown, MA, USA). The reaction mixture without BSA and DNA polymerase was initially heated to 95°C for 5 min. Following addition of BSA and DNA polymerase, 35 cycles were performed, consisting of denaturation at 94°C for 1 min, annealing at 55°C for P1 and P7 or 53°C for fU5 and AGY2 for 1 min, extension at 72°C for 1.5 min, with an extension step at 72°C for 5 min for the final cycle. PCR products were analysed by electrophoresis through a 1% (w/v) agarose gel in 1× Tris-acetate-EDTA (TAE) buffer (Sambrook *et al.*, 1989), stained with ethidium bromide.

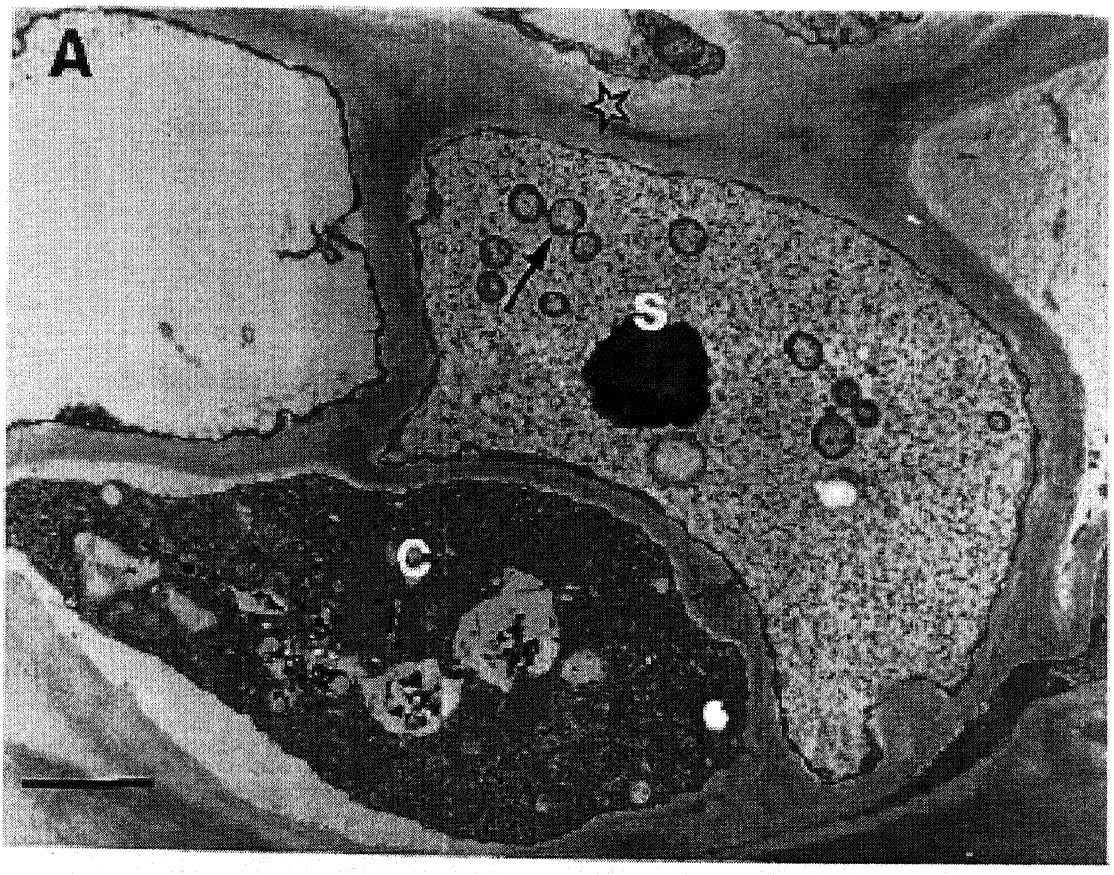
3.3 Results

3.3.1 Visualisation of phytoplasma in yellow crinkle-affected tissue

Phytoplasma cells were observed in the cytoplasm of mature sieve cells within phloem of leaf material displaying symptoms typical of the disease yellow crinkle (Fig. 3.1A). The PpYC phytoplasmas were round in shape and up to 500 nm in diameter. These phytoplasma cells were characterised by a single unit membrane surrounding ribosome-like granules, and a central nucleoplasmic net of fibrils (presumably DNA, Fig. 3.1B).

3.3.2 Anatomy of dieback-affected tissue

Other than the internal symptom of laticifer browning, no anatomical changes were observed in the dieback-affected tissues examined using bright field microscopy (in leaf, Fig. 3.2), compared with corresponding tissues from healthy plants. The areas of tissue necrosis that characterise dieback were anticipated to demonstrate loss of cellular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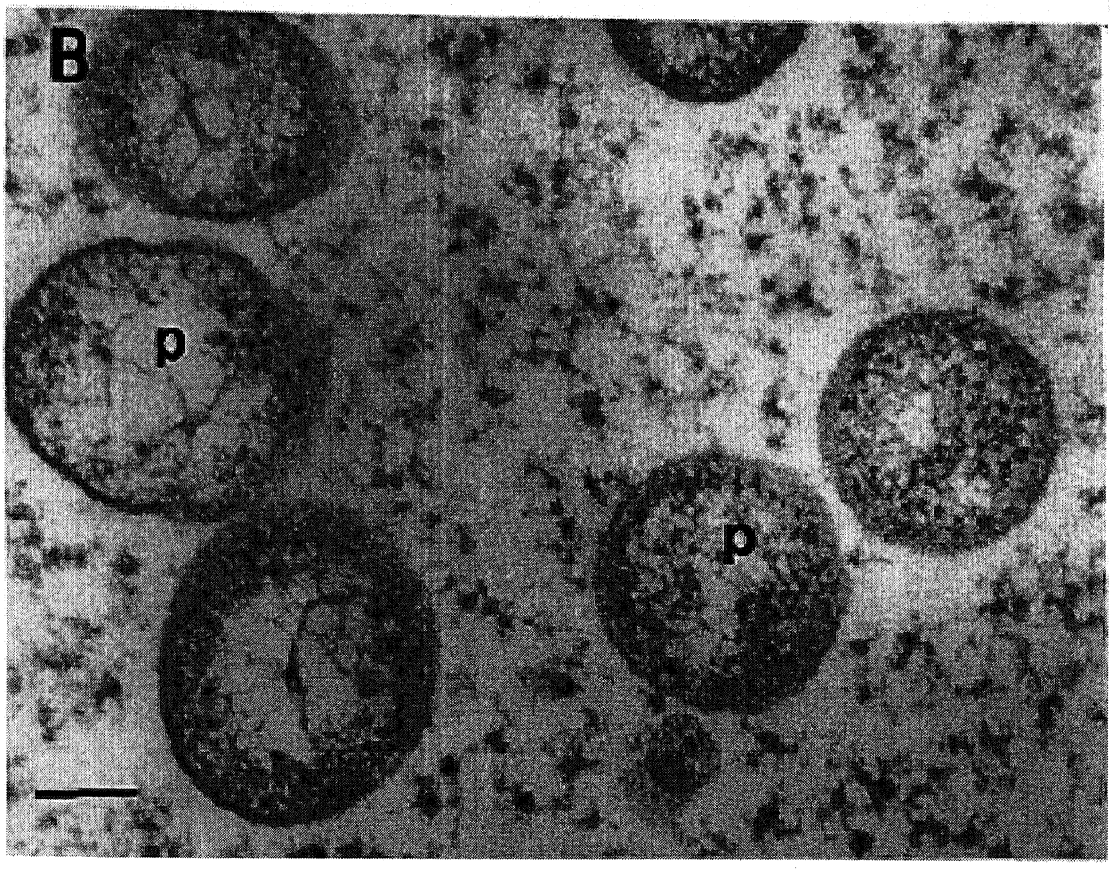
B
P
P

Figure 3.1. Transmission electron micrograph of phytoplasma cells within a mature sieve element of the phloem of minor veins of a yellow crinkle-affected papaya leaf. (**A**) Mature sieve element (S) containing phytoplasma cells (arrow), adjacent to a companion cell (C) containing numerous mitochondria. The sieve cell is characterised by thickened primary cell walls (star) and a cytoplasm occupied by small fibrils (phloem protein). Note plasmodesmata between the sieve element and the companion cell. (**B**) Phytoplasma cells (p) are characterised by a peripheral cytoplasm containing ribosomes, and a central region of fibrillar material, presumed to contain DNA. Bar represents 2 μm in (A) and 0.25 μm in (B).

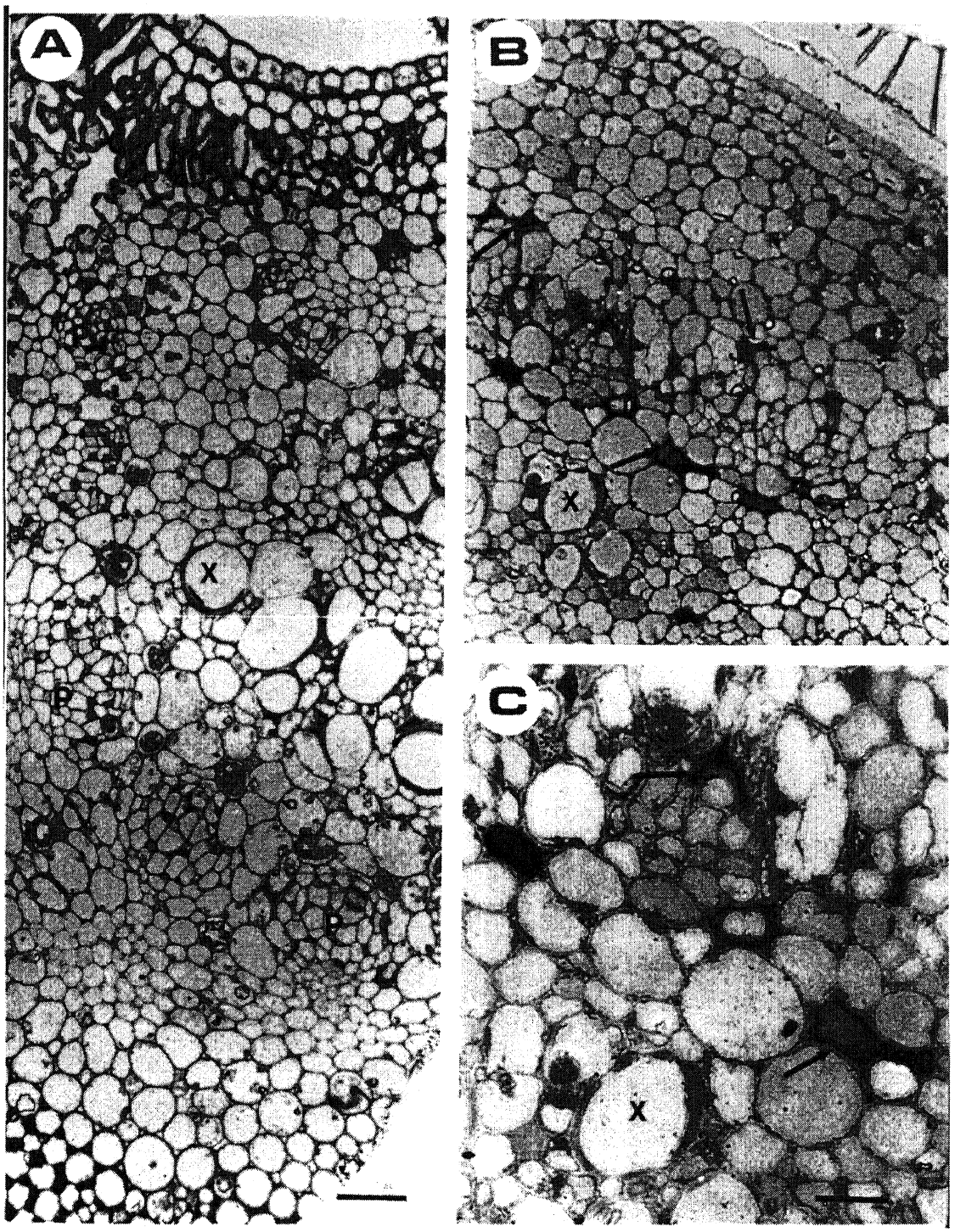

Figure 3.2. Bright field micrographs of papaya leaf tissue. (**A**) Healthy leaf; bundles of phloem (P) adjacent to xylem (X) in a secondary vein. (**B**) Dieback-affected leaf in which discoloured, autofluorescent laticifers were seen in fresh, hand-cut sections. Laticifers (arrows) ramify through the tissue, especially within and adjacent to phloem. Phloem tissue is located adjacent to xylem tissue (X). (**C**) Magnified image of B. Bar represents 50 μm in (A) and (B), and 25 μm in (C).

membrane integrity, but these regions were not sampled in the current study. In the TEM study, roughly spherical membrane-bound structures of up to 1,200 nm in diameter were observed in vacuoles of cells within phloem tissue in sections taken from two blocks. These cells were interpreted as either phloem parenchyma, immature sieve elements, or immature laticifers. One block included secondary veins of leaves with the "X-Y" patterning symptom (Figs. 3.3 and 3.4). The content of the membrane-bound bodies was variable, with some containing granular, ribosome-like electron dense particles (Fig. 3.3), while others were empty (Fig. 3.4B).

Laticifers were noted throughout the papaya plant body, present in both phloem (Fig. 3.2) and xylem tissues. As seen in bright field and epifluorescence microscopy, the visible internal browning associated with the dieback disorder was due to a discolouration of laticifer contents (Fig. 3.2), which was also fluorescent under blue or ultraviolet excitation. As seen in TEM, laticifer cell shape was intact in dieback-affected but not necrotic tissue, and these cells were occluded in parts with an electron dense material, with an apparent loss of latex vesicle integrity (Fig. 3.5). All sieve elements observed appeared healthy (not collapsed).

3.3.3 Distribution of phytoplasma relative to laticifer discolouration

Not all tissues that demonstrated laticifer autofluorescence tested positive for the presence of phytoplasma DNA, and not all tissues that contained phytoplasma DNA demonstrated laticifer autofluorescence. For example, in plant A, with mild tip bending (early visible symptoms of dieback disease), autofluorescence of the laticifers was seen in 11 of 20 samples, including the fruit, an older leaf, and parts of the upper and lower stem tissues (Fig. 3.6A). Phytoplasma DNA was detected in 13 of the 20 samples. Autofluorescence was not seen in four locations that were PCR-positive (2 mid-stem

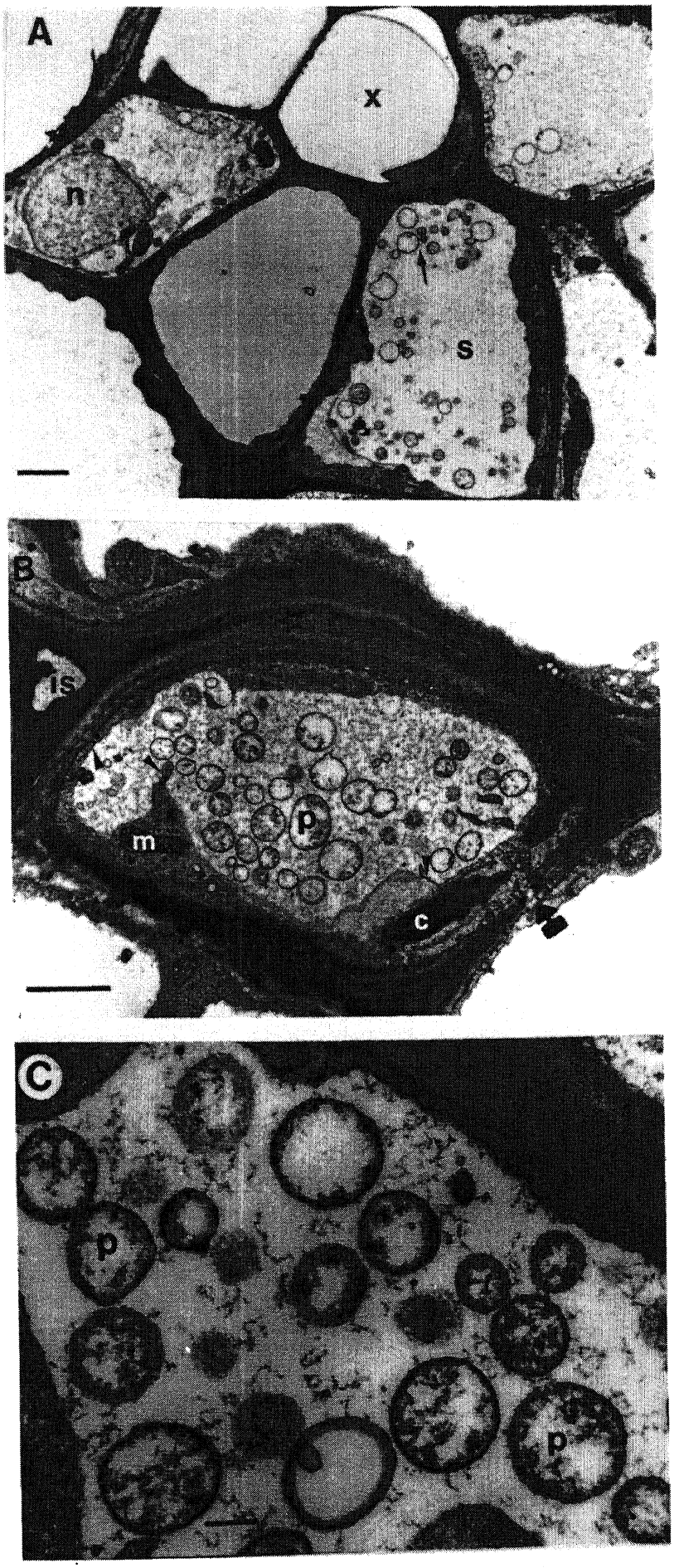
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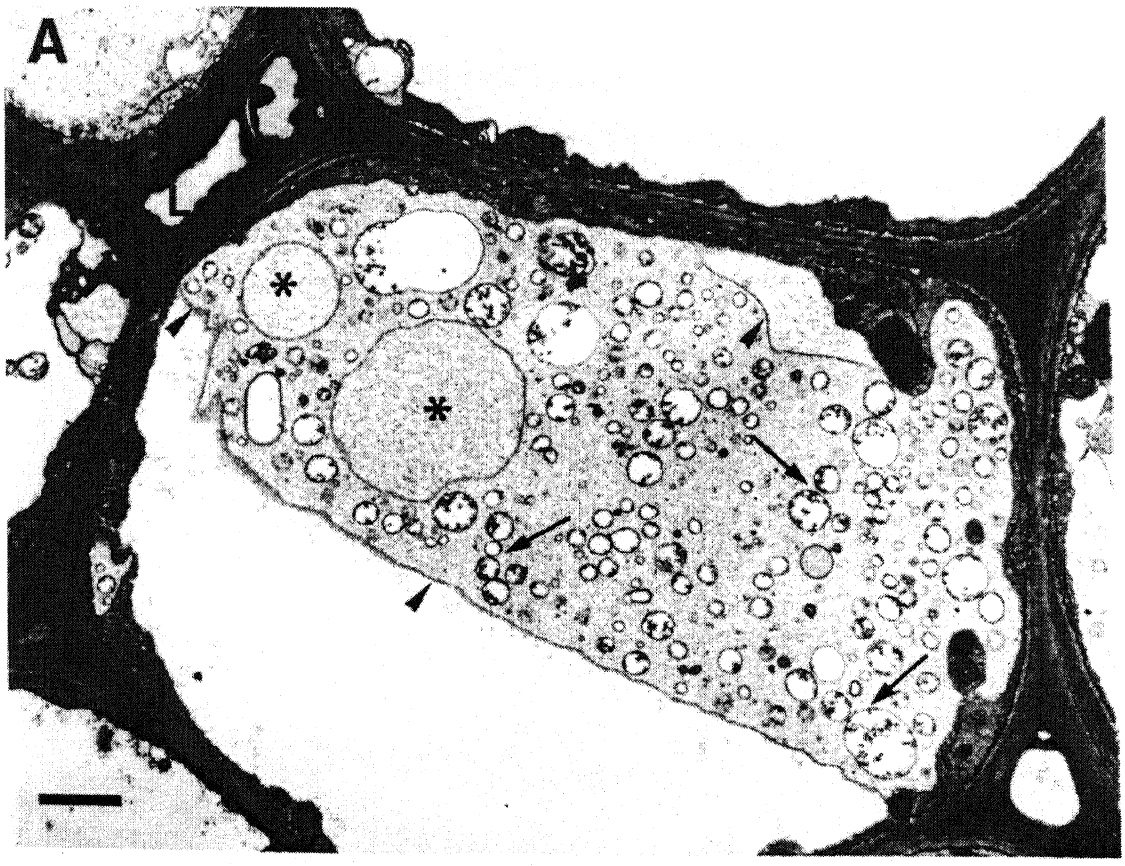
Figure 3.3. Transmission electron micrographs of membrane-bound bodies within cells of phloem tissue of minor leaf veins displaying the "X-Y" water-soaked vein symptom of the papaya dieback disease (adjacent to tissue depicted in Fig. 3.2). **(A)** Mature xylem cell (X) is identified by secondary wall development. Cell containing membrane-bound bodies (arrow) may be either a parenchyma cell, an immature sieve element (S), or an immature laticifer. Similar nearby cell possesses a nucleus (n). **(B)** Cell containing membrane-bound, phytoplasma-like bodies (p) within a vacuole (tonoplast membrane marked by arrowheads) is interpreted as an immature sieve element or an immature laticifer because of thickened cell walls (star). Thinning of the cell wall at the position of a plasmodesmatal field (arrow) may represent the formation of a pore. **(C)** Membrane-bound, phytoplasma-like bodies (p) contained within the cell vacuole are bounded by a single membrane. Contents of these bodies are variable, ranging from denser than that of the surrounding vacuole to empty. Bar represents 2 μm in (A) and (B), and 0.5 μm in (C).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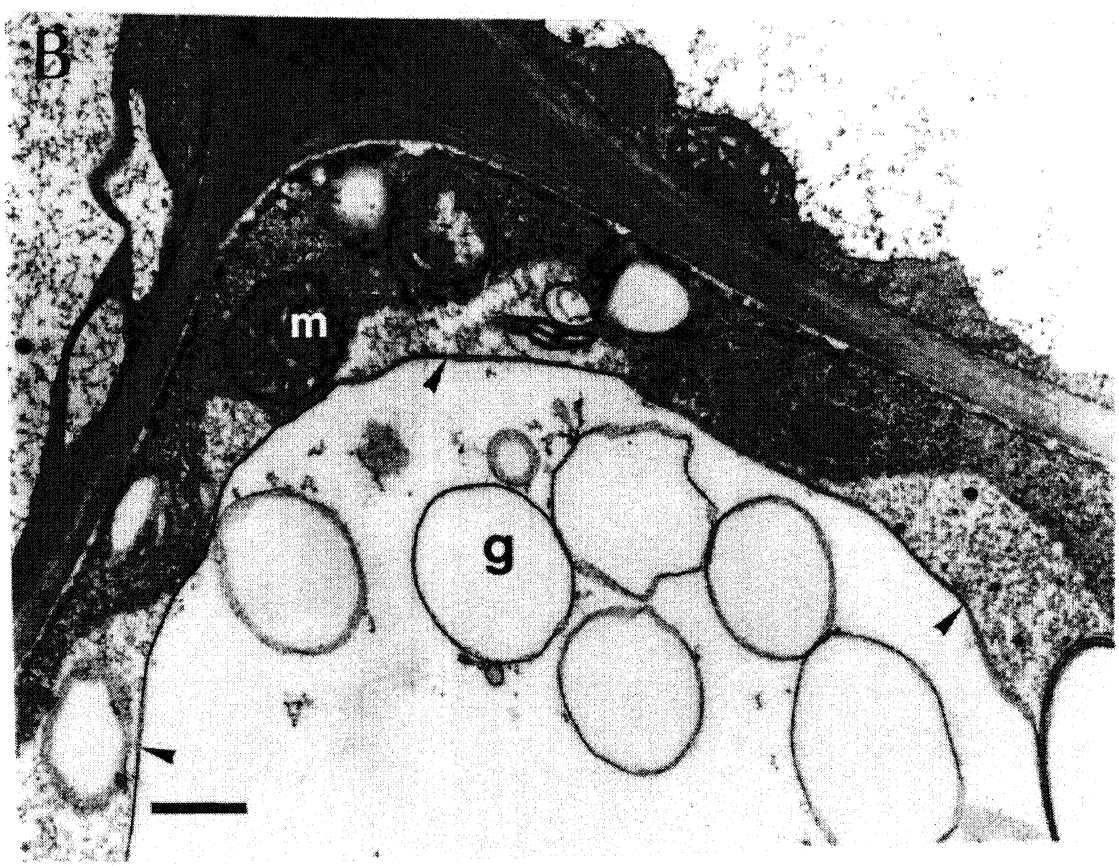
B
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Figure 3.4. Transmission electron micrograph of membrane-bound bodies within cells of the phloem of minor leaf veins displaying the "X-Y" water-soaked vein symptom of the papaya dieback disease. **(A)** Tonoplast (arrowheads) has parted from the cytoplasm, presumably as a tissue-processing artefact, but showing clearly the localisation of the membrane-bound bodies (arrow) within a vacuole. Larger membrane-bound inclusions (asterisks) in the vacuole have different contents to the phytoplasma-sized bodies (arrow). **(B)** Detail of cell containing vesicles which lack inclusions (g) within a vacuole (tonoplast, arrowhead), with healthy mitochondria (m) nearby. Bar represents 2 μm in (A) and 1 μm in (B).

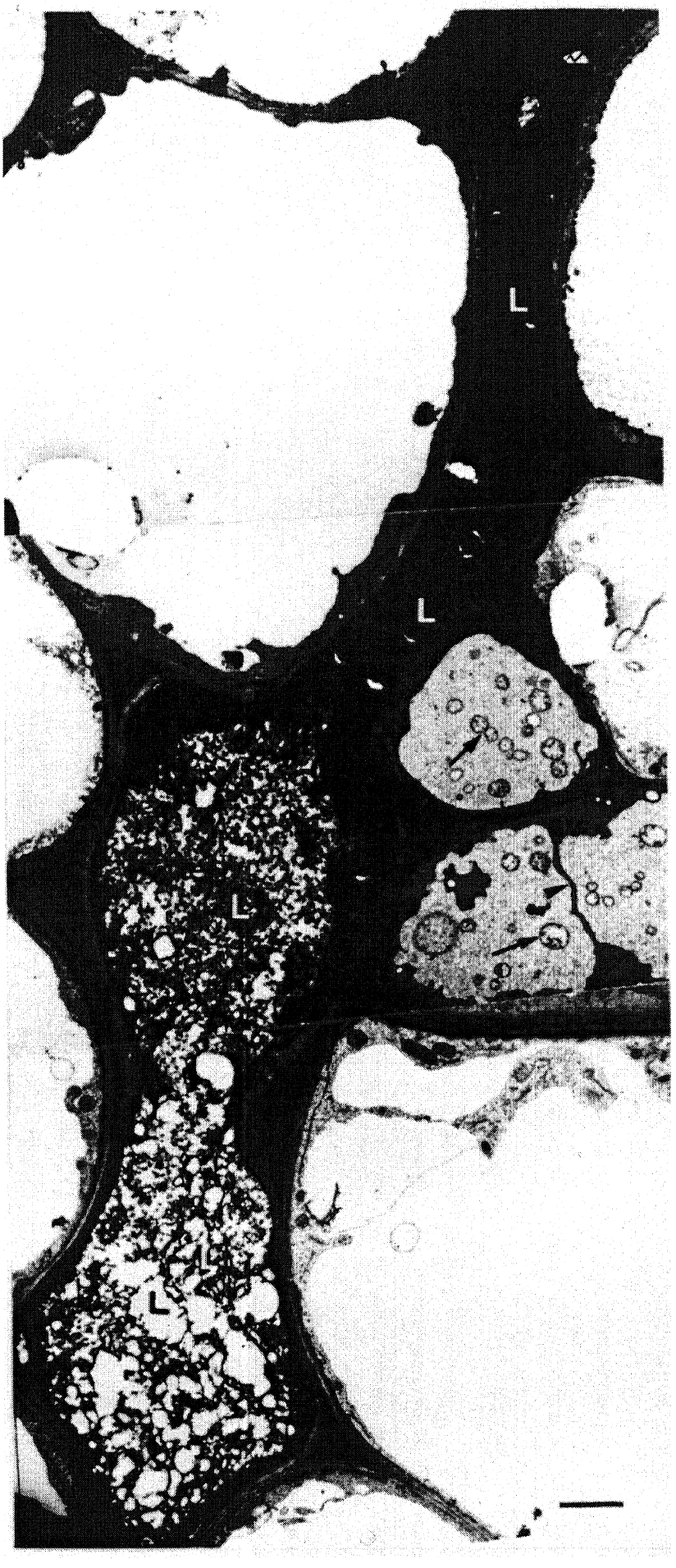
L
L
L
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Figure 3.5. Transmission electron micrograph of laticifer (L) within phloem tissue of dieback-affected papaya plant, adjacent to cells containing membrane-bound bodies (arrows) within vacuoles. A cytoplasmic bridge through a vacuole is visible (arrowhead). Bar represents 3 µm.

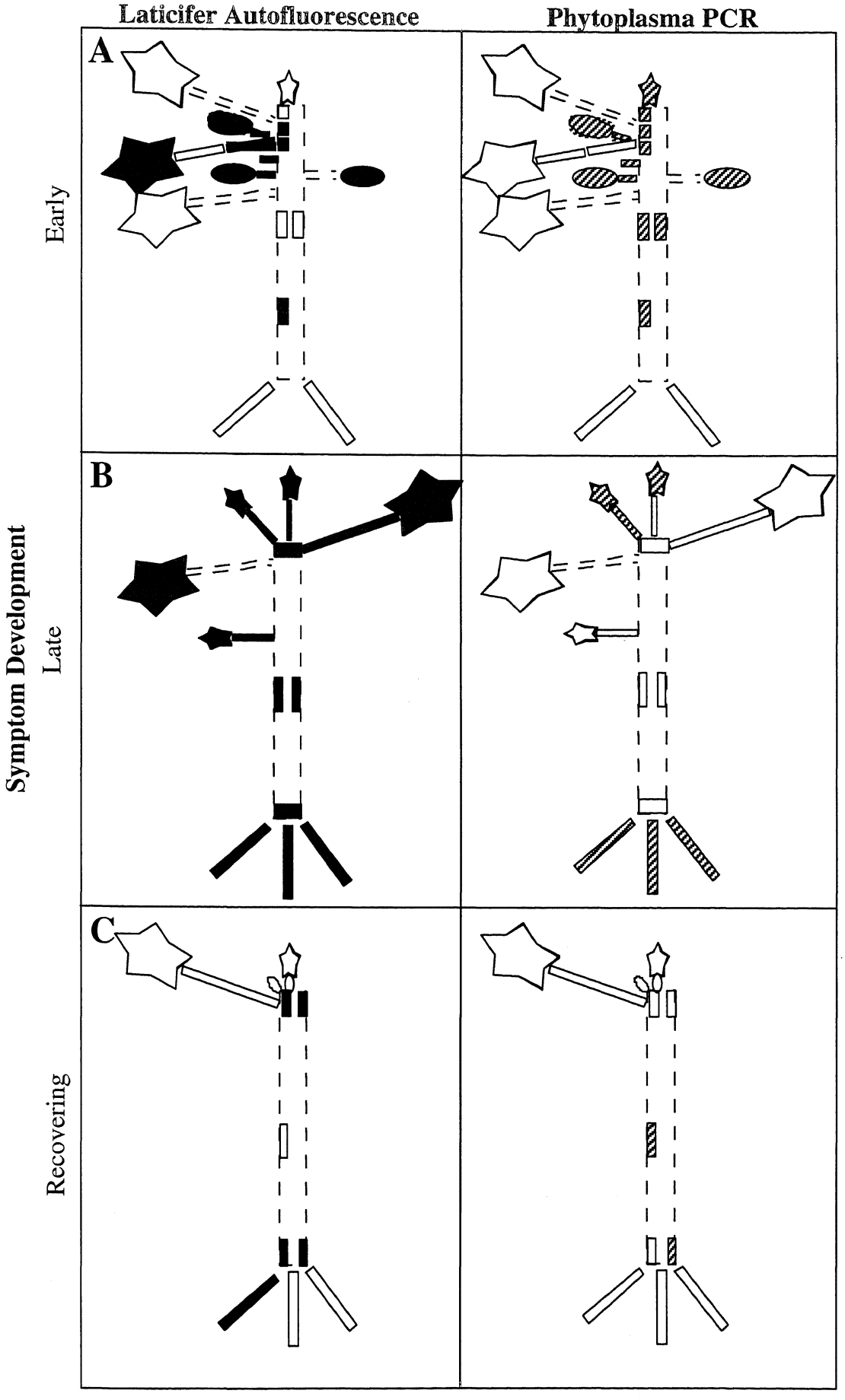
Laticifer Autofluorescence
Phytoplasma PCR
A
B
C
Early
Late
Recovering
Symptom Development

Figure 3.6. Diagrammatic representation of the distribution of laticifer autofluorescence (left-hand panels) and dieback-associated phytoplasma DNA, as detected by polymerase chain reaction (PCR) using the primer pair fU5-AGY2 (right hand panels) in three dieback-affected papaya plants. Plants were destructively sampled at three stages of symptom development: (**A**) very mild stem tip bending; (**B**) obvious dieback symptoms, foliage yellowing, strong tip bending, some tip necrosis; and (**C**) following the dieback occurrence, stem tip and leaves dead. Diagram presents a stylised representation of a plant (small stars as immature leaves, large stars as mature leaves, bars associated with stars as petioles, dashed rectangle as stem, ovals as fruit, bars at base of "stem" as roots). Solid lines represent the regions of the plant sampled. Shaded areas represent a positive result (either autofluorescence or a PCR product) while unshaded areas represent a negative result.

samples, 1 upper stem sample, and 1 sample consisting of material from four expanding leaves), while phytoplasma DNA was not detected in two locations that showed laticifer autofluorescence (mature leaf and its petiole, Fig. 3.6A).

In plant B, with tip necrosis, all 16 tissue samples examined (including root samples) had laticifer autofluorescence (Fig. 3.6B). However, phytoplasmas, as indicated by PCR, were present only in the roots and the immature leaves of the crown (Fig. 3.6B). In plant C, which appeared to be recovering from the disease, autofluorescence of the laticifers was restricted to the upper and lower portion of the stem and in the root section taken from the "bent" side of the plant (Fig. 3.6C). Phytoplasma DNA was only detected in the lower parts of the stem (Fig. 3.6C).

3.3.4 Distribution of phytoplasma relative to stage of symptom development

In plants with mild tip bending (very early visible symptoms of dieback disease), phytoplasma DNA was detected in expanding leaves, flowers, and the upper stem (Fig. 3.6A and Table 3.1). In plants with obvious visible symptoms (crown leaf chlorosis, some apical necrosis), phytoplasma DNA could be detected in the mid-lower stem and even within roots (Fig. 3.6B and Table 3.1). Following death of the stem tip and most leaves, phytoplasma DNA could still be detected in the stem, although the extent of its presence was decreased relative to the preceding stage (Fig. 3.6C and Table 3.1). Mature leaves were free of phytoplasma DNA at all stages of the disease progression. Lateral shoot material that grew from the main trunk after death of the upper stem was free of phytoplasma DNA (Table 3.1).

Table 3.1 Distribution of laticifer autofluorescence and phytoplasma DNA as detected by PCR using the primer pair fU5-AGY2 within three papaya plants affected by dieback, on three sampling days.

	Day[b]		
Tree, sample[a]	1	5	27
1			
Crown leaves	+/–	+/+	...
Symptomatic crown leaves	+/–	+/–	...
Crown petiole	+/–	...	...
Mature leaves	+/–	+/–	...
Stem apex	...	...	+/–
Upper stem	+/–	+/–	+/+
Mid stem	–/–	+/–	+/+
Lower stem	–/–	–/–	+/–
2			
Crown leaves	+/–	+/–	...
Symptomatic crown leaves	...	+/–	...
Crown petiole	+/+	+/–	...
Mature leaves	–/–	+/–	...
Dead immature fruit	...	...	+/–
Stem apex	...	...	+/+
Upper stem	+/+	+/+	+/+
Mid stem	...	–/+	+/–
Lower stem	–/–	–/–	–/+
3			
Crown leaves	+/–	+/–	...
Symptomatic crown leaves	...	+/–	...
Crown petiole	+/+	...	...
Mature leaves	+/–	+/–	...
Flower	...	+/–	...
Stem apex	...	...	+/+
Upper stem	+/+	+/+	+/+
Mid stem	–/+	+/+	+/+
Lower stem	–/–	–/–	–/–
Regrowth	...	...	+/–

[a] Leaf lobes and cores of stem material were collected.

[b] On Day 1 (16 Oct 1997), plants exhibited very mild stem tip bending (equivalent to panel A in Fig. 3.6). On Day 5, the plants displayed obvious dieback symptoms (foliage yellowing, strong tip bending, some tip necrosis) (equivalent to panel B in Fig. 3.6). The third sampling occurred when stem tip and leaves were dead (equivalent to panel C in Fig. 3.6). Test results = autofluorescence/PCR product; positive and negative results (either autofluorescence or a PCR product) are indicated as + and –, respectively; ... indicates data not available.

3.4 Discussion

3.4.1 Electron microscopy visualisation of phytoplasma

Gowanlock *et al.* (1976) observed phytoplasma cells within sieve elements of leaves of yellow crinkle-affected papaya plants, using TEM. This observation was corroborated in the present study. The phytoplasma cells associated with yellow crinkle were healthy, as indicated by the presence of structures interpreted as ribosomes and DNA fibrils. The pathogen was limited to cells identified as mature sieve elements because of thick cell walls and the lack of a nucleus.

In contrast, phytoplasma cells were not observed in mature sieve elements of dieback-affected tissues. The only membrane-bound structures of appropriate size and shape to phytoplasma cells observed in dieback-affected tissue were located within the vacuole of nucleated cells within phloem tissue.

Membrane-bound bodies within vacuoles of phloem parenchyma of *Nicotiana rustica* infected with aster yellows phytoplasma were tentatively interpreted as degenerate phytoplasma (Hirumi & Maramorosch, 1972). However, Esau *et al.* (1976) and McCoy (1979) dispute all reports of phytoplasma cells in vacuoles and parenchyma cells (cells lacking sieve pores). The presence of vesicles within vacuoles was attributed to autophagic activity, and it was noted that phytoplasma cells should be distinguishable from autophagic vesicles on the basis of the size of the ribosomes present in the membrane bound bodies (i.e. procaryotic compared with eucaryotic ribosomes, Esau *et al.*, 1976). As the inclusions within the membrane-bound bodies in dieback-affected papaya were not clearly different from those of cytoplasmic ribosomes (Fig. 3.3), and as these structures were only seen in vascular tissue displaying the "X-Y" patterning symptom (i.e. in tissue beginning to undergo tissue breakdown), it is suggested that

autophagic activity is a plausible explanation for the presence of the membrane-bound bodies. Alternatively, the membrane-bound bodies may be latex vesicles within a parenchyma cell beginning to differentiate as a laticifer.

The cellular location of the phytoplasmas detected by the PCR technique within tissue of plants affected by the dieback disease therefore remains unresolved. The organism may be either present in high densities in very few sieve elements, making it difficult to locate when sectioning, or present in very low densities, making it difficult to distinguish from other cellular inclusions. Immunocytochemistry or *in situ* hybridisation studies at the light microscope level would demonstrate localisation at high densities in a few sieve elements, in the former case, while immunocytochemical studies at the electron microscope level would be necessary to demonstrate low density distribution, in the latter case. Jones *et al.* (1974) concluded that phytoplasma cells were best viewed by electron microscopy in sections of peach leaves displaying early symptoms of X-disease, since the numbers of phytoplasma cells decreased as symptom severity increased. Based on the results presented in this thesis chapter, it is recommended that future attempts to visualise the dieback-associated phytoplasma using TEM concentrate on tissues in which phytoplasma DNA can be detected, but which do not display external symptoms.

3.4.2 Phloem and laticifer anatomy in dieback-affected tissue

Phytoplasma diseases are often accompanied by anatomical change, culminating in necrosis of phloem tissues. For example, in Italian grapevine yellows, a flavescence dorée (FD)-like disease, sieve elements and the associated companion cells often become necrotic and collapse, and are filled with an osmiophilic material (Credi, 1994). Sieve element degeneration may represent a plant defence strategy, explaining the

localised appearance of symptoms in affected vines and the observation of natural symptom remission (Credi, 1994).

However, sieve element collapse is not a universal feature of phytoplasma diseases. For example, when *Vicia faba* plants were inoculated with the FD phytoplasma, root tissues demonstrated high titre without phloem cell collapse or phytoplasma cell degeneration, while shoot tissues demonstrated low titre and sieve element degeneration (Lherminier *et al.*, 1994). In observations presented in this chapter, the phloem of dieback-affected tissue was not structurally affected until the point at which the whole tissue became necrotic. This accords with the observation of Aleemullah and Walsh (1996) that the phloem of dieback-affected tissue was functional, as judged by the movement of the symplastic tracer, carboxyfluorescein.

In contrast, Harding and Teakle (1988) reported necrotic phloem cells in otherwise anatomically healthy tissue. The observations presented in this chapter reinforce the interpretation of Aleemullah and Walsh (1996) that the micrograph of "necrotic phloem cells" presented by Harding and Teakle (Fig. 6 in Harding & Teakle, 1988) is an image of laticifers within the phloem. Laticifers give the appearance of necrotic cells, being reminiscent of a collapsed cell filled with necrotic contents. However, the laticifers observed in dieback-affected tissue (Figs. 3.2 and 3.5), although discoloured as seen in bright field microscopy, were not different in terms of cell shape to those seen in asymptomatic plants.

Laticifers were occluded with an electron-dense (in TEM study), autofluorescent material and suffered an apparent loss of latex vesicle integrity (compare Fig. 3.5 with micrograph of healthy laticifer, Fig. 5A in Davis *et al.*, 1996). These symptoms were

also reported for papaya bunchy top disease (Davis *et al.*, 1996), but are not characteristic of papaya ring spot virus (Davis *et al.*, 1996), PpM, or PpYC diseases. Thus, while the symptom is not a specific response to the dieback disease, it is not a "general" stress response.

Phytoplasma DNA was not detected in all tissues displaying laticifer autofluorescence (Fig. 3.6 and Table 3.1). It can be concluded that, although laticifer discolouration is a useful indicator of the presence of the PpDB phytoplasma in a plant, it is not correlated with the presence of the phytoplasma within a given tissue. Presumably, diseased regions produce a (chemical) signal, which causes laticifer discolouration and possibly other symptoms (leaf yellowing, xylem tyloses, Aleemullah & Walsh, 1996) throughout other parts of the plant.

3.4.3 Observations on the spread of phytoplasma within the plant body, with reference to organ photoassimilate "sink" status

If phytoplasmas are localised within sieve elements, presumably they will tend to move with the bulk flow of phloem sap, moving generally away from photoassimilate source organs (mature leaves) to sink organs (apices, flowers). Mature leaves are sources of photoassimilate to the plant, typically exporting recently fixed carbon by day and remobilised carbon reserves (starch) by night. This hypothesis is consistent with the report that the phytoplasma associated with lethal yellowing disease of palms has rarely been found (in TEM studies) in mature leaves (Parthasarathy, 1974). Similarly, Lherminier *et al.* (1994) used an indirect ELISA to demonstrate that FD phytoplasmas were present only in the apical part of the plant (point of inoculation) and the roots soon after infection. High titre noted in some sink regions was ascribed to active multiplication, rather than simply passive accumulation. Also, in the current study,

phytoplasma DNA was detected in sink areas of the dieback-affected papaya plants, but not in the mature source leaves (Fig. 3.6).

3.4.4 Speculation on the involvement of a toxin

In phytoplasma diseases involving a high titre of the pathogen, the physiology of the plant may be altered due to physical blockage of the sieve elements by, and resource diversion to, the phytoplasma cells. In other cases, where the titre of phytoplasma is low, tissue death may result from phytotoxin activity (X-disease of stone fruits and pear decline, Douglas, 1993). The effect of the toxin may be localised to tissues near the phytoplasma, with phytoplasma concentration correlated to symptom severity (Douglas, 1986; Kuske & Kirkpatrick, 1992a), or the toxin may be transported within the plant to cause symptom expression at a distance from the pathogen (Nakashima *et al.*, 1994; Nakashima & Hayashi, 1995).

The expression of external and internal papaya dieback symptoms in mature leaves when phytoplasmas could not be detected is consistent with the role of a phytotoxin, produced or induced by the phytoplasma. To reach the mature leaves, this "toxin" must be transported within the xylem.

3.4.5 Selection of tissue for in-field, continuous monitoring of dieback phytoplasma

For diseases such as pear decline (Seemüller *et al.*, 1984), apple proliferation (Seemüller *et al.*, 1984), ash yellows (Sinclair *et al.*, 1989), X-disease (Douglas, 1986), and blueberry stunt (Schaper & Converse, 1985), root tissue is the most reliable, year-round source of material for detection of phytoplasmas. Phytoplasma cells overwinter

in the roots and subsequently multiply, re-entering the shoot system via the phloem in spring (Douglas, 1986).

In contrast, the PpDB phytoplasma was detected in the roots of papaya only at the height of symptom expression, at which stage shoot apical death was imminent (Fig. 3.6). In a plant recovering from a dieback infection, no phytoplasma DNA was detected in roots. Phytoplasma DNA was not detected in mature leaves, in contrast to leaves that were not yet fully expanded. Immature leaves are sinks for photoassimilate, and therefore the likely destination of phytoplasmas in the phloem. Immature leaves may also represent a preferred feeding target for the phloem-feeding insects that are likely to transmit the phytoplasma. Leaf lobes can be sequentially removed with relatively little disturbance to the plant.

3.4.6 Conclusion: A description of the papaya dieback disease

From the data presented in this chapter, it is proposed that the PpDB phytoplasma spreads through mature sieve elements, following phloem sap flow, but the conditions in the sieve elements of papaya are not conducive to proliferation of this phytoplasma. Further, due to its genetic makeup, the PpDB phytoplasma may not be capable and/or does not need to proliferate for successful continuance of its population. In contrast, the PpYC phytoplasma, as deduced from the numbers observed by TEM, does proliferate in papaya. A hypersensitive response is apparently triggered by the PpDB phytoplasma, with host tissue death ensuing.

It can be inferred that tissue necrosis results in the destruction of PpDB phytoplasma cells, since the distribution of phytoplasma in the stem decreased with external symptom progression, and leaf samples taken from new lateral shoots of plants which

had recovered from dieback tested negative for PpDB phytoplasma DNA (this study and Chapter 4). With low cell numbers in host tissue, and rapid host tissue death, the PpDB phytoplasma is unlikely to be transmitted by insect vectors from papaya plants. It therefore seems that papaya is a dead-end host.

CHAPTER 4

Estimation of minimum presymptom residency (incubation or lag) period and persistence of phytoplasmas in papaya plants

4.1 Introduction

Crop protection management of PpDB, PpYC and PpM would benefit from knowledge of the presymptom residency (incubation or lag) period between inoculation of papaya plants with phytoplasmas and symptom expression, the insect vectors of the phytoplasmas, and alternate plant hosts (i.e. potential "reservoirs" of phytoplasma outside papaya plantations). Current management is limited to ratooning or removal and destruction of yellow crinkle- and mosaic-affected plants, and ratooning of dieback-affected plants (Glennie & Chapman, 1976; Simmonds, 1965). Ratooning consists of cutting back plant stems to a height of 0.75 m or less as soon as external symptoms of any of the three phytoplasma diseases are recognised, in an attempt to reduce spread of the pathogen throughout the plant and the plantation. Lateral shoots are allowed to grow on these stumps. The new stems should be stable and not liable to break under the weight of shoot and fruit growth. Ratooning therefore helps to replace lost production potential. This management strategy is based on the assumption that the phytoplasma is localised to the upper shoot.

Of the three diseases, PpDB and PpYC have the most seasonal distribution in terms of symptom expression (Elder *et al.*, accepted 2001; Glennie & Chapman, 1976; Simmonds, 1965). Two hypotheses are consistent with the observation that dieback epidemics occur in two periods within the year, October to November and March to April (Aleemullah & Walsh, 1996; Elder *et al.*, accepted 2001; Glennie & Chapman,

1976; Harding, 1989): (i) plants may be infected throughout the year, but symptom expression occurs only during the two periods, due to a change in environmental and/or plant physiological conditions which evokes a toxicity within the disease complex; or (ii) the disease has a short incubation period, with inoculation preceding the two periods of symptom expression due to increased insect vector activity. These hypotheses can similarly be proposed for the increased incidence of yellow crinkle between November and March.

The insect vector or vectors of the phytoplasmas associated with PpDB, PpYC and PpM have not been identified, although it has been noted that the periodic epidemics of PpYC usually occur after hot, dry weather, which favours the breeding and movement of the leafhopper *Orosius argentatus* (Peterson *et al.*, 1993). The *Phormium tenax* (New Zealand flax) yellow leaf (PYL) phytoplasma, which is closely related, genetically, to the PpDB phytoplasma (Chapter 2, Gibb *et al.*, 1998), is vectored by a planthopper, *Oliarus atkinsoni* (Liefting *et al.*, 1997).

Two previous studies have allowed an approximate estimation of the lag period of the PpYC and PpDB diseases. Greber (1966) noted expression of big bud symptoms on tomato plants 7 to 9 weeks following transfer of infective dodder from yellow crinkle-affected papaya to healthy tomato plants. Expression of yellow crinkle symptoms on papaya plants was noted 9 to 13 weeks following transfer of infective dodder from tomato plants exhibiting big bud symptoms (Greber, 1966). Harding (1989) noted dieback symptoms within 5 weeks of transplanting asymptomatic, glasshouse-reared, papaya plants to the field.

It has been suggested that PpYC and PpM may result from a disease complex involving the same phytoplasma strain, differentiated by other factors such as another pathogen, age of host plant, or level of inoculum at time of inoculation (Gibb *et al.*, 1996). Comparison of the lag period would be useful in the characterisation of the two diseases, but an estimate for the PpM phytoplasma has not previously been determined.

Knowledge of the time of inoculation, as opposed to the time of symptom expression, is useful in the quest to identify insect vectors of the phytoplasmas. The aim of this study was to improve the estimate of the minimum residency periods of the three phytoplasmas in relation to the disease symptoms of papaya plants. A reliable means of transmitting the PpDB phytoplasma to papaya plants has not been established; therefore, a field study approach was adopted, involving regular monitoring of a group of plants within a commercial papaya plantation for the presence of phytoplasma. The carry-over of phytoplasma into lateral shoot growth of plants ratooned following observation of external symptoms was also monitored, allowing comment on the efficacy of ratooning as a management strategy for control of these diseases. Results presented in Chapter 3 demonstrated that weekly sampling of the lobes of young crown leaves from the same plant would allow reliable detection of phytoplasmas, while minimising disturbance to the plant itself.

4.2 Materials and Methods

4.2.1 Sample collection

A total of 60 field-grown papaya plants (tissue culture clones of a dioecious variety 'Hybrid 14', Drew & Vogler, 1993), consisting of every fourth female plant within rows of approximately 30 plants within a commercial plantation at Yarwun, central Queensland, Australia (Site 2 in Elder *et al.*, accepted 2001), were sampled every week

from September 1996 until July 1997 so as to include the spring and autumn dieback "outbreak" periods. Lobes of six immature (not fully expanded) leaves were sampled from each plant. Immature leaves were demonstrated to be a reliable indicator tissue for the presence of the PpDB phytoplasma within the plant (Chapter 3). Leaf samples were frozen at –20°C until analysed. One-third of each leaf lobe was subsampled from each sample. This material was pooled across ten samples, giving six combined samples for each week of sampling. When a positive PCR result was recorded, or when visible disease symptoms were noted, the individual samples and all previous samples of the affected individual were analysed.

Diseased plants were immediately ratooned at approximately 0.75 m height, following current grower practice. Leaf lobes of lateral shoot regrowth material from papaya plants that had been ratooned following dieback (11 plants), mosaic (9 plants), and yellow crinkle (7 plants) were sampled in July and October 1997 and January 1998. Stem material was also sampled from some of these plants (two dieback, one mosaic, and two yellow crinkle). Leaf lobes of regrowth material and stem tissue from six papaya plants that had not been ratooned following dieback were also sampled in February 1998.

4.2.2 PCR Amplification of DNA

The methods for the extraction of total nucleic acids from papaya leaf midribs tissue and PCR with primers pairs P1-P7, for generic phytoplasma-specific detection, and fU5-AGY2, for PpDB-phytoplasma-specific detection, are described in Chapter 3.

4.2.3 RFLP analysis

The P1 and P7 amplimers (10 µl) were digested separately using the restriction endonucleases *Alu* I and *Tru* 9 I in their respective buffers, as supplied by the manufacturer (Boehringer Mannheim GmbH). Digestions were incubated over-night at 37°C. The reactions were stopped by heating at 65°C for 20 min. The fragments were separated by electrophoresis through a 3% agarose gel and visualised by staining with ethidium bromide. Known dieback, yellow crinkle, and mosaic samples were used as positive controls.

4.3 Results

4.3.1 Disease incidence - external symptoms

A total of 21 of the 60 papaya plants displayed symptoms of phytoplasma-associated diseases over the 11-month study period. Of those 21, 15 plants exhibited dieback symptoms and 6 exhibited mosaic symptoms. No yellow crinkle was observed in this group of plants, although nearby plants displayed symptoms typical of this disease. The majority of dieback cases (11 of 15) occurred within the periods September to October and April to May, while mosaic cases occurred between April and June.

4.3.2 Disease incidence - PCR detection

From plants with symptoms of the mosaic disease a DNA product of the appropriate size was amplified with the phytoplasma-specific primers (P1 and P7) but not with the "*Candidatus* P. australiense"-specific primers (fU5 and AGY2). In contrast, from plants with symptoms of dieback a DNA product of the appropriate size was amplified with fU5 and AGY2. However, PCR with the primers P1 and P7 was unreliable, giving either faint DNA product or no product. Gibb *et al.* (1998) reported poor amplification of phytoplasma DNA from dieback material with P1 and P7, and suggested that this was

due to a low titre of phytoplasma associated with this disease. Of the 15 dieback symptomatic plants, two did not amplify with either primer pair. DNA extracted from papaya plants that did not exhibit symptoms during the 11-month study (38 plants) gave no PCR product with either primer pair.

Using the "*Candidatus* P. australiense"-specific PCR, amplimers were obtained from tissue samples collected either the week of, or the week prior to, visual symptom expression of dieback. In contrast, DNA associated with the PpM phytoplasma was first detected between 3 and 11 weeks prior to visual symptom expression. First detection within the monitored plants of the PpM phytoplasma associated with mosaic occurred over a 6-week period from 27 March to 7 May, with symptom expression noted over a wider time span (11 weeks, 17 April to 3 July, Table 4.1).

4.3.3 Persistence of phytoplasmas

Leaves from lateral shoots growing on stems of papaya plants which had suffered dieback failed to give a PCR product with either set of primers (P1 and P7; fU5 and AGY2) in 15 cases (nine plants ratooned, six plants left intact). Tissue from the main trunk of the plant, sampled from the six non-ratooned plants, also failed to give PCR product. In two dieback-ratooned plants, stem tissue gave a product with the P1 and P7 primers, while leaf material gave products with both primer sets. Where sufficient products existed to undertake a restriction digest (one leaf sample, two stem samples), the profile was identical to the yellow crinkle and mosaic controls.

Regrowth material from three of nine papaya plants that had been ratooned following mosaic symptom expression gave a product with the P1 and P7 primer pair (Table 4.2). In two of these three plants, a lateral shoot exhibited the typical dieback symptom of

Table 4.1. Polymerase chain reaction (PCR) detection of dieback and mosaic phytoplasma in young leaf tissue relative to time of appearance of visual symptoms.

Disease	Date[b]	Weeks prior to visual symptoms[a]												
		0	1	2	3	4	5	6	7	8	9	10	11	12
Dieback	24 Oct 96	+	...	–	–	–	–	–	–	...	...	...	...	...
Dieback	24 Oct 96	+	...	–	–	–	–	–	–	...	...	...	...	...
Dieback	24 Oct 96	+	...	–	–	–	–	–	–	...	...	...	...	...
Dieback	24 Oct 96	+	...	–	–	–	–	–	–	...	...	...	...	...
Dieback	24 Oct 96	+	...	–	–	–	–	–	–	...	...	...	...	...
Dieback	16 Jan 97	–	–	...	...	...	...	...	...	...	...	...	...	...
Dieback	6 Feb 97	+	–	–	–	–	...	...	...	...	...	...	...	...
Dieback	13 Feb 97	+	–	–	–	–	...	...	...	...	...	...	...	...
Dieback	13 Mar 97	–	–	–	–	–	...	...	...	...	...	...	...	...
Dieback	10 Apr 97	+	+	–	–	–	...	–	–	–	–	...	...	...
Dieback	23 Apr 97	+	+	–	–	–	–	–	...	–	–	–	...	...
Dieback	1 May 97	...	+	–	–	–	...	...	...	...	...	...	...	...
Dieback	7 May 97	+	–	–	–	–	–	–	...	...	...	...	...	...
Dieback	22 May 97	+	...	–	...	–	–	–	–	–	–	...	...	...
Dieback	1 June 97	...	+	–	–	–	–	–	...	...	...	...	...	...
Mosaic	17 Apr 97	+	+	+	+	–	–	...	–	–	–	–	...	...
Mosaic	23 Apr 97	+	+	+	+	–	–	–	...	–	...	...	...	...
Mosaic	23 Apr 97	+	+	+	+	+	–	–	...	–	–	–	–	...
Mosaic	15 May 97	+	+	...	+	+	+	–	–	–	...	...	...	...
Mosaic	19 June 97	+	+	+	+	+	...	...	–	...	+	+	+	–
Mosaic	3 July 97	+	+	+	...	...	+	+	...	...	...	...	+	...

[a] Data is derived from lobes of young leaves from 60 female trees, monitored at weekly intervals. + indicates PCR product (fU5 and AGY2 for dieback; P1 and P7 for mosaic); – indicates no PCR product; ... indicates no data available.

[b] Date of symptom appearance.

Table 4.2. Presence of phytoplasma, as indicated by PCR, in lateral shoot regrowth of papaya trees that were ratooned following appearance of external symptoms associated with a phytoplasma-associated disease.

Original disease	Ratooned	No. plants	Time[a]	Tissue sampled	P1 & P7[b]	fU5 & AGY[b]	Comments
Dieback	+	9	0.4-8	Leaf	–	–	In 5 cases, original disease verified by PCR test (plant of monitored group represented in Table 4.1).
Dieback	–	6	4	Leaf	–	–	Leaf and stem samples taken from each plant.
				Stem	–	–	
Dieback	+	1	4	Leaf	–	+	
				Leaf	+ RFLP	+	
				Stem	–	–	
				Stem	+ RFLP	+	
Dieback	+	1	4	Leaf	+	+	
				Leaf	+	+	
				Stem	–	–	
				Stem	+ RFLP	–	
Mosaic	+	6	1-3	Leaf	–	–	In 1 case, original symptoms verified by PCR.
Mosaic	+	1	1	Leaf	+	–	
Mosaic	+	1	4	Leaf	–	–	Different lateral shoots emerging from the one stump; dieback symptoms apparent in *; original symptoms verified by PCR.
				Leaf *	+	+	
Mosaic	+	1	3.5	Leaf	+ RFLP	–	Different lateral shoots emerging from the one stump; dieback symptoms apparent in *.
				Leaf *	+	+	
Yellow Crinkle	+	2	6	Leaf	–	–	–
Yellow Crinkle	+	3	6.5	Leaf	+	–	In 1 case, regrowth displayed claw-like leaves, a symptom characteristic of mosaic.
Yellow Crinkle	+	2	NK	Leaf	–	–	Two samples tested for each tissue.
				Stem	–	–	

[a] Time from symptoms (months); NK = not known.

[b] Two PCRs were performed for each sample, one primed with P1 & P7 (primers specific for all phytoplasmas), and the other with fU5 & AGY2 (primers specific for the "*Candidatus* P. australiense" group of phytoplasmas). + indicates the production of amplimer of the appropriate size in the PCR; – indicates the absence of product; RFLP = RFLP analysis. Where sufficient P1-P7 product supported a restriction digest, the resultant profile was characteristic of that associated with yellow crinkle- or mosaic-associated phytoplasma.

bending of the apex and "X-Y" patterning of the leaves, and gave a product with the "*Candidatus* P. australiense"-specific PCR (fU5 and AGY2). In both cases, however, another lateral shoot on the same stump gave no product with the "*Candidatus* P. australiense"-specific PCR. In one of these cases, the other lateral shoot gave a PCR product with the P1 and P7 primer pair and, when digested, gave the same profile as the yellow crinkle and mosaic controls. Regrowth material from three of seven papaya plants that had been ratooned following yellow crinkle symptom expression gave a PCR product with the P1 and P7 primer pair, while all seven samples, including stem samples, failed to give a product with the fU5 and AGY2 primer set (Table 4.2).

4.4 Discussion

4.4.1 Incubation period - from infection to symptom appearance

The PpDB phytoplasma was detected only one week before visual symptoms, while the PpM phytoplasma was detected up to eleven weeks before visual symptoms were apparent (Table 4.1). These periods represent an estimate of minimum incubation time or lag period, because phytoplasma DNA may not be detected due to an uneven distribution of the phytoplasma cells within the plant body (Gibb *et al.*, 1996; Lee & Davis, 1992), and the sensitivity of the PCR may not be sufficient to detect phytoplasma DNA immediately upon inoculation.

While the PpYC phytoplasma has been transmitted between hosts by dodder (Greber, 1966), the PpDB and PpM phytoplasmas have not been experimentally transmitted between host plants, by insect, grafting, or dodder. In transmission studies of other phytoplasma diseases, phytoplasma are detectable by PCR or DAPI about two to three weeks after phytoplasma inoculation, while the time to visual symptom expression varies considerably (Chen & Lin, 1997; Douglas, 1986; Kuske & Kirkpatrick, 1992a;

Lherminier *et al.*, 1994; Nakashima & Hayashi, 1995; Sinclair & Griffiths, 1995). Assuming a similar two- to three-week lag period between inoculation and phytoplasma DNA detection, there may be a total delay of approximately three to four weeks from inoculation to onset of visual symptoms for the dieback disease, and five to fourteen weeks for the mosaic disease.

The duration of an incubation period will also be influenced by plant vigour. For example, Greber (1966) reported a shorter incubation period for yellow crinkle in papaya grown under favourable (greenhouse) conditions relative to a field planting (nine to twelve and eleven to thirteen weeks, respectively). Nevertheless, the estimate of incubation period for the PpDB disease obtained in the current study is consistent with Harding's (1989) deduction of less than five weeks, based on the minimum time for symptoms to appear in plants moved from insect-proof to open field conditions. The incubation period of nine to thirteen weeks reported for PpYC (Greber, 1966) is similar to that estimated for PpM in the current study.

It was shown that by the time of the first symptom expression of dieback (stem tip bending), phytoplasma cells were well dispersed within the host, although not present in high titre, because cells could not be observed via TEM (Chapter 3). The observation of a short period between first detection and symptom expression noted in the current study, is consistent with the rapid spread of the PpDB phytoplasma within the plant body following an inoculation event (leafhopper feeding), coupled with a limited multiplication capacity. The spread of phytoplasma cells within the plant may be limited by movement within the phloem, or by the number of phytoplasma cells. Certainly, the number of phytoplasma cells associated with the PpDB disease is low (Chapter 3). The rate of phloem solute movement axially within the plant is in the order

of 30 cm h^{-1}, depending on source-sink relationships (Minchin & Troughton, 1980), sufficient for movement of soluble phloem contents from the upper stem to the lower roots within one day in a mature papaya plant. Phytoplasma cells are presumably carried with this flux, although restricted by passage through the sieve plate pores. Phytoplasma cells must constrict to pass these pores, and it has not been established if this is a passive or active process. An improved understanding of the process of movement and multiplication of phytoplasma within the phloem is required to interpret epidemiological information of the type presented in this study.

4.4.2 Time of infection

Knowledge of the time of infection, as opposed to the time of symptom development, is important in determining the insect vectors and possible environmental triggers for the diseases. The incidence of visible symptoms of PpDB peaks at two periods during the year, October to November and late March to late May (Aleemullah & Walsh, 1996; Elder *et al.*, accepted 2001). In contrast, the incidence of visible symptoms of PpYC peaks within one period during the year, varying between November and March (Elder *et al.*, accepted 2001; Simmonds, 1965), and the incidence of PpM is generally evenly spread throughout the year (Elder *et al.*, accepted 2001).

During this study, the earliest detection of the PpM phytoplasma in individual plants varied from late March until early May (six cases; 100% of total detected; Table 4.1). During this period, five cases (38% of total) of PpDB phytoplasma were detected. This period of overlap is consistent with the possibility that the same insect transmits both phytoplasmas. However, PpM phytoplasma DNA was not detected in plants in October, when there were five cases (38%) of dieback.

PpDB, PpYC and PpM can occur within the first season in papaya plantations established in new growing areas. It can therefore be inferred that a reservoir of disease agents exists in other vegetation. Co-transmission of PpDB, PpYC or PpM could occur if the phytoplasmas were present in the same reservoir plant, or were in different plants that were all hosts of a common vector. The lack of co-incidence of DNA detection and symptom expression between PpM and PpDB in papaya during October suggests the involvement of different alternate hosts, at least during this time of the year, and possibly different vectors for the two diseases.

In parallel studies, Elder *et al.* (accepted 2001) monitored the incidence of insects on papaya on the same property on which the current study was conducted. Further, some of the insect monitoring was conducted within the same papaya plot (Site 2 in Elder *et al.*, accepted 2001) and during the same time period. Elder *et al.* (accepted 2001) found seven species of planthopper and 13 species of leafhopper on papaya plants. Relatively high numbers of the leafhopper species *Zygina honiloa* and *Austroasca alfalfae* were associated with periods of dieback incidence, however, PCR testing failed to detect phytoplasma DNA in specimens of these insects. The absence of nymphs on papaya plants indicated that leafhoppers and planthoppers do not breed on papaya plants, and most likely prefer other plant species for feeding and breeding. Elder *et al.* (accepted 2001) hypothesised that PpDB and PpYC phytoplasmas are transmitted by leafhoppers or planthoppers transported into plantations by weather fronts or troughs that involve a north to south air movement, generally occurring in spring (September-November). PpDB and PpYC outbreaks occur in years with dry conditions in late winter and early spring, as the insects are attracted to the green papaya plantations surrounded by unattractive dry, brown vegetation. The insects do not stay long on the papaya plants as they are not a favoured host.

Based on his own observations, and those of others (Glennie & Chapman, 1976; Simmonds, 1937), that dieback outbreaks tended to occur when wet weather was followed by one to two months of dry weather and then further wet weather at the time of appearance of dieback, Harding (1989) suggested that the initial wet weather period encouraged the growth of "weed" plant species that could be hosts for pathogens and their insect vectors. If the subsequent dry weather was detrimental to the health and growth of weed plants, feeding insects could have been forced to migrate to other plants, such as papaya. Also, Harding (1989) suggested that the wet weather that occurred at about the time of dieback outbreaks encouraged increased growth rate of papaya plants, thus making them more susceptible to pathogenic diseases.

4.4.3 Persistence of infection

The presence of phytoplasma within stem regrowth may represent either a carryover from the original infection, with phytoplasma surviving within the ratooned stem, or a secondary infection of the new shoot material. Reported cases involving carryover of phytoplasma involve slow decline disorders, more comparable to PpYC and PpM than PpDB. For example, in X-disease (Douglas, 1986), blueberry stunt (Schaper & Converse, 1985), apple proliferation and pear decline (Seemüller *et al.*, 1984), and ash yellows (Sinclair *et al.*, 1989), the shoot, but not root, of the affected plant is reported to become free of phytoplasma during the winter. Phytoplasma cells are thought to reinfect the shoot from the root, via the phloem, in the new growing season.

The PpDB phytoplasma was detected throughout the plant body within one week of noting visible symptoms (Chapter 3), only two to three weeks after first detection of phytoplasma within the plant. Distribution of phytoplasma DNA within the plant

decreased as shoot necrosis progressed (Chapter 3). This result was interpreted as a hypersensitive response by the plant, causing the death of phytoplasma throughout the plant body. Consistent with this hypothesis is the fact that, in six cases, phytoplasma DNA was not present in either the main stem or regrowth of plants that were not ratooned (dead apex still present). The two cases in which phytoplasma DNA was detected in regrowth of dieback-affected plants are interpreted as subsequent infection events of the new shoot growth.

The higher incidence of detection of phytoplasma DNA within regrowth of plants which had suffered from yellow crinkle or mosaic is consistent with the survival of PpYC and PpM phytoplasma within the stem and roots, and subsequent reinfection of lateral shoot growth. However, phytoplasma DNA was not detected in regrowth material of all of the papaya plants initially infected with the PpYC and PpM phytoplasmas (Table 4.2), suggesting either uneven distribution or some remission of the phytoplasma within the stump. Studies of the distribution of phytoplasma within the host plant during the progression of mosaic and yellow crinkle diseases would test these interpretations.

It can be concluded that the grower action of ratooning as visible symptoms of any of the three diseases become apparent does not always have the intended effect of preventing phytoplasma cells from reaching the lower stem, though it must reduce the number of such cells. However, the ratooning of dieback-affected plants is warranted to promote lateral shoot growth within 0.75 m of ground level, replacing the lost production potential. In contrast, if PpYC and PpM phytoplasma survive within the trunk and roots of the plants, ratooning is not an effective strategy.

4.4.4 Dual infection

Gibb *et al.* (1998) have recently reported the use of the PCR primer rSPLLS (specific for subclade iii phytoplasmas, Schneider & Gibb, 1997) with P1 to allow specific detection of the PpYC or PpM phytoplasma. This will facilitate future epidemiological studies by using a one step differential detection of the PpYC/PpM and PpDB phytoplasmas. In the current study, the P1-P7 and fU5-AGY2 primer sets did not allow unique identification of the PpYC and PpM phytoplasma in the presence of the PpDB phytoplasma. Therefore, restriction enzyme analysis of the P1-P7 product was undertaken to differentiate between the two types of phytoplasma.

In both plants ratooned following appearance of dieback symptoms, in which phytoplasma DNA was detected in re-growth and main stem (Table 4.2), only some tissues gave a PCR product with the fU5-AGY2 primer pair, and the profile of the restriction digest of the P1-P7 product was characteristic of the PpYC or PpM phytoplasmas. These observations are consistent with a dual infection of the plant with PpDB and PpYC or PpM phytoplasmas. A case of dual infection of papaya with PpDB and PpYC was also recently reported by Gibb *et al.* (1998).

Similarly, in each of two plants that developed mosaic symptoms and were ratooned, a lateral shoot was noted with dieback symptoms, and this material tested positive with both primer sets (Table 4.2). A second shoot from one of these plants tested positive with the P1 and P7 primers and not with the "*Candidatus* P. australiense"-specific PCR, and the profile of the restriction digest of the P1-P7 product was characteristic of PpYC and PpM. We conclude that a dual infection had occurred, with secondary infection by the PpDB phytoplasma.

In one plant that exhibited PpYC symptoms and was ratooned, lateral shoot regrowth exhibited symptoms of mosaic (Table 4.2). This observation is consistent with either the hypothesis that a single phytoplasma is involved in these diseases, with differential symptom development triggered by other factors, or that two infection events occurred, one for each disease.

CHAPTER 5

Transmission of phytoplasmas to the experimental host periwinkle (*Catharanthus roseus*) using the parasitic vine dodder (*Cuscuta australis*)

5.1 Introduction

The study of papaya dieback, yellow crinkle and mosaic would benefit significantly from the ability to transmit the associated phytoplasmas from the papaya host plants to experimental hosts. This is particularly important in the case of dieback because of the severity and rapid progression of the disease in papaya. In previous transmission work, Greber (1966) used the dodder *C. australis* to transmit the causal agent of papaya yellow crinkle from papaya to tomato (*Lycopersicon esculentum*), white clover (*Trifolium repens*) and jimson weed (*Datura stramonium*). Symptoms typical of phytoplasma diseases were observed in the recipient plants, including big bud disease symptoms in tomato, 7-10 weeks after inoculation. Greber (1966) also used dodder to TBB agent from naturally infected tomato to papaya, resulting in the appearance of typical yellow crinkle symptoms 9-13 weeks after inoculation.

Greber (1966) also attempted graft transmission of the PpYC disease agent from diseased papaya plants to healthy papaya plants. These attempts were unsuccessful because the infected scions became necrotic and died before forming a union. In contrast, scions from healthy papaya plants were comparatively easy to establish. There is no previous report of the transmission of phytoplasmas from yellow crinkle-affected papaya to periwinkle, although the closely related tomato big bud (TBB) phytoplasma has been transmitted by *C. australis* from tomato to periwinkle (R. I. Davis *et al.*, 1997).

Harding (1989) attempted graft transmission of the PpDB disease agent by grafting scions from stems, roots and leaf mid-veins of dieback-affected papaya plants to stems, roots and leaf mid-veins of healthy papaya plants in a glasshouse. These attempts were unsuccessful, as scions from diseased plants died within 3-4 days and scions from healthy plants died within 5-6 days. Also, Greber (1995) reported that tissue taken from near the necrotic areas of stem failed to graft to healthy stock, while patch buds taken from more distant parts and scions from plants that had recovered from dieback did not result in development of dieback in recipient stock plants. Attempts to transmit the PpDB agent using dodder have not previously been reported, although Harding (1989) did suggest attempting transmission of the PpDB agent by using dodder in light of the failure of graft and insect transmission experiments.

The focus of transmission experiments reported in this thesis was papaya dieback, since the PpYC phytoplasma has previously been transmitted to experimental hosts using dodder, and "*Candidatus* Phytoplasma australiense" has not been experimentally transmitted from any of the known host species (grapevine, papaya, strawberry and garden bean) in Australia.

Based on PCR and RFLP, the TBB-type phytoplasma had previously been detected in gerbera (*Gerbera jamesonii*) plants exhibiting phyllody (R. I. Davis *et al.*, 1997). This gerbera disease was also observed to be chronic, with symptoms progressing from initial virescence in recently matured flowers, to phyllody in subsequently formed flowers, then shoot proliferation and little-leaf symptoms in later stages. Preliminary PCR testing for the study in this thesis resulted in strong and consistent amplification of phytoplasma DNA from leaves and flowers of gerberas exhibiting virescence and

phyllody, indicating that phytoplasma cells were likely to be present in high titre. It was therefore decided to use this gerbera disease as a model system to test the dodder transmission methods.

5.2 Materials and Methods

5.2.1 Dodder transmission methods

Healthy, uninfected dodder (*C. australis*) was obtained from K. Gibb (Northern Territory University) and was trained onto healthy young periwinkle (*C. roseus*) plants that had been raised from seed and kept in an insect-proof glasshouse at Central Queensland University, Rockhampton. Once the dodder had established itself on the initial periwinkle hosts, strands were trained across to stems of other healthy periwinkle plants that were to be used as recipient plants for the phytoplasma transmission experiments.

Two methods of establishing a dodder bridge between donor plants and recipient periwinkle plants were attempted (Table 5.1). The "cut strand" method, as described by Greber (1966) in PpYC and TBB transmission experiments, involved placing the cut ends of 5 to 10 strands, 10-15 cm in length, of healthy dodder in vials of water and training the strands around the stem, petioles and peduncles of diseased plants. If the dodder formed haustoria and attached to the donor plant, usually within a week, it was then trained around the stem of a healthy recipient periwinkle plant to form a "stable bridge". The second method involved forming an "inverse bridge" by training terminal lengths of dodder already established on healthy periwinkle plants, directly to the stem, petioles and peduncles of diseased plants. To minimise the distance between donor and recipient phloem, the periwinkle stem and the donor plant stem, petiole or peduncle were placed alongside each other and bound together by twining the dodder around

Table 5.1. Strategies and methods used to transmit phytoplasmas from papaya plants with dieback, yellow crinkle or mosaic diseases to periwinkle, using dodder as a vector.

Strategy	**Method**	**No. of diseased plants used in experiments**			**Totals**
		Dieback	**Yellow crinkle**	**Mosaic**	
In field	Inverse bridge	3	...	...	3
	Cut strands	7	3	7	17
Cut stem	Inverse bridge	5	1	3	9
	Cut strands	15	1	1	17
Transplanted	Inverse bridge	4	...	...	4
	Cut strands	4	...	...	4
Transferred pot	Inverse bridge	18	1	...	19
	Cut strands	11	...	...	11
Totals		**67**	**6**	**11**	**84**

them. Within a week after the dodder formed haustorial connections with the donor plant, the mature leaves of the recipient periwinkle plants were removed to encourage the bulk flow of phloem sap to the recipient periwinkle.

5.2.2 Dodder transmission of phytoplasma from gerbera

Five hybrid gerbera plants exhibiting symptoms of virescence and phyllody were obtained from a nursery in Bundaberg, Queensland. The gerbera plants were transplanted from the field, and maintained in the glasshouse at CQU, Rockhampton. Dodder was trained to the leaf petioles and flower stalks of the gerbera plants, using the cut strand method, for two plants, and the inverse bridge method for three plants. Samples of gerbera leaf, flower stalk and petal tissue were collected for PCR testing and TEM, to confirm the presence of phytoplasmas.

5.2.3 Dodder transmission strategies for papaya diseases

Initial attempts were made to establish dodder on diseased papaya plants in the field using the cut strand method (Greber, 1966). Three yellow crinkle, two mosaic, and two dieback affected papaya plants in a commercial plantation at Yarwun, central Queensland, were selected. Since none of the dodder strands established on the papaya plants and did not survive more than two weeks, it was suspected that warm and dry weather conditions had an adverse affect on the dodder. The inverse bridge method was then attempted with three dieback-affected plants in the field, near Rockhampton, Queensland. The pots containing recipient periwinkles were tied to the stems of the papaya plants. Although some of the dodder formed haustoria and attached to the papaya stem and petioles, the periwinkle plants suffered from water stress, as water supply was insufficient between weekly visits to the field site.

It was decided that the diseased papaya plants needed to be transferred from the field to the glasshouse, so that the dodder, papaya and periwinkle plants could be more closely monitored. Three strategies were used (Table 5.1). First, field plants showing disease symptoms were cut at least 80 cm below the apex and transported to the glasshouse where the cut stems were placed in separate 10 L plastic buckets filled with water. Both cut strand and inverse bridge methods of dodder transmission were attempted with these cut stems. Although dodder haustoria were often produced and attached through the epidermis of the papaya stems, there was also rapid degeneration of the papaya plant, particularly in the case of dieback, and from external visual inspection (i.e. dodder haustoria were easily detached from papaya) it appeared that the haustoria did not form any significant connection with the internal papaya tissues. It was concluded that the stress resulting from cutting the stems of the diseased plants needed to be reduced, and that this could be done by transferring the whole plant from the field to the glasshouse.One strategy was to uproot and transplant diseased field plants, less than 2 m tall, to large pots in the glasshouse (Table 5.1). For the third strategy, 152 healthy papaya plants (Hybrid 29) were raised in the glasshouse in pots, 30 cm in diameter, then when the plants were approximately 1.0 m tall, 90 plants were planted in a commercial plantation at Yarwun and 62 plants were planted in the field near Rockhampton. The plants were planted without removal from pots and they were supplied water by trickle (Yarwun) or drip (Rockhampton) irrigation. When disease symptoms were observed during weekly inspections, the symptomatic plants were removed from the field with their pots intact and transported to the glasshouse for dodder transmission (Table 5.1).

One healthy Hybrid 29 papaya plant raised and kept in the glasshouse was used in a trial to test how efficiently dodder established on papaya. The cut strand method and the inverse bridge method were both tested on the same healthy papaya plant.

5.2.4 Nucleic acid extraction and PCR

Total nucleic acids were extracted from papaya (stem, petiole or leaf midribs), periwinkle (leaf midribs or petals), gerbera (leaf, flower stalk or petals), and dodder strands as described in Chapter 3.

PCR was performed as described in Chapter 3, except total PCR volumes for each reaction were 50 µL. PCRs were performed using either the generic phytoplasma-specific primer pair P1 and P7 (Table 2.1) or the "*Candidatus* P. australiense"-specific primer pair fU5 and AGY2. The fU5-AGY2 primer pair was used for specific detection of the PpDB phytoplasma (Chapter 3).

5.2.5 RFLP analysis of PCR products

Restriction endonuclease digestion of the P1-P7 PCR product was used to differentiate the PpYC and PpM phytoplasmas from the PpDB phytoplasma. The P1-P7 PCR products were digested separately using the restriction endonucleases *Alu* I or *Rsa* I (Boerhinger Mannheim). Fifteen microlitres of P1-P7 PCR product was digested with 4 U *Alu* I at 37°C for 4 h, and the reaction was stopped by heating at 65°C for 20 minutes. The digested fragments were separated by electrophoresis through a 2% (w/v) agarose gel for 3 h in 1× TAE buffer at 5 V/cm. Twenty microlitres of P1-P7 PCR product was digested in a total volume of 25 µL with 20 U *Rsa*I at 37°C for 4 h, and the reaction was stopped by heating at 65°C for 20 minutes. The digested fragments were separated by electrophoresis through a 5% (w/v) polyacrylamide gel for 4 h in 1×TBE buffer at 8 V/cm (R. I. Davis *et al.*, 1997). The gels were stained with ethidium bromide and viewed on a UV transilluminator.

5.2.6 Transmission Electron Microscopy

Small pieces, 2 mm in length, of small leaf veins and virescent flower petals from gerbera plants exhibiting virescence and phyllody, were prepared for TEM as described for papaya tissue in Chapter 3, using a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) with 150 mM sucrose and 2 mM calcium chloride for fixation. Thin sections were viewed using a J.E.O.L. 101 transmission electron microscope. Virescent petals from stunted flowers of recipient periwinkle plants, in which phytoplasmas were detected by PCR, were sampled for transmission electron microscopy. Pieces of virescent flower petals, 2 mm^2 in area, were prepared as described by Gowanlock (1998), using a mixture of 3% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) for fixation. Thin sections were viewed using a J.E.O.L. 1010 transmission electron microscope.

5.3 Results

5.3.1 Transmission of "*Candidatus* Phytoplasma australasiense" from gerbera

Most (70%) cut strands trained onto two phytoplasma-infected, glasshouse-maintained gerbera plants withered before haustoria established strong connections. Two or three cut strands did establish on the gerbera plants but continued growth was limited and insufficient to train the strands on to recipient periwinkle plants. Using the inverse bridge method, dodder strands established on the other three infected gerbera plants within two to four days. On one recipient periwinkle plant, green pigmentation in mature flowers and small green flowers were first observed 76 days after the dodder strands were trained on to the gerbera plant.

Using PCR primers P1 and P7, DNA fragments, approximately 1.8 kb in length were amplified from DNA extracts of flower petal and leaf tissue of all five gerbera plants

exhibiting virescence and phyllody. A 1.8 kb PCR product was also obtained from DNA extracts of leaves and flowers of the recipient periwinkle plant that exhibited virescence. The *Alu* I and *Rsa* I RFLP profiles of the P1-P7 products obtained from the gerbera and periwinkle plants were identical to those of TBB, and different from the profiles for SPLL-V4 and PpDB (Fig. 5.1). The transmitted phytoplasma can thus be identified as a strain of "*Candidatus* Phytoplasma australasiense" (Chapter 2).

5.3.2 Transmission of phytoplasmas from papaya

Dodder trained onto the potted healthy papaya plant in the glasshouse established haustorial connections with papaya stem and petioles within two to four days of training the dodder strands onto the papaya plant. A smaller proportion of cut strands established on the papaya than strands trained from a healthy periwinkle (inverse bridge method). However, all strands that did attach to the papaya stem and petioles established many strong haustorial connections and grew vigorously.

Out of a total of 84 papaya plants exhibiting dieback (67 plants), yellow crinkle (6 plants) or mosaic (11 plants) symptoms (Table 5.1), phytoplasma cells were transmitted from only one plant. This plant initially exhibited dieback symptoms in the field.

The inverse bridge method appeared to be more successful than the cut strand method for encouraging the establishment of dodder on papaya stems. Dodder strands remained attached for up to four weeks before degradation of the dieback-affected papaya tissues became too severe to support the growth of dodder. Some dodder strands trained to papaya stems using the inverse bridge method did establish within a week and exhibited some growth, however, symptoms did not develop in the periwinkle plants, and phytoplasma DNA was not detected in extracts from these plants.

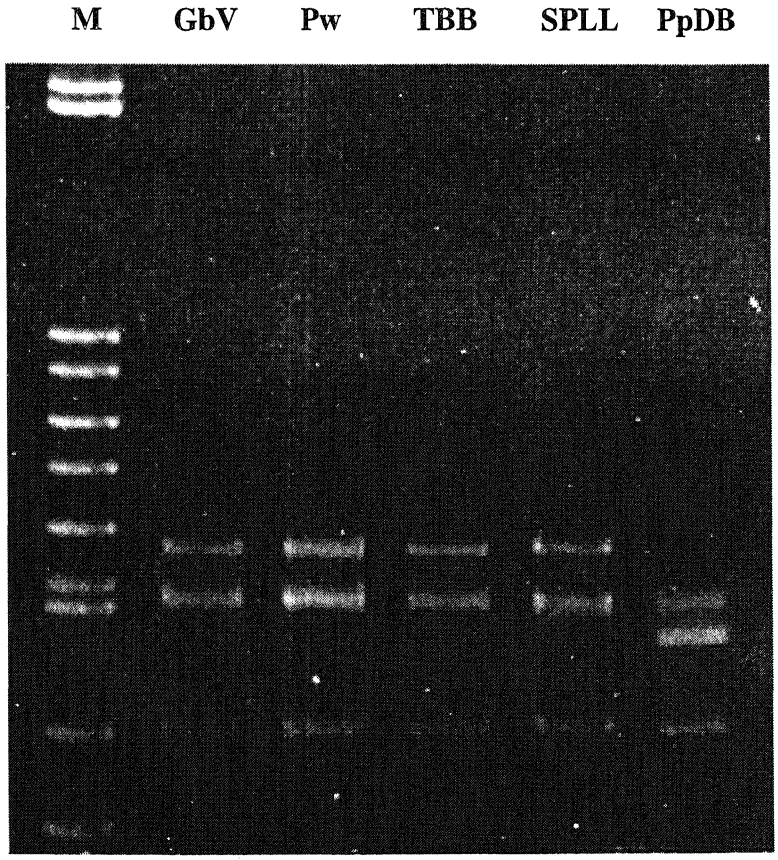

Figure 5.1 *Rsa* I RFLP profiles of P1-P7 PCR products of DNA extracts from gerbera exhibiting virescence (GbV), periwinkle infected with phytoplasma from gerbera (Pw), periwinkle infected with tomato bug bud phytoplasma (TBB), periwinkle infected with sweet potato little leaf variant-V4 phytoplasma (SPLL), and papaya affected by dieback (PpDB). Lane M contains the size marker Probase 50 (Progen Industries, Brisbane, Australia)

Dodder attachment and establishment was more rapid and successful with the transplanted plants than with the cut stems. Most dodder strands that firmly attached to the papaya stem also exhibited at least some growth. Seven of the eight transplanted dieback plants had severely declined, due to disease progression, within five weeks of transplantation to the glasshouse, and there was no evidence of phytoplasma infection in the periwinkle plants that were attached to them by dodder.

Among the papaya plants that were raised in pots in the glasshouse and transferred to the field, dieback symptoms were first noticed 65 days after transfer to the field. Transmission was not achieved from any of these dieback-affected potted plants when they were transferred back to the glasshouse. The transferred potted plants did survive longer than the transplanted plants that were uprooted from the field, in some cases up to six weeks before severe dieback of the stem occurred. Dodder also attached and established more easily on these plants than on the transplanted plants and the cut stems.

One of the transplanted dieback-affected plants recovered from the initial disease and survived for almost seven months. It is from this plant that successful transmission of phytoplasma cells was achieved. In the field, this plant exhibited early papaya dieback symptoms, such as bent growing tip, chlorosis of young crown leaves, and dark green water-soaked streaking on stem and petioles, and was therefore transplanted to the glasshouse. DNA extracts of stem and leaf midvein samples, taken at the time of transplantation, tested positive using PCR primer pair fU5-AGY2. These extracts were not tested using PCR primer pair P1-P7, as the purpose of the fU5-AGY2 PCR was to confirm the presence of the PpDB phytoplasma. Within three days of training cut strands of dodder to the papaya plant, haustoria had formed and attached to the papaya petioles. Within 14 days after transplantation the dieback symptoms were advanced in

the papaya plant. Some dodder strands appeared to have attached firmly to immature leaves, however there was no sign of growth. Two of these dodder strands tested negative using PCR primers fU5 and AGY2, while a positive result was obtained for the papaya leaf petiole to which the two dodder strands were attached. Primer pair P1-P7 was not used to test the dodder or papaya leaf petiole.

Forty-nine days after transplantation to the glasshouse, it appeared that the progression of dieback had stopped. Some small immature crown leaves around the growing point and approximately two thirds of the upper stem had survived. These leaves and the upper stem developed dark green streaks. The leaves also exhibited some chlorosis and lateral shoots had developed from the upper stem. Fifty-four days after the papaya plant was transplanted to the glasshouse, cut strands of dodder were trained onto the symptomatic petioles and lateral shoots, and twined around stems of two healthy periwinkle plants in pots. This was repeated 19 days later with another set of cut strands of dodder.

Virescent mature flowers (approximately 40 mm in diameter) and green, stunted flowers (approximately 10 mm in diameter), with abnormally short corolla tubes (Fig. 5.2B), were first noticed on both periwinkle plants 133 days after initial transplantation of the papaya plant. This observation was 79 days after dodder was first trained between the papaya and periwinkle plants and 60 days after the second set of dodder was trained between the papaya and periwinkle plants. PCR using primers P1 and P7 amplified a DNA fragment approximately 1.8 kb in length, from DNA extracts of leaves, flowers and attached dodder from the two recipient periwinkle plants. Using PCR primers fU5 and AGY2, there were no amplification products obtained from the same plant tissue samples. *Alu* I and *Rsa* I RFLP analysis of the P1-P7 products from both periwinkles

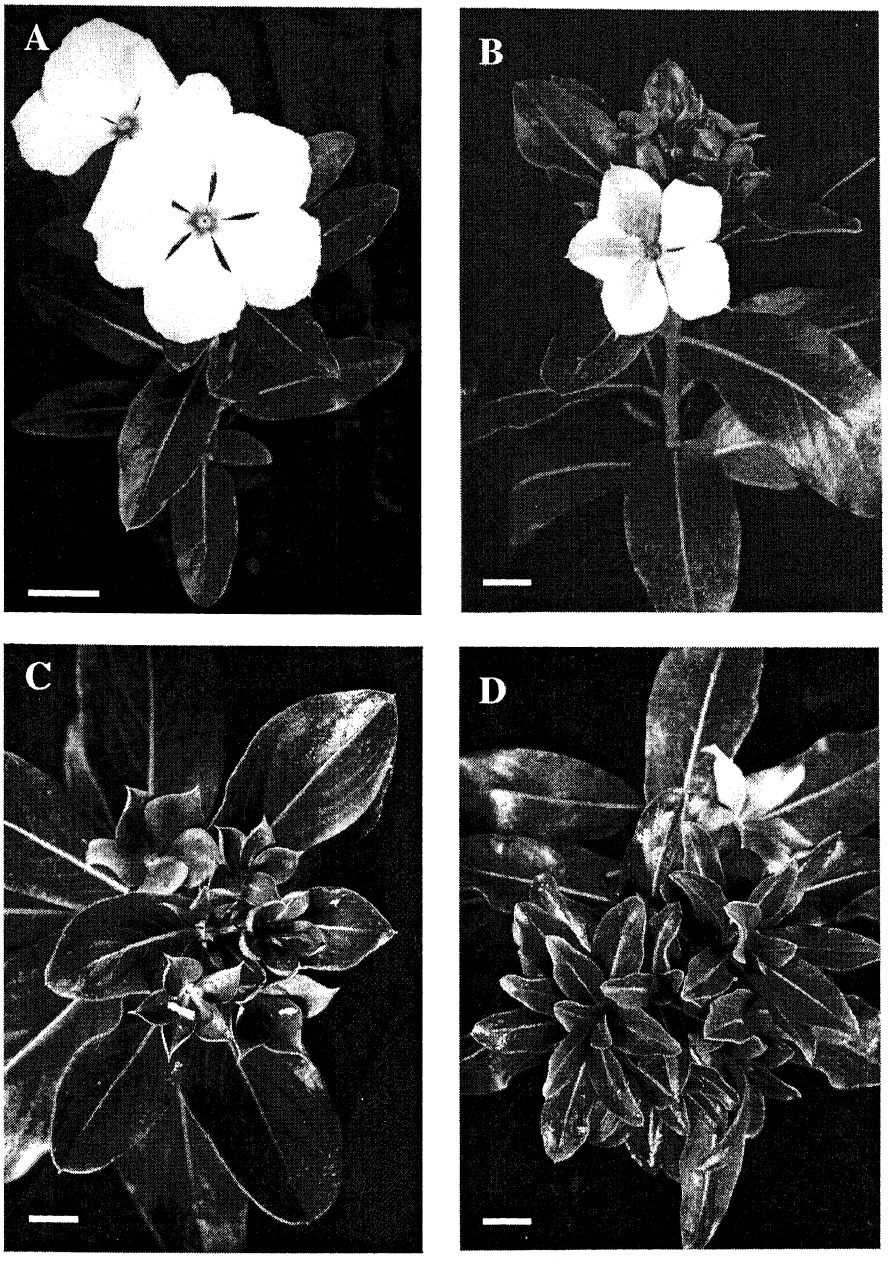

Figure 5.2 Shoots of periwinkle plants showing normal flowers on a healthy plant (A) and progressive symptom development in periwinkle plants infected with the phytoplasma transmitted from papaya; (B) virescence in large white flowers and green stunted recently developed flowers, (C) all new flowers are green and stunted, and (D) development of bunched stunted leaves instead of flowers. Bar in each photograph represents approximately 1 cm.

revealed a profile identical to that of PpYC, PpM and TBB, but different to that of SPLL-V4 and PpDB (Fig. 5.3). The transmitted phytoplasma can thus be identified as a strain of "*Candidatus* Phytoplasma australasiense".

After the first observation of virescence in the recipient periwinkles, DNA extracts from stem and axillary bud samples of the donor papaya plant were tested using PCR primer pairs P1-P7 and fU5-AGY2. The axillary bud tested positive with P1-P7 and negative with fU5-AGY2. The stem samples tested negative for both primer pairs. *Rsa* I RFLP analysis of the P1-P7 PCR product from the axillary bud, revealed a profile identical to that obtained for the two recipient periwinkle plants, reference samples of PpYC, PpM, and TBB, and different from the profiles for SPLL-V4 and PpDB.

Within one month of the initial observation of virescence, most new flowers on both periwinkle plants were stunted and green (Fig 5.2C). Within four months from the initial observation of virescence, new shoot growth on the recipient periwinkle plants exhibited advanced phyllody and stunted leaves (Fig. 5.2D). After initial transmission of phytoplasma from papaya to periwinkle plants, further transmission to other periwinkle plants was achieved by training infected dodder growing on the original recipient periwinkles to other healthy periwinkle plants. Virescence was first noticed on recipient periwinkle plants 40 days after infective dodder was trained to them.

5.3.3 TEM

Round to pleomorphic phytoplasma cells, ranging in diameter and length from 50 to 500 nm, were observed in the sieve elements of green flower petals of the recipient periwinkle plants involved in the single case of transmission from a papaya plant (Fig. 5.4A). Phytoplasma cells were observed in at least one sieve element in each vascular

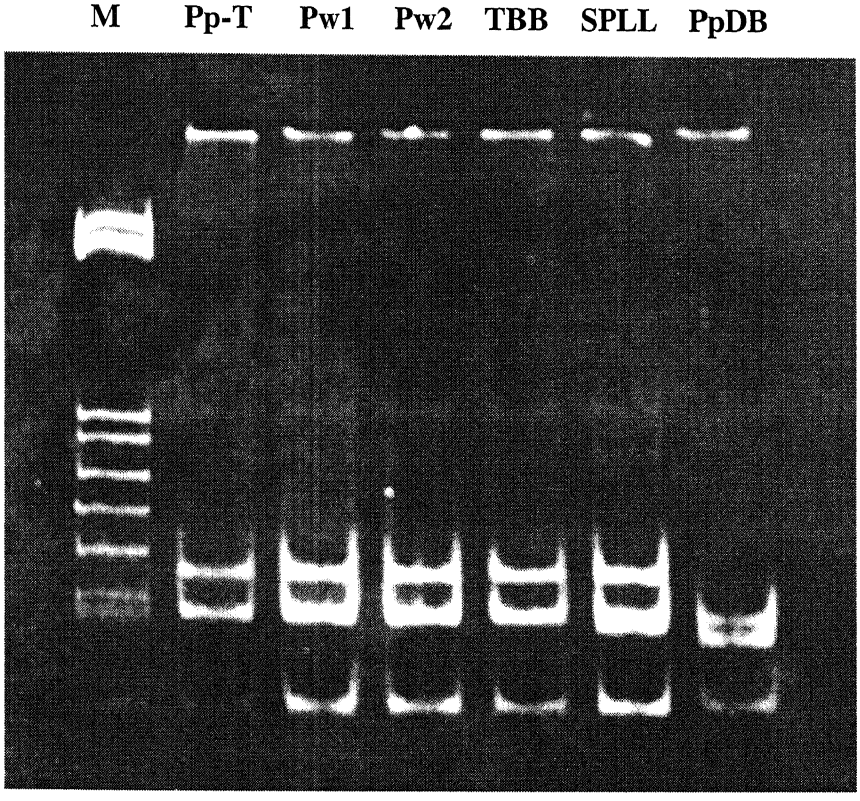

Figure 5.3 *Rsa* I RFLP profiles of P1-P7 PCR products of DNA extracts from transplanted papaya from which phytoplasma was transmitted (Pp-T), periwinkles infected with phytoplasma from transplanted papaya (Pw1 and Pw2), periwinkle infected with tomato bug bud phytoplasma (TBB), periwinkle infected with sweet potato little leaf variant-V4 phytoplasma (SPLL), and papaya affected by dieback (PpDB). Lane M contains the size marker Probase 50 (Progen Industries, Brisbane, Australia)

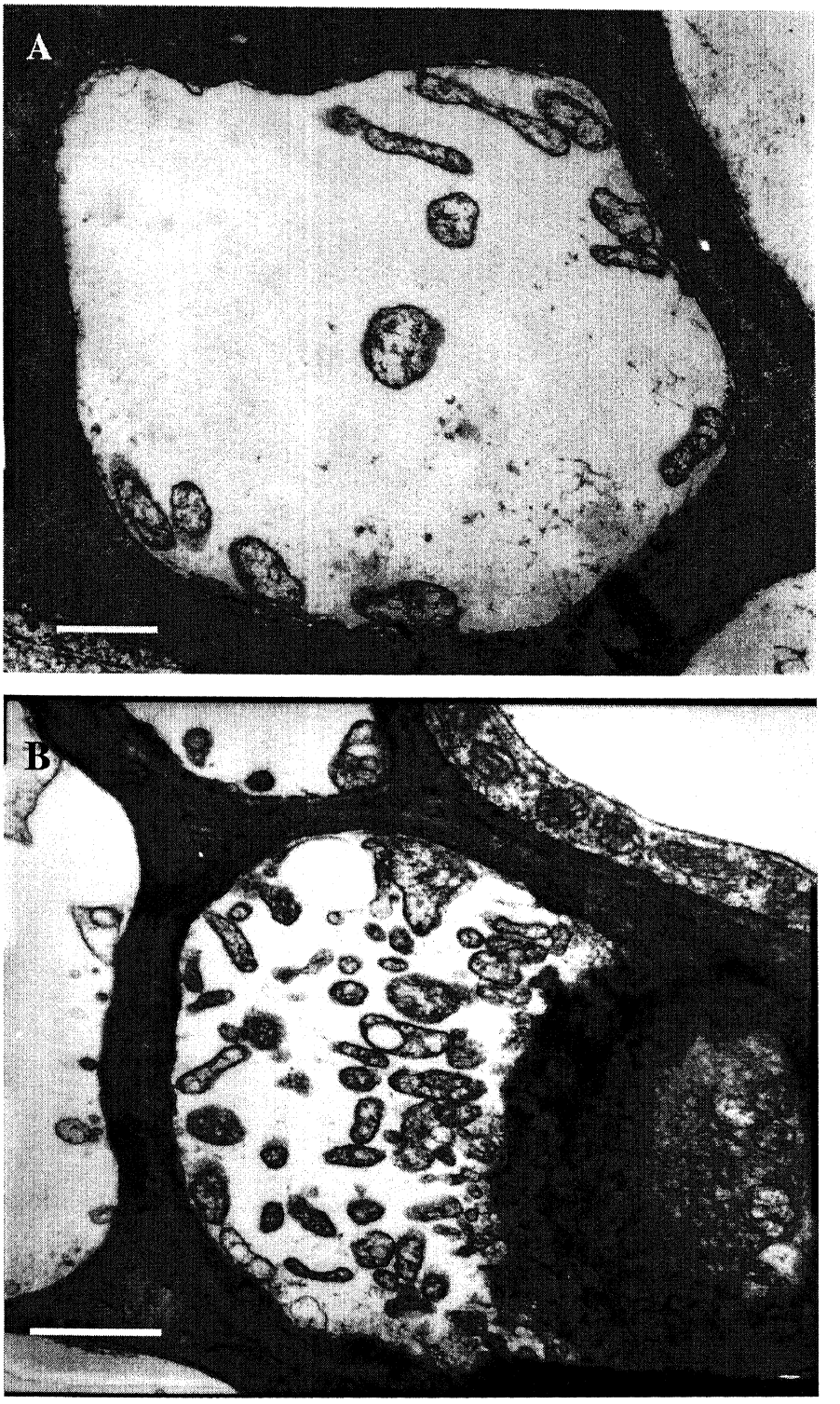

Figure 5.4. Transmission electron micrographs of phytoplasma cells within sieve elements of veins of virescent flower petals of periwinkle plants infected with "*Candidatus* Phytoplasma australasiense" transmitted from papaya. **(A)** Cross section of mature sieve element. Bar represents 500 nm. **(B)** Section through sieve element near sieve plate. Bar represents 1 µm.

bundle. Greater numbers of phytoplasma cells were observed at sieve plates (Fig. 5.4B). Round to pleomorphic phytoplasma cells were also observed in sieve elements of leaves and flower petals of virescent gerberas (Fig. 5.5).

5.4 Discussion

5.4.1 Transmission of "*Candidatus* P. australasiense" from gerbera

Gerbera virescence phytoplasma transmission experiments were conducted as a model system for transmitting phytoplasmas via dodder from naturally infected plants. The inverse bridge method was more effective than the cut strands method for the establishment of dodder bridges between donor and recipient host plants. This was further highlighted by the transmission of phytoplasmas from one out of three gerbera plants that were connected to periwinkle plants by the inverse bridge method. From these experiments, it appears that phytoplasma transmission using the inverse bridge method was achieved because phytoplasma cells were present at high titre in the gerbera phloem (as revealed by TEM), disease progression was slow, and dodder continued to grow vigorously while it parasitised the gerbera plants.

5.4.2 Inability to transmit the PpDB phytoplasma

The initial attempts to transmit phytoplasmas from dieback, yellow crinkle and mosaic affected papaya plants in the field, to periwinkle plants, used the cut strand method described by Greber (1966) for the transmission of the yellow crinkle phytoplasma to tomato, white clover and *D. stramonium* plants. Greber (Greber, 1966) reported ready establishment and growth of the dodder on a yellow crinkle-affected papaya plant in the field. In the current study, it is suspected that the failure of dodder to establish and grow on papaya plants in the field was due to warm and dry conditions. Establishment

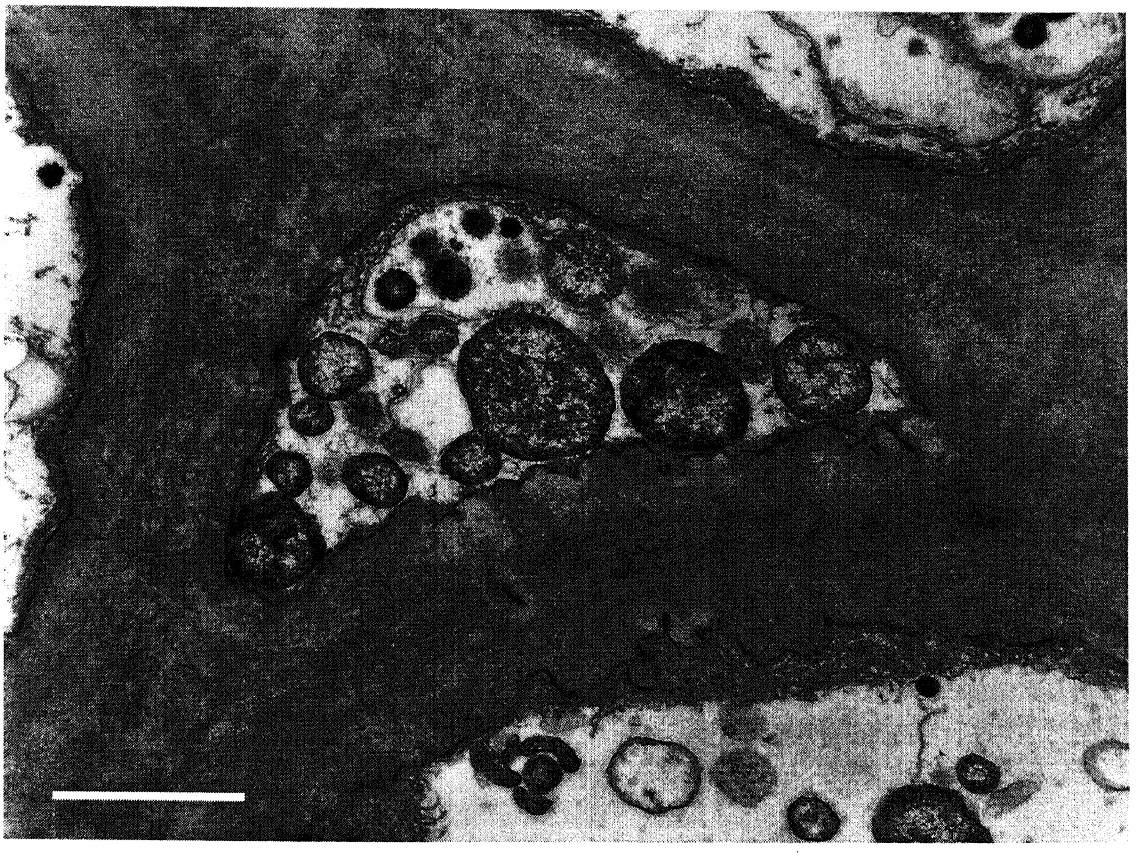

Figure 5.5. Transmission electron micrograph of phytoplasma cells within sieve elements of a vein of a virescent flower petal from gerbera plant infected with "*Candidatus* Phytoplasma australasiense". Bar represents 1 μm.

of dodder on papaya plants in the field using the inverse bridge method was also unsuccessful. In Italy, Credi and Santucci (1992) experienced problems during attempts to transmit phytoplasmas from grapevines affected, by a FD-type grapevine yellows disease, to periwinkle using *C. campestris* and the inverse bridge method. Although 70% of the periwinkle plants died due to unfavourable field conditions, dodder did establish well on the grapevines. Cuttings of grapevine shoots parasitised by dodder were taken to the glasshouse to establish dodder bridges to healthy periwinkle plants. Also, cuttings of infected grapevine were taken to the glasshouse to establish inverse bridges with dodder already established on healthy periwinkle plants. Credi and Santucci (1992) only achieved successful transmission when experiments were conducted in the glasshouse.

Transplanting diseased papaya plants from the field proved to be more beneficial for establishing dodder than the cut stem strategy, mainly because the papaya plants survived for longer periods of time after transfer from the field. It was from one of eight dieback-affected papaya plants transplanted from the field that successful transmission of a phytoplasma was achieved. Similarly, Maixner *et al.* (1994) were able to transmit phytoplasmas from a transplanted grapevine with symptoms of Vergilbungskrankheit (VK), to periwinkle plants via dodder.

The cut papaya stem technique most likely failed due to the rapid progression of stem necrosis associated with dieback, resulting in poor haustorial connection of the dodder and the inability to establish a phloem bridge. Since yellow crinkle and mosaic are more chronic diseases than dieback, it might be expected that a transmission protocol using cut stems could still be developed for these diseases.

Although phytoplasmas were not transmitted from papaya plants that were transferred in pots from the field, this strategy did demonstrate that the diseased papaya plants survived longer. This was likely to be the result of reduced root trauma and overall stress on the plant compared with that which occurred in the cut stem and transplantation strategies. Since dodder established and grew relatively easily on the healthy potted papaya plant, it can be concluded that the diseased papaya plants were not favourable hosts for the establishment and growth of dodder.

5.4.3 Transmission of "*Candidatus* Phytoplasma australasiense" from papaya

This is the first report of a "*Candidatus* P. australasiense" strain being transmitted directly from papaya to periwinkle using dodder. The PCR and RFLP results indicate that the papaya plant, from which "*Candidatus* P. australasiense" was transmitted, had a mixed population of "*Candidatus* P. australiense" and "*Candidatus* P. australasiense". The effects of the PpDB phytoplasma infection apparently masked those of "*Candidatus* P. australasiense", as the plant was selected in the field because it exhibited typical external symptoms of dieback. It is possible that the PpDB phytoplasma population was eliminated during dieback disease progression, as described in Chapter 3, and "*Candidatus* P. australasiense", having already been established in the plant when it was transplanted from the field, was the only species of phytoplasma remaining in the papaya plant when dodder bridges were established to the periwinkle plants. Another possibility is that *C. australis* may not be a suitable transmission vector for the PpDB phytoplasma. Differential transmissibility of different phytoplasma types by the same species of dodder has been reported by Carraro *et al.* (1991).

The results of the transmission attempts correspond with observations presented in Chapters 3 and 4, regarding the virulence, distribution, and persistence of the PpDB,

PpYC and PpM phytoplasmas in papaya plants. Since the progression of dieback is rapid, it was suggested that the PpDB phytoplasma is highly virulent and induces an acute hypersensitive response that limits phytoplasma multiplication and reduces phloem transport (Chapter 3, Guthrie *et al.*, 2001). The PpDB phytoplasma has only ever been detected by PCR one to two weeks before first appearance of external symptoms, and intact phytoplasma cells have not yet been observed in papaya plants that exhibit external dieback symptoms (Chapters 3 & 4). Since papaya plants with dieback were selected or collected for transmission experiments only when external symptoms appeared, it is likely that the total numbers and concentrations of viable phytoplasma cells were already low, and phloem transport was already severely affected. The combination of these two factors alone would significantly reduce the probability of (i) establishment of a phloem link between papaya and dodder, and (ii) translocation of intact or viable phytoplasma cells into the dodder and recipient periwinkle plant. Although PpYC or PpM phytoplasmas were not transmitted from papaya plants with yellow crinkle (six plants) or mosaic (11 plants) symptoms, successful transmission of "*Candidatus* P. australasiense" from the dual infected papaya plant indicates that this phytoplasma is more persistent in papaya than the PpDB phytoplasma, as was also concluded in Chapter 4.

5.4.4 Suggestions for future transmission work

The three major challenges with transmitting the dieback phytoplasma from papaya using dodder are the establishment of viable dodder connections with diseased plants, the size of papaya plants, and the rapid progression of the disease.

The environmental conditions at the two central Queensland field sites proved to be unfavourable for the survival of the dodder, therefore it is recommended that future in-

field establishment of dodder on papaya plants should be conducted in locations with a cooler and more humid climate, such as southeast Queensland. Also, future transmission experiments should be conducted in controlled glasshouse conditions where possible. Another consideration for future work would be to trial other dodder species. Previous investigations have demonstrated different transmission efficiencies of a single phytoplasma type by two different dodder species (Marcone *et al.*, 1997), and different transmission efficiencies of two different phytoplasma types by a single species of dodder (Carraro *et al.*, 1991). In their attempts to transmit the phytoplasma associated with root (wilt) disease of coconut in India, Sasikala *et al.* (1988) reported that three different dodder species failed to parasitise coconut plants, but phytoplasma transmission using a species of dodder laurel, *Cassytha filiformis*, was successful.

The procedure presented in this chapter involving the growth of papaya plants in pots in the field and transferring the plants to the glasshouse while still in their pots is recommended for reducing plant stress when moving the plants to the glasshouse. One possible strategy would be to raise papaya plants in pots in the glasshouse, establish dodder on them, then when the dodder is growing vigorously, transfer the potted plants to the field and transfer them back to the glasshouse when dieback symptoms are observed.

The severity and rapid progression of dieback appears to be the most significant limiting factor in transmission attempts of the PpDB phytoplasma. So, although it would be ideal to transmit the PpDB phytoplasma from naturally infected papaya plants, the best alternative is to transmit from other plant species naturally infected with "*Candidatus* P. australiense". The same or closely related strains of "*Candidatus* P. australiense" have also been detected in Australia in grapevines (*Vitis vinifera*) affected by grapevine

yellows (R. I. Davis *et al.*, 1997), garden bean (*Phaseolus vulgaris*) with witches' broom symptoms (Schneider *et al.*, 1999), and strawberry (*Fragaria* × *ananassa*) with green petal and lethal yellows diseases (Padovan *et al.*, 1998; Padovan *et al.*, 2000). The same or closely related strains of "*Candidatus* P. australiense" have been detected in New Zealand in flax (*Phormium tenax*) with yellow leaf disease (Chapter 2), strawberry with lethal yellows disease (M.T. Andersen *et al.*, 1998), and cabbage tree (*Cordyline australis*) with sudden decline disease (Andersen *et al.*, 2001).

In Australia, strawberry plants naturally infected with the "*Candidatus* P. australiense" could be an alternative transmission source for the phytoplasma. Green petal and lethal yellows diseases of strawberry plants are relatively longer lasting or more chronic diseases when compared with papaya dieback. This would allow more time for multiplication of phytoplasma cells and the establishment of dodder bridges before the degradation of phloem tissue. Future strategies for transmitting the "*Candidatus* P. australiense" to periwinkle should include transplanting naturally infected strawberry plants from the field to the glasshouse. Strawberry and bean plants raised in pots and transferred to the field to be used as bait plants could be transferred back to the glasshouse, with pot intact, when symptoms appear, or when the phytoplasma is detected by PCR during routine sampling.

The case of double infection of a papaya plant, presented in this chapter, has highlighted the importance of being able to detect co-resident phytoplasma species and strains. Since these transmission experiments were performed, Gibb *et al.* (1998) have reported the use of a PCR primer rSPLLS (Schneider & Gibb, 1997) that allowed one-step differentiation of PpYC and PpM phytoplasmas from PpDB phytoplasma without the need for RFLP analysis. It is recommended that in future, field-collected plants to be

used in transmission, epidemiology and within-plant distribution studies should be tested for mixed infections of different phytoplasma species and strains.

CHAPTER 6

Summary and conclusions

Collectively, papaya dieback, yellow crinkle and mosaic continue to be important problems for the Queensland papaya industry, particularly in central and southeast Queensland. Individually, dieback is the most economically important disease, followed by yellow crinkle, while mosaic has relatively low importance. Although all three phytoplasma-associated diseases were investigated or considered during the work for this thesis, the main emphasis was directed toward dieback because of its overriding importance and severity, and the lack of specific knowledge about the disease, relative to yellow crinkle and mosaic.

Prior to this study, the phytoplasmas associated with papaya dieback (PpDB), yellow crinkle (PpYC) and mosaic (PpM) had been differentiated and classified with other known phytoplasmas. Based on RFLP analysis of PCR-amplified 16S rDNA (R. I. Davis *et al.*, 1997; Gibb *et al.*, 1996) and limited sequence analysis (White *et al.*, 1997), PpDB was classified as a subclade xii phytoplasma (stolbur group) and PpYC and PpM were classified as subclade iii phytoplasmas (peanut witches' broom or faba bean phyllody group). In this study, DNA sequence analysis of the near-complete 16S rDNA of PpDB, PpYC and PpM allowed a more detailed comparison with other phytoplasmas including their phylogenetic positions. The phylogenetic analysis confirmed the earlier classifications of the PpDB, PpYC and PpM. The more detailed sequence and phylogenetic analyses provided evidence to support the inclusion of PpDB in the species "*Candidatus* Phytoplasma australiense". Phytoplasma strains from grapevine, strawberry and garden bean in Australia, and from flax, strawberry and cabbage tree in

New Zealand have also been included in "*Candidatus* Phytoplasma australiense" (Chapter 2, Andersen *et al.*, 2001; M.T. Andersen *et al.*, 1998; R. E. Davis *et al.*, 1997; Padovan *et al.*, 2000; Schneider *et al.*, 1999).

The DNA sequence and phylogenetic analyses presented in this thesis revealed that PpYC and PpM are most closely related to the TBB, SPLL, PnWB, SPWB and SUNHP phytoplasmas, and are distinct from the FBP and WBDL ("*Candidatus* Phytoplasma aurantifolia") phytoplasmas. Thus, it was proposed that the PpYC, PpM, TBB, SPLL, SPWB, PnWB and SUNHP phytoplasmas be classified together in the new species "*Candidatus* Phytoplasma australasiense", with TBB as the type strain. TBB and SPLL have extensive plant host and geographic ranges in Australia, but have not yet been reported in any other country. PnWB, SPWB and SUNHP occur in southeast Asia, but have not been reported in Australia.

It is acknowledged that these classifications and taxa are only provisional, due to the limited genetic, biological and biogeographical information on which they are based. As more phytoplasmas are discovered and characterised, and more phytoplasma genes are sequenced and analysed, the relationships of currently known and yet to be discovered phytoplasma strains will be better understood. It is likely that it will be necessary to redefine and rename many currently proposed phytoplasma taxa. For example, De La Rue *et al.* (1999) found three types of phytoplasma associated with papaya yellow crinkle (TBB, SPLL, and cactus witches' broom, CWB, from Indonesia) and two with papaya mosaic (TBB and SPLL), based on 16S rDNA RFLP profiles. This highlights the need to define and name species that can be identified in any plant or insect host. The survey of Schneider *et al.* (1999) updated the earlier survey of Australian phytoplasma diseases by Davis *et al.* (1997), with the discovery of eight

phytoplasma types (based on 16S rDNA RFLP analysis) not previously reported. Some of these recently discovered Australian strains are closely related to strains reported only from southern Asia. For example, vigna little leaf (ViLL) from the Northern Territory and sorghum grassy shoot (SGS) from Western Australia are closely related to SCWL (subclade v) from Thailand, and bonamia little leaf (BoLL) from Western Australia is closely related to FBP from Sudan and WBDL from Oman (subclade iii, "*Candidatus* Phytoplasma aurantofolia"). The name "*Candidatus* P. australasiense" was proposed for this thesis before Schneider *et al.* (1999) reported the occurrence of other phytoplasma strains with geographic ranges from Australia to southern Asia. These recently discovered Australian phytoplasma strains therefore demonstrate that names of broad geographic regions should be avoided for new and revised species names.

Phytoplasma taxonomy has only recently started to advance significantly. This is a result of the application of molecular biological techniques to the study of phytoplasmas, which had previously been limited by the inability to isolate and culture phytoplasma cells axenically. Currently, phytoplasma taxonomy is heavily based on rRNA gene sequence comparisons. However, sequences of other genes, such as ribosomal protein genes and the *tuf* gene, are now being characterised for many phytoplasmas for the purpose of refining phytoplasma taxonomy and identification (Table 1.1). Obviously, with recent advances in high throughput DNA sequencing techniques, projects aimed at sequencing whole phytoplasma genomes will provide information that will significantly enhance phytoplasma taxonomy.

The anatomical and PCR-based detection studies presented in this thesis have resulted in a better understanding of the role of the PpDB phytoplasma in the plant disease. It is

proposed that PpDB phytoplasma cells are introduced to papaya plants by a leafhopper or planthopper insect vector, most likely feeding on phloem tissues of young leaf petioles and stem. Once introduced to the papaya phloem, the phytoplasma cells move with the bulk flow of the phloem sap to sink tissues, such as the fruit and young leaves and stem. Over the course of possibly two to three weeks, the phytoplasma cells undergo limited multiplication and continue to spread into sink tissues, until they reach levels detectable by PCR. At some stage the phytoplasma produces, or induces the production of, a xylem-mobile toxin or other metabolite that initiates the observed hypersensitive response that is manifested as the rapid symptom development observed within the week following first detection by PCR. By this stage, the phytoplasma cells appear to be well dispersed in immature leaves, fruits, stem and roots, but not mature leaves. However, the phytoplasma cells appear to be in low concentration throughout the plant at all stages. The tissue necrosis in the regions of disease symptoms is likely to result in the degeneration and death of phytoplasma cells. The progressive tissue necrosis may destroy much or all of the papaya stem resulting in plant death, or it may stop and the plant recovers to produce new lateral shoots. In both cases it appears that the phytoplasma population is completely destroyed, as lateral shoots produced after recovery from a dieback infection are not infected by any residual phytoplasma cells. Thus it is unlikely that papaya acts as a source of inoculum for further spread of the PpDB phytoplasma. New infections must again be introduced by insects that have fed on alternative, reservoir host plants.

The practice of papaya farmers cutting back the stem when dieback symptoms appear is justified, in that it should limit further spread of the phytoplasma into the lower stem and roots, and generally it does halt the basipetal progress of severe tissue necrosis. In contrast, it appears that yellow crinkle and mosaic are associated with a chronic

phytoplasma infection, in which the phytoplasma must multiply to higher concentrations and do so over a longer period than the PpDB phytoplasma to induce disease symptoms. Also, in contrast to dieback-, yellow crinkle- and mosaic-affected plants can survive for many months before final death, and the phytoplasma population survives throughout the rest of the life of the plant, as evidenced by persistence in ratooned plants and subsequent infection of new lateral shoots. It follows that on commercial plantations, yellow crinkle- and mosaic-affected plants should be removed. Although these plants may not necessarily be sources of inoculum for further spread of the phytoplasmas, they are no longer viable for fruit production.

Although attempts to transmit the PpDB phytoplasma via dodder failed, the experiments described in this thesis did provide further evidence that phloem transport in dieback-affected plants is severely affected by the time external symptoms are observed. Although demonstration of Koch's postulates requires that a causal agent be isolated from the diseased host and inoculated into a healthy host to produce the same symptoms, it is difficult in the case of dieback due to the severe phloem dysfunction. Future attempts at transmitting the PpDB phytoplasma from papaya would need to aim to transmit the phytoplasma well before external symptoms appear. The most likely strategy would be to have dodder already colonised on field grown papaya plants up to five weeks before an expected "dieback period", that is before September and before March.

Transmission of the PpDB phytoplasma from papaya may not be necessary if the aim of transmission is simply to maintain a source of phytoplasmas for future work. Since the PpDB phytoplasma belongs to "*Candidatus* P. australiense", strains genetically identical or similar to the PpDB phytoplasma could be transmitted from strawberry plants with

green petal or lethal yellows diseases, as these appear to be chronic diseases in strawberry. "*Candidatus* P. australiense" may therefore be more easily transmitted from strawberry plants than from papaya plants.

The studies presented in this thesis have provided new information about why infections of papaya plants with PpDB, PpYC or PpM phytoplasmas are difficult to treat. Disease control strategies should focus on prevention of infection. This can be achieved by preventing the vector insects from feeding on the plants and/or cultivating plants that are resistant to phytoplasma infection. Elder *et al.* (accepted 2001) failed to determine the leafhopper or planthopper vectors of the PpDB and PpM phytoplasmas. However, in an experiment in which papaya plants were enclosed in an aphid-proof net structure, Elder *et al.* (accepted 2001) found that papaya plants protected by the netting never developed dieback, yellow crinkle or mosaic symptoms and were never infected by phytoplasmas, whereas plants outside of the net structure were affected by dieback, yellow crinkle and mosaic, and infected with the associated phytoplasmas. While identifying the vector insect species and alternative, reservoir plant species of the PpDB, PpYC and PpM phytoplasmas is important, efficient and economic phytoplasma disease prevention may be achieved in the short term by growing papaya plants under insect-proof netting.

An alternative disease prevention strategy is to develop disease resistant papaya plants, either by conventional breeding methods or by engineering transgenic plants. While tolerance or resistance has been reported for some phytoplasma diseases (Carraro *et al.*, 1998; Sinclair *et al.*, 1997; Thomas & Mink, 1998), previous attempts to identify, maintain and/or breed dieback-tolerant or dieback-resistant lines of papaya have been unsuccessful (Greber, 1995). Now that "*Candidatus* P. australiense" has been

associated with dieback, the development of a reliable experimental transmission method would allow papaya plant breeders to challenge plants in breeding programs for the development of dieback resistant lines. Another approach may be the development of transgenic papaya plants that produce phytoplasma-specific antibodies (or plantibodies). Le Gall *et al.* (1998) engineered tobacco plants that expressed plantibodies specific for the stolbur phytoplasma and subsequently demonstrated that these transgenic plants were resistant to infection by the stolbur phytoplasma. The development of phytoplasma-resistant papaya plants by genetic engineering or conventional breeding would be medium to long-term solutions to prevention of papaya dieback, yellow crinkle and mosaic.

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